Nephropathy and Elevated BP in Mice with Podocyte-Specific NADPH Oxidase 5 Expression

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ABSTRACT
NADPH oxidase (Nox) enzymes are a significant source of reactive oxygen species, which contribute to glomerular podocyte dysfunction. Although studies have implicated Nox1, -2, and -4 in several glomerulopathies, including diabetic nephropathy, little is known regarding the role of Nox5 in this context. We examined Nox5 expression and regulation in kidney biopsies from diabetic patients, cultured human podocytes, and a novel mouse model. Nox5 expression increased in human diabetic glomeruli compared with nondiabetic glomeruli. Stimulation with angiotensin II upregulated Nox5 expression in human podocyte cultures and increased reactive oxygen species generation. siRNA-mediated Nox5 knockdown inhibited angiotensin II–stimulated production of reactive oxygen species and altered podocyte cytoskeletal dynamics, resulting in a Rac-mediated motile phenotype. Because the Nox5 gene is absent in rodents, we generated transgenic mice expressing human Nox5 in a podocyte-specific manner (Nox5pod+). Nox5pod+ mice exhibited early onset albuminuria, podocyte foot process effacement, and elevated systolic BP. Subjecting Nox5pod+ mice to streptozotocin-induced diabetes further exacerbated these changes. Our data show that renal Nox5 is upregulated in human diabetic nephropathy and may alter filtration barrier function and systolic BP through the production of reactive oxygen species. These findings provide the first evidence that podocyte Nox5 has an important role in impaired renal function and hypertension.


Albuminuria is a clinical marker of kidney dysfunction that arises in most glomerulopathies and is associated with poor prognoses for ESRD, hypertension, and cardiovascular mortality. Changes to the podocyte (e.g., foot process effacement, hypertrophy, detachment, and loss) underlie the development and progression of albuminuria and thereby highlight the critical role for these cells in upholding the glomerular filtration barrier.1,2 Therefore, identifying factors that induce podocyte injury and loss is essential to understanding the mechanisms of filtration barrier dysfunction. Of the many factors implicated in podocyte dysfunction, excessive production of reactive oxygen species (ROS; oxidative stress) may be particularly important.3–5 Although sources of ROS are numerous, the NADPH oxidase (Nox) family of enzymes yields significant superoxide production in the

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Nox5 is expressed in human podocytes

Consistent with a potential role in diabetic nephropathy, immunodetectable Nox5 was observed in glomeruli from diabetic renal biopsies but not nondiabetic samples (Figure 1B). Furthermore, Nox5 did not colocalize with TGF-β, which is produced mainly by mesangial cells in diabetic glomeruli, thereby suggesting that Nox5 induction was primarily in podocytes. Immunofluorescent localization with the podocyte marker nephrin was consistent with Nox5 expression in podocytes of diabetic glomeruli (Figure 1B). Nox4 expression was apparent in both nondiabetic and diabetic glomeruli, with greater expression observed in the latter (Supplemental Figure 1). In contrast, low levels of Nox1 and -2 were observed in glomeruli, with little difference in expression between diabetic and nondiabetic samples (Supplemental Figure 1). RT-PCR of RNA from conditionally-immortalized human podocytes (hPODs) revealed ample Nox5 transcript (Figure 1C). Nox1 and -4 were also detected in hPODs along with minimal Nox2 expression (Figure 1C). Examination of Nox5 splice variants by RT-PCR identified Nox5β as the predominant species in hPODs (Figure 1D). Likewise, Nox5β was the principal variant detected in human embryonic kidney cells and human proximal tubule epithelial cells. Low levels of Nox5α were detected in human embryonic kidney cells and proximal tubule epithelial cells but not hPODs, whereas Nox5β and -γ were absent from all cell lines examined (Figure 1D).

