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Activation of endothelial NAD(P)H oxidase accelerates early glomerular injury in diabetic mice

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Increased generation of reactive oxygen species (ROS) is a common denominative pathogenic mechanism underlying vascular and renal complications in diabetes mellitus. Endothelial NAD(P)H oxidase is a major source of vascular ROS, and it has an important role in endothelial dysfunction. We hypothesized that activation of endothelial NAD(P)H oxidase initiates and worsens the progression of diabetic nephropathy, particularly in the development of albuminuria. We used transgenic mice with endothelial-targeted overexpression of the catalytic subunit of NAD(P)H oxidase, Nox2 (NOX2TG). NOX2TG mice were crossed with Akita insulin-dependent diabetic (Akita) mice that develop progressive hyperglycemia. We compared the progression of diabetic nephropathy in Akita *versus* NOX2TG-Akita mice. NOX2TG-Akita mice and Akita mice developed significant albuminuria above the baseline at 6 and 10 weeks of age, respectively. Compared with Akita mice, NOX2TG-Akita mice exhibited higher levels of NAD(P)H oxidase activity in glomeruli, developed glomerular endothelial perturbations, and attenuated expression of glomerular glycocalyx. Moreover, in contrast to Akita mice, the NOX2TG-Akita mice had numerous endothelial microparticles (blebs), as detected by scanning electron microscopy, and increased glomerular permeability. Furthermore, NOX2TG-Akita mice exhibited distinct phenotypic changes in glomerular mesangial cells expressing α-smooth muscle actin, and in podocytes expressing increased levels of desmin, whereas the glomeruli generated increased levels of ROS. In conclusion, activation of endothelial NAD(P)H oxidase in the presence of hyperglycemia initiated and exacerbated diabetic nephropathy characterized by the development of albuminuria. Moreover, ROS generated in the endothelium compounded glomerular dysfunctions by altering the phenotypes of mesangial cells and compromising the integrity of the podocytes.

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Diabetic nephropathy is a major microvascular complication of diabetes, and is a leading cause of end-stage renal disease.[1] Previous studies indicate that 30–40% of patients with diabetes mellitus develop overt nephropathy[2] by mechanisms other than hyperglycemia that involve genetic factors,[3] reactive oxygen species (ROS),[4] and advanced glycation end products.[5] Moreover, factors such as derangements in lipid profile, abnormal levels of nitric oxide (NO), protein kinase C, and kinin, as well as hypertension, contribute to the pathophysiology of diabetic nephropathy.[6] However, there are yet other pathogenetic mechanisms of diabetic nephropathy that remain to be explored.

Endothelial dysfunction is a consistent finding in patients with diabetes.[7] Endothelial damage and microcirculatory impairment are the early pathogenetic events in diabetic nephropathy in patients with hyperglycemia.[8] Endothelial dysfunction can be defined as increased ROS with reduced bioavailability of endothelial NO. Evidence suggests that in patients with diabetes increased ROS are a common denominator that links diverse pathogenic mechanisms that are involved in the progression of diabetic microvasculopathy.[9] We reported that nicotinamide adenine dinucleotide phosphate-oxidase (NAD(P)H oxidase) and the uncoupling of endothelial NO synthase (eNOS) contribute to glomerular ROS production by diabetic rats.[10] This is associated with albuminuria, apparently caused by endothelial dysfunctions that is seen in diabetic Zucker fatty rats.[11] However, to our knowledge, there is little direct evidence

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indicating whether or not endothelial-derived generation of ROS in diabetic rats induces albuminuria with exacerbation of diabetic nephropathy.

In the present study, we hypothesized that activation of endothelial NAD(P)H oxidase, in the presence of diabetes, initiates and worsens the outcome of diabetic nephropathy, in particular, the development of albuminuria. To test this hypothesis, we cross-bred endothelial-targeted NAD(P)H oxidase (Nox) 2 overexpression (NOX2TG) mice[12] with Akita mice.[13] The latter acquire insulin-dependent diabetes mellitus caused by a spontaneous point mutation in *Ins2* gene. We demonstrate here that activation of endothelial NAD(P)H oxidase caused albuminuria and accelerated glomerular damage at an early stage of diabetes in Akita mice, indicating that endothelial-derived ROS exacerbates the progression of diabetic nephropathy.

MATERIALS AND METHODS

Mice

The Animal Research Committee of Kawasaki Medical School approved all the procedures included in the protocols (No. 11-005) that are described in this report. The studies were conducted according to the Guidelines provided by the Care and Use of Laboratory Animals of Kawasaki Medical School, which follows the National Institutes of Health Guide (NIH Publication No. 80-23, revised 1996). Mice were housed in a room with controlled temperature and humidity with a 14–10 h light–dark cycle and provided with unrestricted access to standard laboratory animal chow and tap water. C57BL/6 control (wild-type (WT)) mice and Akita mice (*Ins2*$^{Akita/Ins2+}$, C57BL/6 background) were obtained from Japan SLC (Shizuoka, Japan). One of us (KC) provided NOX2TG mice (C57BL/6 background), which harbor a human *NOX2* transgene that is primarily expressed in the endothelium under the control of the mouse *Tie-2* promoter.[12] We verified the TIE-2 expression in renal glomeruli of transgenic mice that harbors the gene encoding green fluorescent protein, which is expressed under the control of the mouse *Tie-2* promoter (STOCK Tg(TIE2GFP) 287Sato/J; The Jackson Lab, Bar Harbor, ME, USA) (Supplementary Figure S1).

We did not detect significant glomerular structural changes in NOX2TG mice at 30 weeks of age compared with WT mice (Supplementary Figure S2). Male Akita mice were cross-bred with female NOX2TG mice to generate offspring carrying the *NOX2* transgene and Akita traits (NOX2TG-Akita). Experiments were carried out using male mice of four groups: WT, NOX2TG, Akita, and NOX2TG-Akita group. Blood pressure, blood glucose, and urinary albumin excretion were assayed at 6, 8, and 10 weeks after birth. Blood pressure was measured using the tail-cuff method with an automatic sphygmomanometer (BP98A; Softron, Tokyo, Japan). Blood samples were obtained using venipuncture of the tail. Blood glucose was measured using a Terumo Finetouch glucose analyzer (Terumo, Tokyo, Japan). Urine samples were collected 24 h after fasting the mice in metabolic cages. Urinary albumin was quantified using Albuwell M (Exocell, Philadelphia, PA, USA), and it was expressed as the ratio of albumin to creatinine.[14] For pathological and biochemical analyses, mice 6 weeks of age were anesthetized and then killed using sevoflurane inhalation. Certain NOX2TG-Akita mice were treated with a peptide inhibitor (gp91TAT)[15] of NAD(P)H oxidase (10 mg/kg per day, via an osmotic pump) when they were 6–8 weeks old and killed at 8 weeks.

Histopathological Examination

Sections (4 μm thick) were prepared from renal tissue samples embedded in paraffin, and deparaffinized kidney sections were stained with periodic acid-Schiff (PAS) reagent to evaluate glomerular injury. Images of glomeruli were acquired using a BZ-9000 All-in-one Fluorescence Microscope (Keyence, Osaka, Japan). The severity of glomerular injury was evaluated according to a mesangial matrix score from 0 to 4 as described previously.[16] Twenty random glomeruli per mouse were blindly evaluated by a renal pathologist who was not informed of the nature of the samples. Ten mice from each group (WT, NOX2TG, Akita, and NOX2TG-Akita group) were evaluated to calculate the mean score.