Podocyte Nox5 is induced and activated by factors in the diabetic milieu

Previous reports showed increased ROS production in podocytes exposed to high glucose (HG). Furthermore, because podocytes express AT1Rs, AngII may induce Nox5 expression and activity in human podocytes. As determined by quantitative RT-PCR, Nox5 mRNA expression was significantly increased in response to AngII at both 2 and 8 hours (Figure 1E). Stimulation with HG media for 2 hours failed to increase Nox5 mRNA; however, 10 hours after stimulation, Nox5 mRNA was apparent in both nondiabetic and diabetic glomeruli, with little difference in expression between diabetic and nondiabetic samples (Supplemental Figure 1). RT-PCR of RNA from conditionally-immortalized human podocytes (hPODs) revealed ample Nox5 transcript (Figure 1C). Nox1 and -4 were also detected in hPODs along with minimal Nox2 expression (Figure 1C). Examination of Nox5 splice variants by RT-PCR identified Nox5β as the predominant species in hPODs (Figure 1D). Likewise, Nox5β was the principal variant detected in human embryonic kidney cells and human proximal tubule epithelial cells. Low levels of Nox5α were detected in human embryonic kidney cells and proximal tubule epithelial cells but not hPODs, whereas Nox5β and -γ were absent from all cell lines examined (Figure 1D).

To assess whether AngII-dependent increases in Nox5 protein expression resulted in a corresponding increase in ROS, superoxide production was assayed using lucigenin-based fluorescence. Unstimulated and vehicle-treated controls
AngII induced a significant increase in ROS production at all time points assayed (Figure 2A). Similarly, HG induced a significant increase in ROS at 10 hours (Figure 2B). Although not statistically significant compared with AngII or HG alone, costimulation with HG+AngII increased ROS production in an additive manner, with an increase of 167.7±9.0% of control (Figure 2B). To determine if AngII-mediated ROS production was Nox5-dependent, small interfering RNA (siRNA) oligonucleotides were used to knock down expression of either Nox5 or p22phox, a subunit required for activity of Nox1, -2, -3, and -4. Knockdown of Nox5 and p22phox was confirmed by Western blot (Figure 2D). Nox5 knockdown significantly blunted ROS production in response to AngII (91.2±4.2% of control), whereas scrambled control oligonucleotides (scrs) were without effect (126.4±20.2% for scr versus 121.1±9.6% for control) (Figure 2C); p22phox knockdown did not affect AngII-stimulated ROS production (125.3±10.0% for p22phox versus 126.4±20.2% for scr), suggesting that Nox5 activity predominates over other Nox isoforms in this context (Figure 2C).

Heterologous Nox5 Expression Generates ROS and Alters Cytoskeletal Dynamics

To examine the role of Nox5 in podocyte function in an animal model, transgenic mice expressing Nox5 in a podocyte-specific manner were generated. As proof of principle for this approach, conditionally immortalized mouse podocytes (mPODs) were transduced with an adenovirus (ad) to express Nox5<sup>b</sup>. Nox5 expression was verified by Western blotting and immunofluorescence (Figure 2, E and F). Cells infected with adNox5<sup>b</sup> displayed greater basal ROS production than adGFP-infected cells (116.9±3.2% of control for adNox5<sup>b</sup> versus 87.5±5.4% of control for adGFP) (Figure 2G). Furthermore, ROS generation in mPODs expressing adNox5<sup>b</sup> was responsive to AngII (148.3±16.9% of control) in a manner similar to human podocytes (Figure 2G).

adNox5<sup>b</sup>-infected mPODs exhibited reduced surface area, more numerous cellular projections, and lamellipodia compared with uninfected and adGFP-infected cells (Figure 3A). Staining with fluorescein-conjugated phalloidin revealed a reduction in stress fibers and greater numbers of peripheral actin aggregates in Nox5-expressing cells (Figure 3, A–H). Incubation of Nox5-expressing mPODs with the Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) inhibitor KN-93, which is known to reduce Nox5 activity, and the broad spectrum Nox inhibitor displayed minimal baseline ROS production (Figure 2A). AngII induced a significant increase in ROS production at all time points assayed (Figure 2A). Similarly, HG induced a significant increase in ROS at 10 hours (Figure 2B). Although not statistically significant compared with AngII or HG alone, costimulation with HG+AngII increased ROS production in an additive manner, with an increase of 167.7±9.0% of control (Figure 2B). To determine if AngII-mediated ROS production was Nox5-dependent, small interfering RNA (siRNA) oligonucleotides were used to knock down expression of either Nox5 or p22phox, a subunit required for activity of Nox1, -2, -3, and -4. Knockdown of Nox5 and p22phox was confirmed by Western blot (Figure 2D). Nox5 knockdown significantly blunted ROS production in response to AngII (91.2±4.2% of control), whereas scrambled control oligonucleotides (scrs) were without effect (126.4±20.2% for scr versus 121.1±9.6% for control) (Figure 2C); p22phox knockdown did not affect AngII-stimulated ROS production (125.3±10.0% for p22phox versus 126.4±20.2% for scr), suggesting that Nox5 activity predominates over other Nox isoforms in this context (Figure 2C).
diphenyleneiodonium blunted these effects\(^3\) (Figure 3I). Furthermore, KN-93 significantly attenuated ROS production (199.6 ± 19.7% of control for infected + vehicle versus 107.2 ± 2.0% of control for infected + KN-93) (Figure 3J). Consistent with the enhanced lamellipodia presence, Rac1 activity in adNox5-infected cells was elevated compared with controls (Figure 3, K and L).

**Figure 2.** AngII induces Nox5-dependent ROS production in human podocytes. Nox5-dependent ROS production in human and mouse podocytes. (A) Lucigenin-based ROS assay measuring superoxide production in human podocytes stimulated with vehicle (Veh; white columns) or 500 nM AngII (black columns) for various times. Values are reported as percent of untreated controls (mean ± SEM; n=3). *P=0.04; **P=0.03; P=0.05 at 2, 8, and 24 hours, respectively. (B) Lucigenin-based ROS assay measuring superoxide production in human podocytes stimulated with control (white columns) or HG (black columns). Values are reported as percent of controls (mean ± SEM; n=3). *P<0.05; **P<0.01. (C) Lucigenin-based ROS assay on human podocytes treated for 8 hours with vehicle (white columns) or 500 nM AngII (black columns) after siRNA knockdown with nontargeting scr-, Nox5-, or p22phox-specific siRNA. Values are expressed as percent of untreated controls (Cont; mean ± SEM; n=3; *P<0.05). (D) Western blots for Nox5 or p22phox in human podocytes transfected with non-targeting scr-, Nox5-, or p22phox-specific siRNA. Untransfected cells are controls. *β-Actin is the loading control. (E) HA-7 Western blot on lysates from uninfected and adNox5βHA (86 kDa)-infected mouse podocytes. (F) Immunofluorescence using HA-7 on uninfected or adNox5βHA-infected mouse podocytes showing expression of Nox5 (green). Scale bar, 100 μm. (G) Lucigenin-based ROS assay comparing superoxide production in adGFP- and adNox5βHA-infected mouse podocytes treated for 8 hours with vehicle (white columns) or 500 nM AngII (black columns). Values are expressed as percent of untreated controls (mean ± SEM; n=3). *P=0.03; **P=0.009.

**Albuminuric and Increased BP in Nox5\(^{pod+}\) Mice**

Having verified that human Nox5β is active in mouse podocytes in **vitro**, we next examined its function **in vivo**. For this purpose, human Nox5β cDNA was cloned directly downstream of an 8.3-kb fragment of the mouse nephrin promoter (mNPHS1) used previously\(^3^4,3^5\) (Figure 4A). After pronuclear injection, 11 Nox5\(^{pod+}\) FVB/n founders were identified by PCR-based genotyping. Three separate founder colony lines were maintained that exhibited similar phenotypic characteristics. Nox5 was readily detectable in the renal cortices of transgenic mice by both Western blotting and RT-PCR (Figure 4, B and C). Immunofluorescence of Nox5 revealed expression in glomerular structures, which is consistent with podocyte-specific localization (Figure 4D). Glomerular Nox5 activity was determined by measuring oxidative stress **in vivo** by dihydroethidium (DHE) staining. DHE-positive glomerular staining was greater for Nox5\(^{pod+}\) mice than non-transgenic (non-tg) littermates (Figure 4E). Weekly spot urine beginning at 6 weeks of age showed elevated albumin to creatinine ratios (ACRs) in Nox5\(^{pod+}\) mice compared with non-tg littermates. By 12 weeks of age, Nox5\(^{pod+}\) mice showed a mean ACR of 687 ± 100 μg/mg compared with 259 ± 70 μg/mg for non-tg littermates (Figure 5A). ACR values progressively rose in Nox5\(^{pod+}\) animals, reaching a mean of 1370 ± 57 μg/mg by 40 weeks of age (Figure 5F). Systolic BP was also monitored by tail-cuff plethysmography. Nox5\(^{pod+}\) mice exhibited significant increases in systolic BP (120 ± 3 mmHg) compared with non-tg littermates (108 ± 2 mmHg) (Figure 5B) at 12 weeks of age. ACR levels directly correlated with changes in systolic BP in Nox5\(^{pod+}\) mice (Figure 5C).