Immunohistochemical Studies

Kidney cryostat sections (3 μm thick) were prepared and subjected to immunohistochemical analyses for the expression of plasmalemmal vesicle-1 (PV-1), podocin, desmin, and α-smooth muscle actin (α-SMA), using the primary antibodies as follows: PV-1 (no. sc-50169; Santa Cruz Biotechnology, Santa Cruz, CA, USA), podocin (no. ab50339; Abcam, Cambridge, MA, USA), desmin (no. D33; Dako, Carpinteria, CA, USA), and α-SMA (no. 1A4; Sigma-Aldrich, St Louis, MO, USA). Deparaffinized kidney sections (4 μm thick) were stained with Texas Red-conjugated lectin from *Lycopersicon esculentum* (tomato lectin) (Vector Laboratories, Burlingame, CA, USA). The sections for the expression of PV-1, podocin, desmin, α-SMA, and tomato lectin outlining the podocytes of the glomerular tuft were evaluated to determine the staining score as described previously.[16] Briefly, staining was scored as follows (score, % cells stained): 0, 0–5; 1, 5–25; 2, 25–50; 3, 50–75; and 4, >75. At least 20 glomeruli were randomly selected from each mouse ($n=10$) to calculate the mean score (total >200 glomeruli). The area encompassing the stained glomeruli was measured using Measurement module/Dynamic Cell Count Software (Keyence).

Isolation of Glomeruli

The glomeruli were isolated by taking advantage of their preferential uptake of Dynabeads (M-450 Tosylactivated; Life Technologies Japan, Tokyo, Japan) as described previously[17] and processed for RNA extraction, measurements of superoxide levels, and NAD(P)H oxidase activity.

Table 1 Analysis of blood glucose concentrations

Group	Blood glucose (mM)		
	6 weeks of age	8 weeks of age	10 weeks of age
WT	9.0 ± 1.9	9.1 ± 1.6	8.7 ± 2.3
NOX2TG	9.4 ± 1.6	9.4 ± 1.6	9.6 ± 1.4
Akita	34.3 ± 4.9[a]	38.9 ± 5.7[a]	46.0 ± 6.8[a]
NOX2TG-Akita	30.0 ± 5.5[a]	40.7 ± 6.4[a]	45.4 ± 8.3[a]

Data are expressed as the mean ± s.e.m., 10 mice per group.
[a]$P < 0.05$ *versus* the respective WT.

Analysis of Superoxide Production

Superoxide production in glomeruli was detected using 2′,7′-dichlorofluorescein (DCF).[18] The isolated glomeruli from each group of mice were incubated for 10 min with RPMI-1640 containing 20 μmol/l DCF diacetate (Molecular Probes, Eugene, OR, USA). Images were obtained using a confocal laser microscope (Leica Microsystems, Tokyo, Japan) at excitation/emission wavelengths of 485/535 nm for DCF. The fluorescence intensity values of 20 different glomeruli isolated from each group were calculated using Leica TCS-NT software (Leica Microsystems), and the mean values are presented.[19]

Real-Time Quantitative PCR

Total RNA was isolated from the glomeruli using TRIzol (Life Technologies Japan). Reverse transcriptase reactions were performed using a Ready-To-Go T-Primed First-Strand Kit (GE Healthcare Japan, Tokyo, Japan) for first-strand cDNA synthesis. Real-time quantitative PCR was performed using the ABI Prism 7700 sequence detection system (Life Technologies Japan). Data were expressed as copy number relative to that of 18 S rRNA. Primers and probes for *Taq*Man analysis of human *NOX2*, mouse *Nox2*, mouse *Nox4*, mouse *P22*, mouse *P47*, and mouse *P67* are included in Supplementary Table S1. TaqMan probes consist of a fluorophore 6-carboxyfluorescein covalently attached to the 5′ end of the oligonucleotide probe and a quencher tetramethylrhodamine at the 3′ end.

Lucigenin Chemiluminescence Assay of NAD(P)H Oxidase Activity

NAD(P)H oxidase activity of glomeruli was measured using lucigenin chemiluminescence (units/min/mg) as described previously.[16]

Scanning and Transmission Electron Microscopy

For transmission electron microscopy, small fragments of the left kidneys were fixed by immersion fixation in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 3 h. Afterwards, kidneys were treated with 1% OsO_4 in 0.1 M cacodylate buffer for 1.5 h, dehydrated with graded ethanol solutions, and embedded in Spurr's low-viscosity resin (Electron Microscopy Sciences, Hatfield, PA, USA). Semithin sections were stained with toluidine blue stain. Tissues were sectioned using an Ultracut UCT Microtome (Leica Microsystems) equipped with a diamond knife, mounted on copper grids coated with a formvar film, and stained with uranyl acetate and lead citrate. Ultrathin sections were analyzed on a transmission electron microscope (Hitachi H-7100 Electron Microscope; Hitachi, Tokyo, Japan).

For scanning electron microscopy, kidney samples were fixed by immersion in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 3 h. After treatment with 1% OsO_4 in 0.1 M cacodylate buffer for 1.5 h, the samples were dehydrated with graded ethanol solutions and immersed in isoamyl acetate solution for 30 min. The kidney samples were dehydrated by critical-point drying with liquefied carbon dioxide. Dried samples were sputter-coated with platinum and examined with a scanning electron microscope (Hitachi S-3400 N electron microscope; Hitachi).

In Vivo Imaging of Macromolecule Filtration

A Laser Scanning Spectral Confocal Microscope (TCS SP2 AOBS MP; Leica Microsystems) was used for *in vivo* imaging of glomerular microcirculation as described previously.[20] For analysis of glomerular permeability, a catheter was used to infuse 70-kDa fluorescein isothiocyanate (FITC)-conjugated dextran (anionic; excitation 494 nm, emission 518 nm; Molecular Probes) through the jugular vein.

Statistical Analysis

Data are expressed as mean ± s.e.m. Differences between groups were assessed for statistical significance using a two-tailed unpaired Student's *t*-test for paired data or one-way analysis of variance for multiple groups. Statistical significance was defined as $P < 0.05$.

RESULTS

Physiological Characterization of Mice

The mean blood glucose concentration of the Akita group was significantly higher compared with that of the WT group and was similar to that of the NOX2TG-Akita group (Table 1). There was no significant difference in mean blood pressure among the groups (Table 2). The NOX2TG-Akita and Akita groups developed albuminuria at 6 and 10 weeks of age, respectively (Figure 1). The NOX2TG groups showed no difference with WT group in blood glucose, blood pressure, and albuminuria.

NAD(P)H Oxidase Expression and Activity

The expression of human *NOX2* mRNA was detected in the glomeruli of only the NOXTG-Akita group only (Supplementary Figure S3a), whereas the mean expression level of mouse *Nox2* mRNA did not differ among the groups (Supplementary Figure S3b). Expression of mRNAs encoding

Table 2 Analysis of systolic blood pressure

Group	Systolic blood pressure (mmHg)		
	6 weeks of age	8 weeks of age	10 weeks of age
WT	101 ± 8	108 ± 10	115 ± 12
NOX2TG	112 ± 8	110 ± 9	110 ± 8
Akita	110 ± 11	111 ± 10	116 ± 13
NOX2TG-Akita	109 ± 9	112 ± 8	116 ± 11

Data are expressed as the mean ± s.e.m., 10 mice per group.

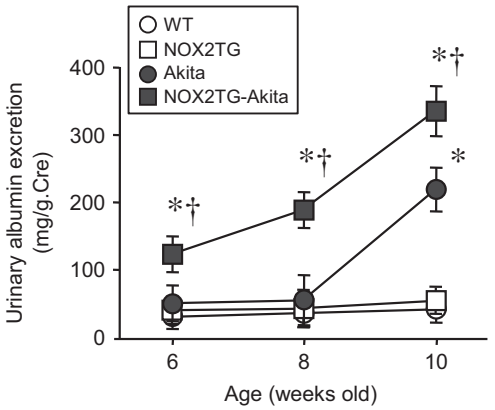


Figure 1 Analysis of urinary albumin excretion of wild-type mice (WT, open circles), endothelial dominant *Nox2* transgenic mice (NOX2TG; open squares), heterozygous Akita mice ($Ins2^{Akita/Ins2+}$, black solid circles), and heterozygous Akita mice ($Ins2^{Akita/Ins2+}$) expressing a *Nox2* transgene (NOX2TG-Akita, black solid squares), 10 mice per group. *$P<0.05$ *versus* WT group; †$P<0.05$ *versus* Akita group.

NAD(P)H oxidase components did not differ between the Akita and NOX2TG-Akita groups (Supplementary Figures S3c, e, and f), except for that of p22phox, which was increased in the Akita and NOX2TG-Akita groups compared with that of the WT group (Supplementary Figure S3d). The highest level of NAD(P)H oxidase activity was detected in the glomeruli of the NOX2TG-Akita group followed in decreasing order by the Akita and WT groups (Supplementary Figure S3g).

Glomerular Production of Superoxide

The production of superoxide in glomeruli was determined by staining isolated glomeruli of mice of each group at 6 weeks of age (Figure 2a). The mean fluorescence intensity of the isolated glomeruli of the NOX2TG-Akita group was significantly higher compared with that of the Akita group (Figure 2b).

Morphology of Glomeruli

PAS staining did not reveal morphological differences between the glomeruli of the WT and NOXTG groups. Akita group showed a slight increase in mesangial matrix deposition (Figure 2c). Mild diffuse expansion of mesangium and a mild increase in mesangial cellularity were observed in NOX2TG-Akita group. The PAS-positive area (mean matrix score) was significantly larger in the Akita and NOX2TG-Akita groups compared with that of the WT group (Figures 2c and d), and the mean matrix score of the NOX2TG-Akita group was significantly higher compared with that of the Akita group.

Characteristics of Glomerular Endothelial Cells

The induction of PV-1 expression coincided with the development of glomerular capillary perturbations and endothelial structural damage.[21] PV-1 was detected in the glomeruli of only the NOX2TG-Akita group at 6 weeks of age (Figures 3a and b). The glomerular glycocalyx, which is a main component of endothelial surface layer (ESL), was evaluated by lectin staining. We used tomato lectin to label *N*-acetyl glucosamine, which residues are present in the surface-bound glycocalyx region.[22] Tomato lectin was detected in the glomeruli of the Akita group (Figure 3c), and the area stained by tomato lectin on the capillary wall was significantly smaller in the NOX2TG-Akita group compared with that of the Akita group (Figure 3d). Figures 3e and f shows representative scanning electron micrographs and transmission electron micrographs of glomerular capillary endothelia, respectively. The Akita group showed endothelial cell activation in the form of an increased number of slightly swollen blebs; however, most of the capillary fenestrae and the ridges remained intact (Figure 3f, upper). In contrast, the NOX2TG-Akita group showed more exuberant activation of endothelial cells, as reflected by the appearance of numerous large numbers of swollen blebs, microparticles and the partial effacement of capillary fenestrae and ridges (Figure 3f, lower).

Glomerular Filtration

Glomerular filtration was determined using intravenous injection of 70-kDa FITC-conjugated dextran[11,20] that is poorly filtered by ESL-preserved endothelium. Leakage of the FITC-labeled dextran into the Bowman's capsule space was undetectable in WT, whereas it was slight and massive in the Akita and NOX2TG-Akita groups, respectively (Figure 4 and Supplementary Movies S1). The leakage was readily appreciated in Supplementary Movies S1, suggesting attenuation of ESL.

Mesangial Cells

Mesangial cells in diverse glomerular diseases become myofibroblast-like, characterized by activation of α-SMA expression.[23] Increased α-SMA expression in mesangial cells reflects cellular hypertrophy.[24] We analyzed the expression pattern of α-SMA to assess the phenotypic changes and hypertrophy in mesangial cells (Figure 5a). Immunostaining of α-SMA showed that it was distributed over the largest area