**Glomerular Filtration Barrier Damage in Nox5\(^{pod+}\) Mice**

Podocyte number was unchanged by Nox5 expression, because Wilm’s tumor-1 cortex mRNA levels in Nox5\(^{pod+}\) mice remained similar to those levels seen in non-tg mice (Figure 5D). However, podocyte injury was suggested, because nephrin levels were significantly reduced in Nox5\(^{pod+}\) mice (Figure 5E). To further assess progressive pathologic changes induced in glomeruli of Nox5\(^{pod+}\) animals, we examined periodic acid–Schiff (PAS)-stained sections of renal cortex. At 20 weeks of age, no gross morphologic...
changes were observed. However, at 40 weeks of age, a trend to larger total glomerular area and decreased glomerular tuft area was observed (Figure 6, A and B). Interestingly, these changes resulted in a $614\text{ }\mu\text{m}^2$ increase in overall Bowmann's space area ($1809\pm59\mu\text{m}^2$ Nox5$^{\text{pod+}}$ versus $1195\pm45\mu\text{m}^2$ non-tg) (Figure 6, C and E–H). Evidence of tubular interstitial fibrosis was also observed in Nox5$^{\text{pod+}}$ animals at 40 weeks of age. This result was indicated by $\alpha$-smooth muscle actin staining in
tubulointerstitial spaces in Nox5pod+ animals, which was limited to vascular structures in non-tg animals. Examination of Nox5pod+ glomerular and podocyte ultrastructure by electron microscopy revealed widespread foot process effacement and fusion as well as mesangial expansion, changes that were not observed in age-matched non-tg littermates (Figure 6, I–L).

Furthermore, electron micrographs revealed a striking increase in GBM thickness for Nox5pod+ mice (Figure 6, I–L) (347.2 ± 5.4 μm Nox5pod+ versus 232.2 ± 2.1 μm non-tg) (Figure 6D).

Nox5pod+ Mice Develop More Severe Renal Damage in Response to STZ-Induced Diabetes

We next examined the potential pathologic role of Nox5 in diabetic nephropathy. Nox5pod+ and non-tg littermates (8 weeks of age) were subjected to low-dose STZ injections and followed for 16 weeks. Albumin levels in 24-hour urine samples were increased in diabetic animals compared with controls, with Nox5pod+ animals having significantly higher albumin excretion rates than non-tg littermates (Figure 7A). Although systolic BP increased slightly in non-tg diabetic animals compared with controls, it did not reach statistical significance at any point (Figure 7B). In contrast, diabetic Nox5pod+ animals had significant elevations in systolic BP at 8 weeks post-STZ compared with nondiabetic non-tg controls (123 ± 4 versus 100 ± 5 mmHg) and 16 weeks post-STZ compared with nondiabetic non-tg and Nox5pod+ controls (137 ± 7 versus 105 ± 8 and 111 ± 7 mmHg, respectively) (Figure 7B). Importantly, systolic BP increases in Nox5pod+ STZ animals did not occur until 4 weeks after the initial increases in albumin excretion in Nox5pod+ versus non-tg animals. Morphologic examination of PAS-stained sections revealed increased glomerulosclerosis (39.2 ± 0.5% versus 34.9 ± 0.7%) in diabetic Nox5pod+ animals compared with non-tg patients with diabetes (Figure 7, C–G). Although systolic BP increased slightly in non-tg diabetic animals (Figure 7A), it did not reach statistical significance at any point (Figure 7B). In contrast, diabetic Nox5pod+ animals had significant elevations in systolic BP at 8 weeks post-STZ compared with nondiabetic non-tg controls (123 ± 4 versus 100 ± 5 mmHg) and 16 weeks post-STZ compared with nondiabetic non-tg and Nox5pod+ controls (137 ± 7 versus 105 ± 8 and 111 ± 7 mmHg, respectively) (Figure 7B).

Importantly, systolic BP increases in Nox5pod+ STZ animals did not occur until 4 weeks after the initial increases in albumin excretion in Nox5pod+ versus non-tg animals. Morphologic examination of PAS-stained sections revealed increased glomerulosclerosis (39.2 ± 0.5% versus 34.9 ± 0.7%) in diabetic Nox5pod+ animals compared with non-tg patients with diabetes (Figure 7, C–G). Although STZ-induced diabetes did result in a decrease in overall podocyte number, which was determined by Wilm’s tumor-1 staining, podocyte loss was not significantly increased in Nox5pod+ animals compared with non-tg controls (Figure 8, A and B). Examination of podocyte ultrastructure by electron microscopy (Figure 8C) revealed that diabetic Nox5pod+ animals displayed a significant increase in GBM width (Figure 8D) compared with non-tg patients
with diabetes (272.3 ± 13.1 versus 226.0 ± 6.9 nm) as well as a trend to increased foot process width (534.6 ± 54.1 versus 422.7 ± 14.5 nm), which is indicative of increased foot process effacement (Figure 8, E and F).

**DISCUSSION**

Renal oxidative stress contributes significantly to the progressive pathologic changes associated with nephropathy. Observations in animal models show that increased renal ROS generation promotes oxidative kidney damage and that treatment with antioxidants, such as N-acetyl cysteine, tempol, ebeselen, and vitamin E, decreases albuminuria and delays the progression of diabetic nephropathy.36–39

As a major cellular source of ROS, the role of Nox family members in mediating the renal complications of diabetes has become increasingly the focus of investigation. However, it remains unclear which of the Nox isoforms is the primary source of ROS in kidney disease. At the cellular level, Nox activity is linked to podocyte effacement through the AT1R, with AngII-induced actin cytoskeletal rearrangement in podocytes occurring in an ROS-dependent manner, which leads to increased cellular motility.5 Our *in vitro* studies show that *de novo* human Nox5 expression in mouse podocytes induces actin cytoskeleton rearrangement, Rac1 activation, and a lamellipodia-rich, ROS-dependent cellular phenotype reminiscent of the phenotype reported in the work by Hsu *et al.*5 It has been suggested that increased motility *in vitro* is analogous to podocyte effacement *in vivo*, an event closely associated with slit diaphragm deterioration and development of albuminuria.40 In agreement with this hypothesis and our *in vitro* results, Nox5* pod+* mice develop albuminuria and podocyte effacement, highlighting a role for Nox5 both at the cellular level and in the broader context of filtration barrier dysfunction.

Several reports have shown that Nox inhibition *in vivo* through apocynin reduces albuminuria and podocytopenia in rodent models of diabetes.15,41 Furthermore, pharmacological inhibition of Nox1 and -4 reduces albuminuria and slows diabetic nephropathy progression in a *db/db* type 2 diabetic model.42 Although these studies support a role for Nox inhibition as a viable treatment for diabetic nephropathy, gene deletion studies of Nox isoforms in animal models have raised questions regarding the role of individual Nox members in diabetic nephropathy. Deletion of either Nox4 or -2 in STZ-induced diabetes is not beneficial and may even be deleterious in the case of Nox4, supporting studies showing that Nox4 may play a protective role in chronic kidney injury and endothelial dysfunction.43–46 However, the role of Nox4 remains controversial in the diabetic setting, because short-term pharmacological Nox4 inhibition reduces renal ROS generation, glomerular hypertrophy, and fibronectin expression.42 Unlike other Nox family members, nothing is known regarding the function of Nox5 in animal models of disease, owing mainly to its absence from the mouse and rat genome. *In vitro* studies show a role for Nox5 in human vascular endothelial cells, where it is responsive to several factors...
present in the diabetic milieu, including AngII, ET-1, and PDGF. Furthermore, other reports indicate that Nox5 is normally absent, but upregulated in the vascular smooth muscle cells of vessels in coronary artery disease and in intramyocardial blood vessels after myocardial infarction. In support of these studies, our findings with human renal biopsies indicate that Nox5 is readily detectable in diabetic but not nondiabetic glomeruli. In contrast, glomerular Nox1 and -2 expressions remained unchanged irrespective of diabetes, whereas Nox4 seemed to be induced in diabetes, particularly in fibrotic glomeruli. Furthermore, our study showed that select diabetic factors, including AngII and TGF-β (data not shown), induced expression and activity of Nox5 in cultured human podocytes. Although incubation with HG had little impact on Nox5 expression at the time points examined, we cannot rule out a role for hyperglycemic conditions in driving podocyte Nox5-generated ROS, because they provided an additive effect when combined with AngII. Alternatively, glucose-dependent ROS may involve other Nox isoforms, such as Nox4.