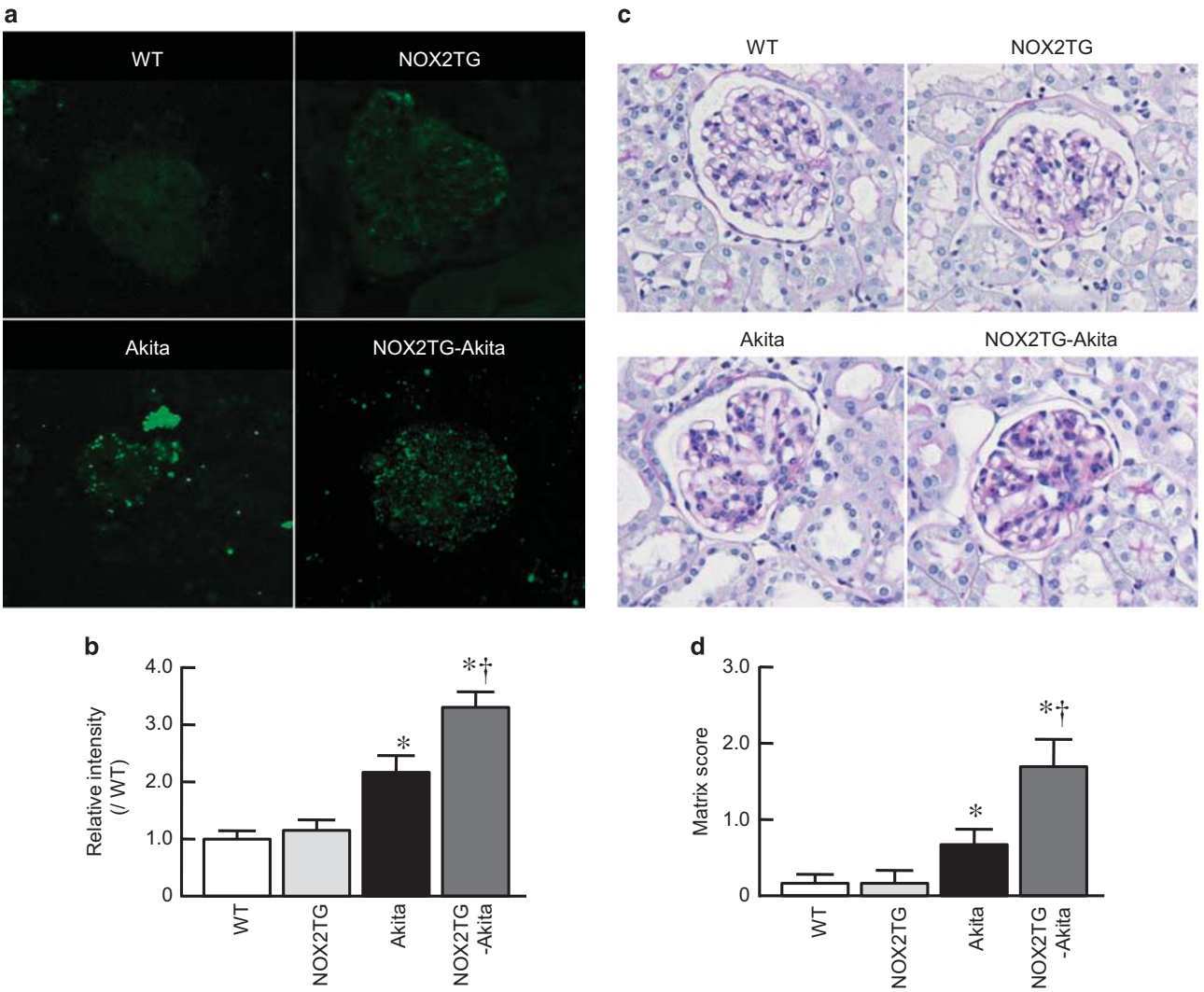


Figure 2 Glomerular superoxide production and morphological changes in glomeruli of mice from various strains at 6 weeks of age. (**a**) Reactive oxygen species (ROS) detected by staining with 2′,7′-dichlorofluorescein (DCF) in isolated glomeruli. Magnification ×400. (**b**) Intensity of DCF staining relative to WT, 10 mice per group. Twenty glomeruli from each mouse were evaluated to calculate the mean intensity. *$P<0.05$ *versus* WT group; †$P<0.05$ *versus* Akita group. (**c**) Periodic acid-Schiff (PAS) staining images of glomeruli. Magnification ×400. (**d**) Glomerular matrix score, 10 mice per group. Twenty glomeruli from each mouse were evaluated to calculate the mean score was calculated. *$P<0.05$ *versus* WT group; †$P<0.05$ *versus* Akita group. NOX2TG, endothelial dominant *Nox2* transgenic mice; NOX2TG-Akita, heterozygous Akita mice (*Ins2*$^{Akita/Ins2+}$) expressing a *Nox2* transgene; WT, wild-type mice.

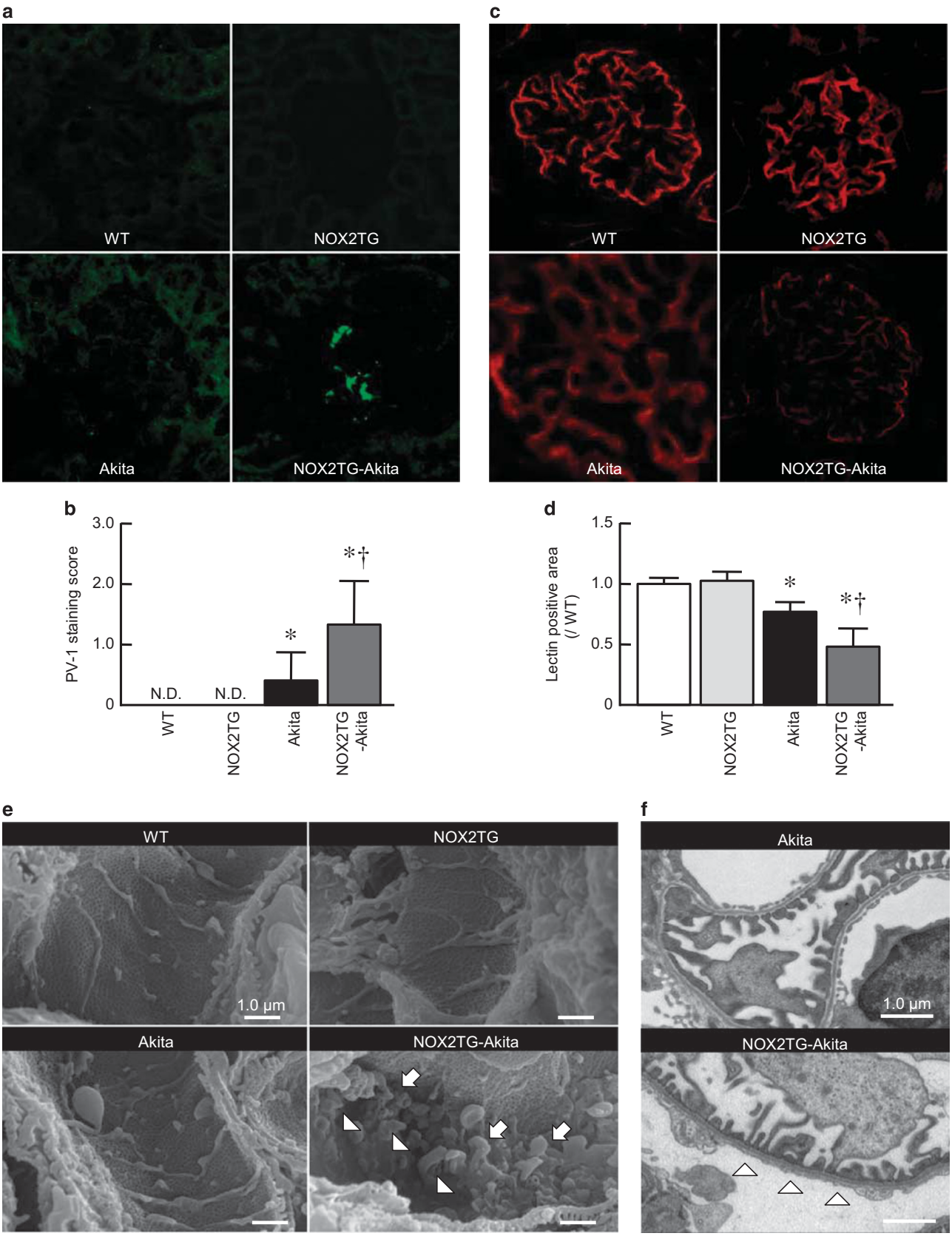
of the glomeruli of the NOX2TG-Akita group compared with those of the other three groups (Figure 5b). The greatest expansion of the mesangial area occurred in the NOX2T-Akita group (Figure 5c).