Based on these observations and our finding that Nox5-derived ROS is involved in podocyte function, we hypothesized that Nox5-induced ROS is critical to the progression of nephropathy. Mouse models have provided significant insight into the early pathologic changes associated with CKD or diabetic nephropathy, including hypertension, GFR decline, and tubulointerstitial fibrosis. Surprisingly, even without induction of diabetes, Nox5 pod+ mice develop many pathologic features at the functional and structural levels that are associated with the early phases of diabetic nephropathy, including albuminuria, mesangial expansion, podocyte effacement, and GBM thickening, as well as later changes not normally observed in most mouse diabetic nephropathy models, including tubulointerstitial fibrosis. Moreover, Nox5 pod+ mice rendered diabetic with STZ exhibited more severe glomerular and tubular pathologies than their non-tg diabetic controls. These data strongly suggest that Nox5-induced ROS production in the podocyte participates in the progression of diabetic nephropathy.

In addition to renal dysfunction, Nox5 pod+ mice exhibited increased BP from 12 weeks of age, which was likewise exacerbated by STZ-induced diabetes. This phenomenon may facilitate the progression of nephropathy in a manner similar to the manner seen in eNOS−/− mice: diabetic nephropathy is
Figure 7. Nox5 exacerbates diabetic kidney damage in an STZ model. (A) ELISA-based albumin normalized to 24-hour urine volume for non-tg (white columns) control (CTRL; n=7), non-tg STZ (n=10), Nox5<sup>pod+</sup> control (CTRL; n=7), and Nox5<sup>pod+</sup> (black columns) STZ (n=12) obtained at indicated time points after STZ injection. Data presented as mean±SEM. *P<0.05. (B) Systolic BP measured by tail-cuff plethysmography for the groups in A. Solid lines are Nox5<sup>pod+</sup>, and hashed lines are non-tg controls. Squares are STZ-injected animals, and circles are control injections. Data are presented as mean±SEM. *P<0.05 versus non-tg controls; #P<0.05 Nox5<sup>pod+</sup> STZ versus non-tg STZ. (C) Total glomerular area, (D) glomerular tuft area, and (E) Bowman’s space area (n=3 mice per group; minimum of 30 glomeruli per mouse). White columns are non-tg, and black columns are Nox5<sup>pod+</sup>. Data are presented as mean±SEM. ***P<0.001. (F) PAS staining on paraffin-embedded kidney sections from non-tg and Nox5<sup>pod+</sup> control and STZ littermates 16 weeks post-STZ. Original magnification, ×400. (G) Percent of total mesangial area exhibiting sclerosis as determined by PAS staining. White columns are non-tg STZ; black columns are Nox5<sup>pod+</sup> STZ. Data are presented as mean±SEM. **P=0.01 (n=3).
exacerbated in conjunction with a moderate increase in systolic BP, leading to a more progressive renal phenotype, including tubulointerstitial fibrosis. Podocyte-expressing Nox5 mice exhibit early increases in systolic BP accompanied by progressive tubulointerstitial fibrosis. Although the exact mechanism that elevates BP remains unclear, it is possible that increased superoxide production resulting from aberrant Nox5 activity may lead to decreased NO bioavailability, with consequent decreased vasodilation and increased vascular resistance and elevation of systolic BP. Alternatively, we cannot rule out ROS-induced activation of the renin-angiotensin system in these mice. Importantly, because albumin leakage developed well before BP increases in our diabetic Nox5pod+ animals, such hypertension is likely secondary to progressive renal dysfunction. Furthermore, these results do not preclude a role for Nox5 in other forms of nephropathy, such as FSGS, in which ROS production has been implicated. Given that enhanced Nox5 activity in podocytes is sufficient to induce filtration barrier dysfunction, it is possible that additional investigation may uncover a role for Nox5 in other forms of CKD.

In summary, we showed that podocyte Nox5 expression is enhanced in human diabetic nephropathy, that Nox5-derived ROS is critically involved in the regulation of podocyte function, and that de novo introduction of Nox5β in mouse podocytes in vivo recapitulates numerous features reminiscent of diabetic nephropathy and exacerbates the progression of renal dysfunction in a diabetic model. These novel data identify Nox5 as an important Nox isoform in the development of nephropathy. Nox5 may emerge as a novel therapeutic target for reducing progression of CKD.