Podocytes

Immunohistochemical analysis detected desmin, a marker of podocyte damage,[25] on the capillaries of the NOX2TG-Akita group (Figure 6a) and occupied an area greater than that of the Akita group (Figures 6a and b). Scanning electron microscopy revealed that podocytes were swollen and showed increased microvilli formation in the NOX2TG-Akita group (Figure 6c). Immunohistochemical analyses revealed that podocin, a slit membrane protein, was present in the capillaries of each group (Supplementary Figure S4a), and there were no significant differences for the area occupied by podocin among the four groups (Supplementary Figure S4b). Interestingly, transmission electron micrographs of the glomerular capillary walls did not reveal podocyte foot-process effacement in the Akita and NOX2T-Akita groups (Supplementary Figure S4c).

Inhibiting NOX2 Activity Repairs the Glycocalyx of NOX2TG-Akita Mice

Treatment of NOX2TG-Akita mice at 6–8 weeks of age with the NOX2-specific inhibitor gp91TAT led to recovery of


a
WT
NOX2TG
Akita
NOX2TG-Akita
c
WT
NOX2TG
Akita
NOX2TG-Akita
b
PV-1 staining score
3.0
2.0
1.0
0
N.D.
N.D.
*
*†
WT
NOX2TG
Akita
NOX2TG -Akita
d
Lectin positive area (/ WT)
1.5
1.0
0.5
0
*
*†
WT
NOX2TG
Akita
NOX2TG -Akita
e
WT
NOX2TG
1.0 μm
Akita
NOX2TG-Akita
f
Akita
1.0 μm
NOX2TG-Akita

tomato lectin staining of the glomeruli and it was similar to that of WT mice (Figures 7a and b). The staining was not detected in untreated mice. The recovery of lectin staining of glomeruli was associated with a significant reduction in the level of albuminuria in NOX2TG-Akita mice, although blood glucose or blood pressure were not altered (Table 3).

DISCUSSION

The data presented here support the hypothesis that enhanced endothelial oxidative stress promotes the onset and progression of diabetic nephropathy in mice. Albuminuria appeared in mice 6 and 10 weeks of age of the NOX2TG-Akita and Akita groups, respectively. Moreover, the level of ROS generated by NAD(P)H oxidase increased in NOX2TG-Akita mice compared with Akita mice, and caused glomerular microabnormalities. In endothelial cells, decreases in the thickness of the ESL, which apparently has a role in glomerular permeability, and it was greater in NOX2TG-Akita mice. Podocyte activation and mesangial cell matrix expansion were increased in NOX2TG-Akita mice, consistent with increased oxidative stress in endothelial cells. These results indicate that activation of endothelial NAD(P)H oxidase, in particular, accelerated the development of diabetic nephropathy.

Vascular NAD(P)H oxidase, mitochondrial dysfunction, and uncoupled eNOS all the three contribute to ROS generation in diabetes as well as in its microvascular complications, for example, diabetic nephropathy. For example, Gorin *et al.*[26] detected an increase in NAD(P)H-dependent ROS generation in the kidney cortex and isolated glomeruli of diabetic rats. In rats with diabetes induced by streptozotocin (STZ), administration of the NAD(P)H oxidase inhibitor apocynin leads to a reduction in renal ROS and may have a potent therapeutic effect on diabetic nephropathy.[27] Deletion of p47 phox[28] or NOX4 (ref. 29) attenuates the progression of diabetic nephropathy. Similarly, we reported that NAD(P)H oxidase activity and uncoupling of eNOS contribute to ROS production in the glomeruli of rats with diabetic nephropathy.[10,30,31] Prolonged NAD(P)H oxidase activation may lead to depletion of intracellular NAD(P)H and impaired ROS scavenging associated with eNOS uncoupling, mitochondrial dysfunction, and diminished defense against antioxidants.[32] Therefore, prolonged

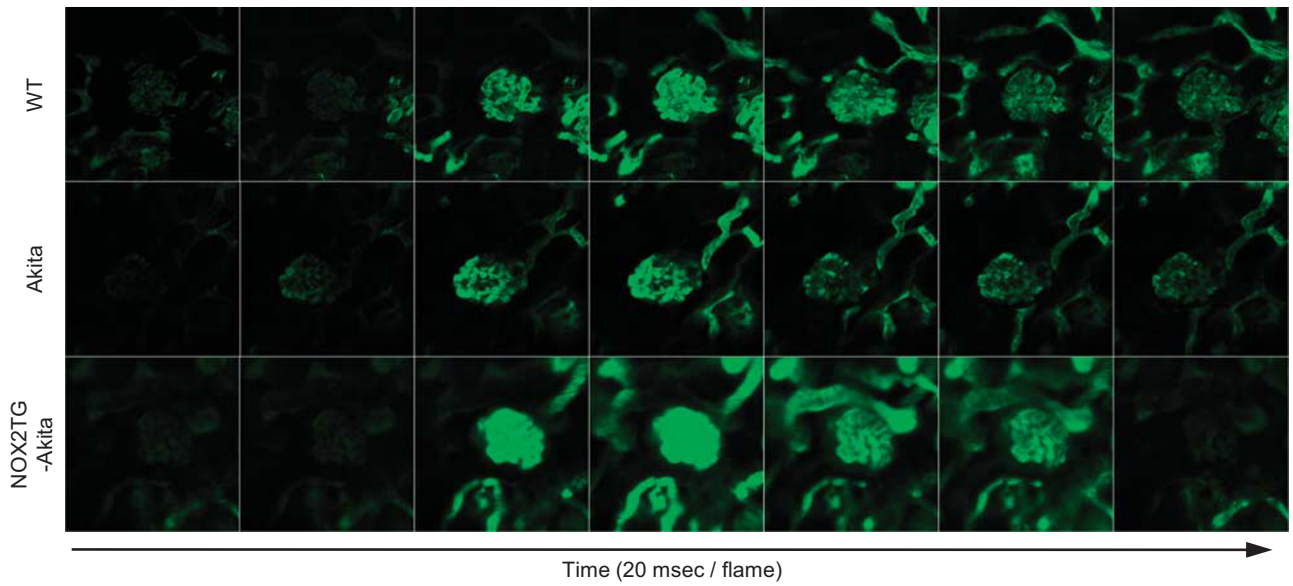


Figure 4 Analysis of glomerular filtration in mice 6 weeks of age. Representative series of images showing filtration of macromolecules in the glomeruli of the indicated mouse group. Green color indicates the localization of 70-kDa fluorescein isothiocyanate (FITC)-labeled dextran. Magnification ×200.

Figure 3 Evaluation of glomerular endothelial injury and morphological changes in mice from various groups at 6 weeks of age. (**a**) Immunohistochemical analysis of plasmalemmal vesicle-1 (PV-1), a marker of glomerular capillary remodeling. Magnification ×400. (**b**) PV-1 staining score, 10 mice per group. Ten glomeruli from each mouse were evaluated to calculate the mean score. $^{*}P<0.05$ *versus* WT group; $^{\dagger}P<0.05$ *versus* Akita group. (**c**) Assessment of glycocalyx of the endothelial surface layer detected by using tomato lectin staining. Magnification ×400. (**d**) Lectin-positive areas compared with WT, 10 mice per group. Ten glomeruli from each mouse were evaluated to calculate the mean area. $^{*}P<0.05$ *versus* WT group; $^{\dagger}P<0.05$ *versus* Akita group. (**e**) Scanning electron micrographs of activated and swollen endothelial cells (arrows) of NOX2TG-Akita mice. Note the partial occlusion and disappearance of fenestrae (arrowheads). Scale bar = 1.0 μm. (**f**) Transmission electron micrographs of glomerular capillary endothelia. Note the partial disappearance of fenestrae in NOX2TG-Akita mice (arrowheads). Scale bar = 1.0 μm. ND, not detected; NOX2TG, endothelial dominant *Nox2* transgenic mice; NOX2TG-Akita, heterozygous Akita mice ($Ins2^{Akita/Ins2+}$) expressing a *Nox2* transgene; WT, wild type.