CONCISE METHODS

Cell Culture

Conditionally immortalized human and mouse podocytes, provided by Dr. Moin Saleem (University of Bristol) and Dr. Karlhans Endlich (University of Heidelberg, Germany), respectively, were grown at 33°C on type I collagen (BD Biosciences)-coated plastic culture dishes in RPMI-1640 medium (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin/streptomycin, and 10 U/ml recombinant γ-interferon. Differentiation was induced by maintaining the cells at 37°C in the above media without recombinant γ-interferon for 10–14 days. Cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin/streptomycin, and 10 U/ml recombinant γ-interferon for 2 days before stimulation with 500 nM AngII (Bachem). For glucose experiments, cells were growth-arrested for 36 hours in RPMI-1640 supplemented with 0.5% FBS, penicillin/streptomycin, and 5 mM d-glucose. Cells were then exposed to either 5 or 25 mM d-glucose for 10 hours using mannitol as an osmotic control before a 30-minute stimulation with 500 nM AngII, where appropriate. For inhibitor

Figure 8. Podocyte loss is not significantly altered in diabetic Nox5pod+ mice. (A) Wilm’s tumor-1 (WT1; green) for glomerular podocytes in non-tg and Nox5pod+ animals subjected to STZ or control (CTRL) injections; 4′,6-diamidino-2-phenylindole was used to stain nuclei. (B) Graphical representation of total WT1-positive nuclei per glomerulus in A. White columns are non-tg, and black columns are Nox5pod+ (n=6 mice per group, with a minimum of 40 glomeruli per mouse). *P<0.05 non-tg STZ versus non-tg CTRL. (C) Electron micrographs of podocytes from non-tg and Nox5pod+ animals subjected to STZ or control injections. (D) GBM thickness for non-tg STZ (white columns) and Nox5pod+ STZ (black columns; n=3). *P=0.04. (E) Average foot process width for non-tg STZ (white columns) and Nox5pod+ STZ (black columns; n=3). (F) Percent foot process effacement for non-tg STZ (white columns) and Nox5pod+ STZ (black columns; n=3). *P=0.04.
experiments, cells were maintained as above and treated with 10 μM KN-93 (Cayman Chemical) and 10 μM diphenyleneiodonium (Invitrogen) for 30 minutes before the addition of 500 nM AngII.

Human Biopsies
Archival biopsies were obtained from the Department of Pathology at the Ottawa Hospital. Nondiabetic samples were chosen from renal transplant recipients who had undergone a protocol biopsy and were determined to have no glomerular lesions, but in some cases, they showed evidence of focal acute tubular necrosis. Biopsies of diabetic patients all showed evidence of glomerular injury without evidence of nondiabetic renal disease. Sections were stained with Nox1, -2, -4, and -5 (Santa Cruz Biotechnology).

RNA Extraction and Quantitative PCR
Snap-frozen cortex was mechanically homogenized using the TP-103 Amalgamator COE Capmixer (GC America, Inc.). Cells were homogenized using Qiashredder columns (Qiagen).

RNA was extracted from snap-frozen kidney cortices of human and mouse podocytes using the Qiagen RNEasy Minikit as per the manufacturer’s instructions. Extracted RNA was converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with 500 ng starting material per reaction as per the manufacturer’s instructions. Quantitative PCR was performed on an ABI Prism 7000 Sequence Detection System using SYBR Advantage (Clontech) according to the manufacturer’s instructions. Primers used are listed in Supplemental Table 1.

Western Blots
For protein extraction from tissue, snap-frozen cortex was mechanically homogenized using the TP-103 Amalgamator COE Capmixer (GC America). The powdered tissue was resuspended in 1× Laemmli lysis buffer of 62.5 mM Tris-HCl (pH 6.8), 2% wt/vol SDS, 10% glycerol, and 50 mM dithiothreitol with protease inhibitors (Sigma-Aldrich) and incubated on ice for 30 minutes. For cells, plates were rinsed in PBS before being scrapped in 1× Laemmli lysis buffer with protease inhibitors and placed on ice for 30 minutes. Extracted proteins were run on appropriate percentage SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked in 5% nonfat dairy milk (NFDM) in 1× PBS containing 0.2% Triton X-100 (PBST) for 45 minutes before the addition of primary antibodies Nox4, Nox5 (1:750), HA-7 (1:2500), and β-actin (1:1000). Membranes were incubated in 5% NFDM in PBST with primary antibody overnight at 4°C. Membranes were washed three times in PBST before incubation with 2% horseradish peroxidase (1:50000) in 5% NFDM in PBST for 45 minutes. Membranes were washed in PBST, incubated with enhanced chemiluminescence (GE Healthcare), and exposed to film.