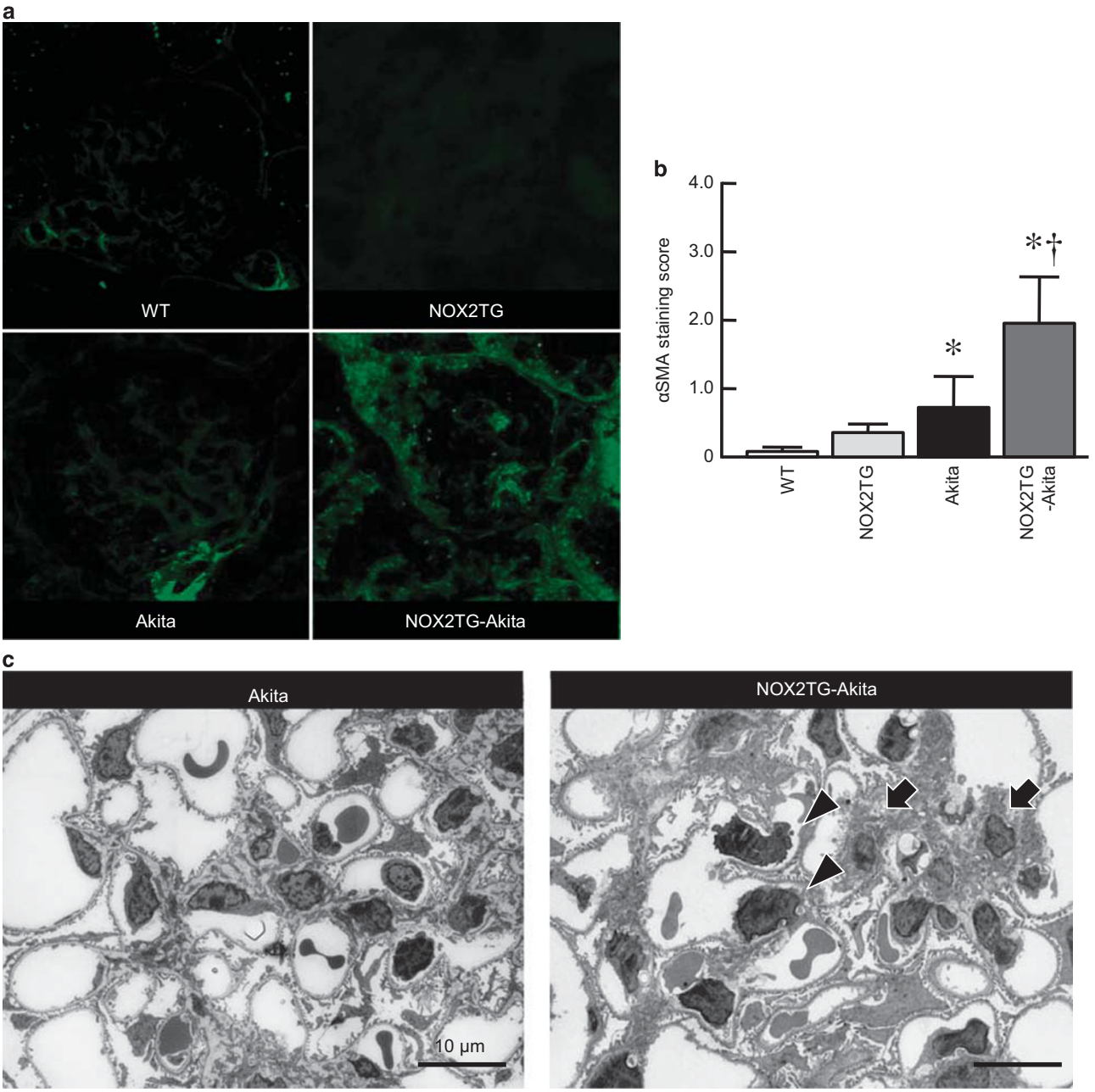


Figure 5 Analyses of mesangial lesions in various groups of mice at 6 weeks of age. (**a**) Immunohistochemical analysis of *α*-smooth muscle actin (*α*-SMA) expression in glomeruli. Magnification ×400. (**b**) *α*-SMA staining score, 10 mice per group. Ten glomeruli from each mouse were evaluated to calculate the mean score. *$P<0.05$ *versus* WT group; $^{\dagger}P<0.05$ *versus* Akita group. (**c**) Electron micrographs of representative glomeruli from Akita and NOX2TG-Akita mice showing notable expansion of the mesangium in the latter group (arrows). Swollen endothelial cells are indicated by the arrowheads. Scale bar = 10 *μ*m. NOX2TG, endothelial dominant *Nox2* transgenic mice; NOX2TG-Akita, heterozygous Akita mice ($Ins2^{Akita/Ins2+}$) expressing a *Nox2* transgene; WT, wild type.

ROS production by endothelial cells may participate in the onset and progression of diabetic nephropathy.

Enhanced production of endothelial ROS and high glucose concentration induce several structural changes in endothelial cells.[33,34] Endothelial dysfunction is associated with the expression of PV-1, a marker of endothelial structural damage and remodeling.[21] PV-1 is not expressed in the mature glomerular endothelial cells but is re-expressed in Thy1.1 nephritis, presumably reflecting a process of restorative remodeling of the glomerular capillary tuft after injury.[21] Although the exact functions of PV-1 are not clear, evidence suggests that it is involved in the control of capillary permeability by acting on the capillary wall[35] and may have a role in the development of diabetic nephropathy and

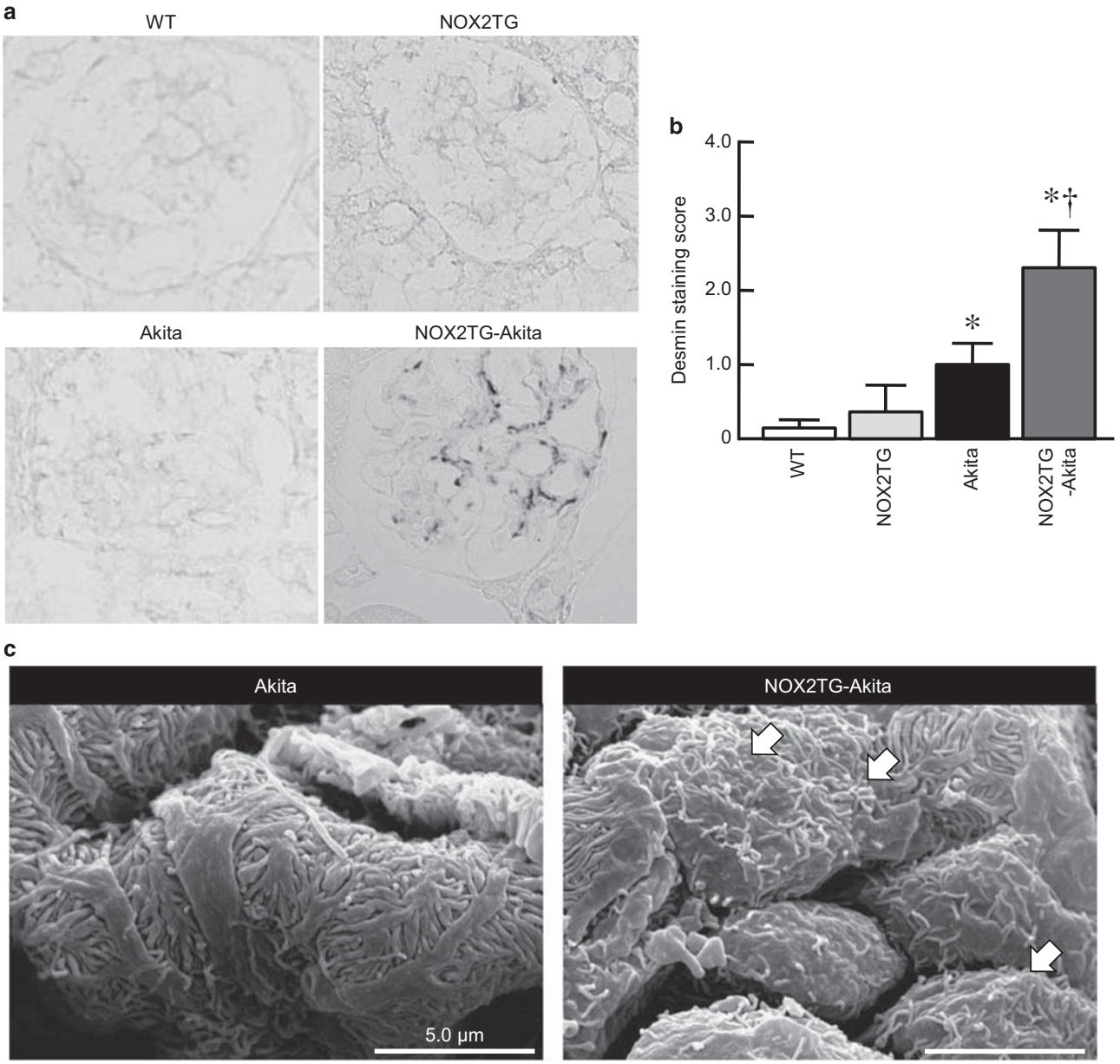


Figure 6 Analysis of podocyte injury and morphological changes in mice at 6 weeks of age. (**a**) Immunohistochemical analysis of desmin expression in glomeruli, which is tremendously increased in NOX2TG-Akita mice group. Magnification ×400. (**b**) Desmin staining score, 10 mice per group. Ten glomeruli from each mouse were evaluated to calculate the mean score. *$P<0.05$ *versus* WT group; $^{\dagger}P<0.05$ *versus* Akita group. (**c**) Electron micrographs depicting ultrastructural changes in podocytes of Akita and NOX2TG-Akita mice. Numerous microvilli-like structures in the podocytes are readily seen (arrows). Scale bar = 5.0 μm. NOX2TG, endothelial dominant *Nox2* transgenic mice; NOX2TG-Akita, heterozygous Akita mice (*Ins2*$^{Akita/Ins2+}$) expressing a *Nox2* transgene; WT, wild type.

albuminuria. In the present study, we detected significantly higher levels of PV-1 on the endothelial cells of NOX2TG-Akita mice compared with those of Akita, NOXT2G, and WT mice.

Consistent with these findings are our observations of hypertrophy and activation of endothelial cells in NOX2TG-Akita mice. Blebs were formed on the membranes of activated or injured endothelial cells, which has been also described to be associated with an increased number of microparticles released into the blood stream.[36] Further, endothelial microparticles from glucose-treated endothelial cells have been reported to increase vascular inflammation and impair endothelial functions by promoting the activation of endothelium.[37] These findings reinforce the idea that the generation of ROS by NAD(P)H oxidase activates the endothelium.

The glomerular endothelium is coated with a polysaccharide-rich layer composed primarily of glycocalyx

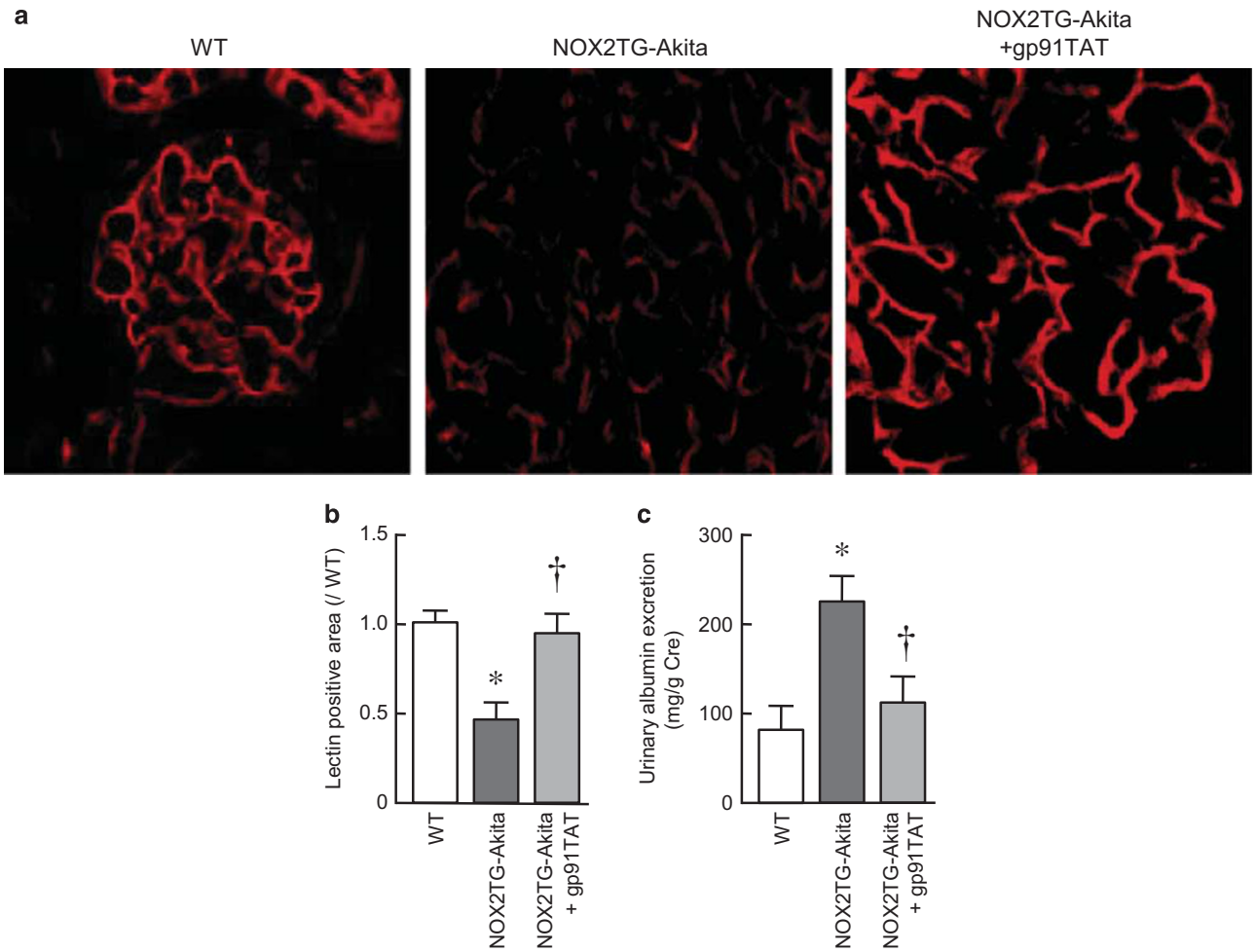


Figure 7 Analysis of the glomerular glycocalyx and urinary albumin excretion in mice NOX2TG-Akita treated with the NOX2 inhibitor gp91TAT. (**a**) Glycocalyx was detected using tomato lectin. Magnification ×400. (**b**) Relative lectin-positive area to WT. Treatment with gp91TAT led to the recovery of the lectin-stained area, 10 mice per group. Ten glomeruli from each mouse were evaluated to calculate the mean area. (**c**) Urinary albumin excretion. Cre, urinary creatinine. *$P<0.05$ *versus* WT group; $^{\dagger}P<0.05$. NOX2TG, endothelial dominant *Nox2* transgenic mice; NOX2TG-Akita, heterozygous Akita mice (*Ins2*$^{Akita/Ins2+}$) expressing a *Nox2* transgene; WT, wild type.

and a loosely attached endothelial cell coat of secreted proteoglycans, glycosaminoglycans, glycoproteins, and plasma proteins, and it is referred to as the ESL.[22,38,39] Reduced thickness of the ESL is associated with increased vascular permeability.[40] We reported previously that ROS-induced glomerular ESL perturbation coincides with increased glomerular vascular permeability and albuminuria in a rat model of metabolic syndrome.[11] Here, we demonstrate that in NOXTG-Akita mice activation of endothelial NAD(P)H oxidase enhanced the reduction of the ESL while increasing the glomerular capillary permeability.

Excessive ROS production activates the transcription of *heparanase* via nuclear translocation of E-twenty six transcription factor.[41] Heparanase has been shown to have a role in the development of proteinuria through the degradation of endothelial glycocalyx enriched with heparan sulfate proteoglycans.[37] Therefore, the reduction of endothelial ROS may mitigate the pathophysiological changes observed in diabetic nephropathy through the downregulation of *heparanase* transcription. In fact, our data show that an NOX2 inhibitor has therapeutic potential, which may have apparently prevented the reduction of glycocalyx on glomerular capillary in rats with experimental diabetes.

In addition to endothelial cell injury, we also show here that overproduction of endothelial ROS-induced mesangial injury in Akita mice with diabetes. Endothelial cells are located adjacent to mesangial cells, and they seem to have direct contact with one another.[42,43] Proliferation of mesangial cells and excessive production of extracellular matrix by mesangial cells are typical findings in the progression of glomerular injury,[44] and mesangial cells undergo phenotypic changes in patients with diabetic nephropathy and in rats with STZ-induced diabetes.[45] Such changes may be partly associated with the activation of endothelial NAD(P)H oxidase and accumulation of ROS. R ROS generated by NAD(P)H oxidases downregulate subfamily C member of

Table 3 Analysis of physiological parameters of NOX2TG mice treated with gp91tat

Parameter	WT	NOX2TG-Akita	NOX2TG-Akita treated with gp91TAT
SBP (mmHg)	115 ± 12	116 ± 13	116 ± 11
BG (mg/dl)	8.6 ± 2.3	34.9 ± 6.8[a]	34.3 ± 8.3[a]

BG, blood glucose; BW, body weight; SBP, systolic blood pressure.
Data are expressed as the mean ± s.e.m., six mice per group.
[a]$P < 0.05$ *versus* WT.

transient receptor potential cation channel in mesangial cells of rats with STZ-induced diabetes.[46] This may explain the alterations in mesangial contractile functions observed in diabetic nephropathy. Thus, NAD(P)H oxidase activation associated with endothelial dysfunction may also lead to induction of phenotypic changes in mesangial cells in the early stages of diabetic nephropathy.

Besides glomerular endothelial and mesangial changes, we also demonstrate that enhanced endothelial ROS production induced podocyte injury in Akita mice with diabetes. In diabetic kidney disease, podocytes and glomerular endothelial cells appear to participate in the initiation and progression of nephropathy.[47–50] Integrated functions of glomerular endothelial cells and podocytes are essential to maintain normal homeostasis of glomerular capillaries.[51] Podocytes maintain the structure of glomerular endothelial cells via the production of vascular endothelial growth factor (VEGF).[52] Moreover, the structure of glomerular endothelial cells is abnormal in podocyte-specific VEGF-deficient mice.[53] In humans with diabetic nephropathy, podocyte loss is associated with reduced endothelial cell fenestration.[47,48] In contrast, diabetic mice with a genetic deficiency of eNOS develop podocyte-specific injury and heavy albuminuria,[54] and glomerular endothelial mitochondrial oxidative stress determines podocyte depletion in segmental glomerulosclerosis in transgenic mice.[55] In view of the above considerations it seems that podocyte–endothelial crosstalk presents new therapeutic opportunities, including the use of endothelial ROS scavengers for the amelioration of diabetic nephropathy.

The observations in this study support the notion that increased endothelial NOX2 sensitizes the kidney to diabetic injury because accelerated renal injury is detected as early as 6 weeks of age in NOX2TG-Akita mice. We also investigated whether endothelial NOX2 transgenic mice showed early progression to advanced diabetic nephropathy. We followed Akita and NOX2TG-Akita mice until 20 weeks. However, the pathological differences between two groups unfortunately disappeared (data not shown). The NOX2 expression was almost same in both Akita and NOX2TG-Akita mice at 20 weeks. High glucose levels in Akita mice might induce the elevation of endothelial Nox2 expression and result in the same degree of diabetic nephropathy lesion. Hence, we cannot make a conclusion on the role of Nox2 in the late phase of diabetic nephropathy, but we believe that our data are important to understand diabetic nephropathy onset because the mechanism of the initiation of diabetic nephropathy has also still been unclear.

In conclusion, it seems that activation of endothelial NAD(P)H oxidase accelerates the initiation as well as the progression of diabetic nephropathy. The present findings highlight the importance of the interactions among endothelial cells, podocytes, and mesangial cells and suggest their importance in the modulation of normal and abnormal endothelial functions. Overall, it is safe to conclude that endothelial dysfunctions associated with increased endothelial ROS production by NAD(P)H oxidase is an important step in the pathogenesis of diabetic nephropathy and this may serve as a potential therapeutic target for the onset of diabetic nephropathy.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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