Lucigenin Assays
Treated cells were harvested in 70 μl ice cold phosphate buffer of 50 mM KH2PO4, 1 mM EGTA, and 150 mM sucrose (pH 7.4) with protease inhibitors; 50 μl cell lysate was added to 175 μl buffer and 1.25 μl 10^{-3} M lucigenin (ENZO Life Sciences). Baseline activity was measured. Cells were stimulated by the addition of 25 μl 1 mM NADPH, and active levels were measured. Baseline activity was subtracted, and adjusted activity was normalized to protein concentration.

**siRNA Knockdown**
Transient transfection of siRNA was performed using HiPerfect Transfection Reagent (Qiagen) as per the manufacturer’s instructions. Briefly, scrambled nontargeting control, Nox5 siRNA, or p22phox siRNA (Santa Cruz Biotechnology) was diluted in serum-free RPMI media and mixed with transfection reagent. The mixture was incubated for 10 minutes at room temperature to allow for lipid–RNA complex formation and then added dropwise to podocytes (2×10^5) seeded on type I collagen-coated 10-cm dishes. Cells were maintained for 48–72 hours before use.

**DHE Staining**
Frozen sections of tissue in optimal cutting temperature were cut to a thickness of 8 μm and placed on glass slides; 50 μl 2 μM dihydroethidium (Sigma-Aldrich) in Krebs solution (pH 7.4) of 11.8 mM NaCl, 0.465 mM KC1, 0.118 mM MgSO4, 0.118 mM KH2PO4, 14.702 mM CaCl2, 25 mM NaHCO3, 5.5 mM D-glucose, and 0.026 mM EDTA was placed on top of each section in a light-protected humidified chamber and incubated at 37°C for 30 minutes. Negative controls were incubated with Krebs only. Staining solution was removed, and slides were rinsed in Krebs solution. Slides were covered slipped using Fluormount G (Southern Biotech) and imaged.

**Viral Preparation and Infection**
Nox5 adenoviral vector obtained from Dr. David Fulton was transfected into 293T cells using Lipofectamine (Qiagen) per the manufacturer’s recommendations. Cells were allowed to round up and detach from the plate, and they were then gently washed from the plate with media. The media/cell suspension was collected in 50-ml Falcon tubes and centrifuged at 1500 rpm for 5 minutes. The supernatant was collected, and the pellet was resuspended in 2 ml serum-free DMEM (Invitrogen). The pellet was flash frozen in liquid nitrogen followed by thawing in a 37°C water bath. The freeze/thaw cycle was repeated a total of four times. The resulting suspension was centrifuged at 1500 rpm for 5 minutes, and the resulting supernatant was added to the already collected supernatant. The supernatant was then used to infect new plates of 293T cells. Viral propagation was continued until sufficient viral supernatant was obtained. Virus was purified using the Fast-Trap Adenovirus Purification and Concentration Kit (Millipore). GFP virus was obtained from Dr. David Park. Purified virus was used to infect podocytes at an moi of 5–20.

**Phalloidin Staining**
Phalloidin staining was performed as previously described. Briefly, cells were fixed with 4% paraformaldehyde and permeabilized in PBST. Cells were then incubated with Alexa Fluor 594 phalloidin (Molecular Probes) to visualize actin cytoskeleton.

**Rac1–Glutathione-S-Transferase Pull-Down Assay**
Pull-down assays were used to determine the state of Rac1 activation in mouse podocytes using the Rac1/CDC42 binding domain of p21-activated kinase coupled to glutathione-S-transferase. For in vitro pull-down assays in mouse podocytes, 500 μg cleared cell lysate...
was immediately added to 80 μg p21-activated kinase coupled to glutathione-S-transferase beads and incubated at 4°C for 45 minutes. Beads were washed, washed four times in PBS, and subjected to Western blotting. Samples were boiled and resolved by 15% SDS-PAGE, transferred to nitrocellulose membrane, and blotted for Rac1 using 1:1000 anti-Rac1 primary antibody (Cytoskeleton). Active (bead-bound) Rac1 was normalized to total Rac1 using 10% of input. Ponceau staining was used to verify loading.

Construct and Generation of Tgs
The human Nox5β cDNA (GeneCopoeia) was cloned into the pCDNA3.0 vector (Promega). An 8.3-kb HindIII/XhoI fragment of the NPHS1 promoter was inserted upstream of the Nox5β start site. An 11.3-kb NPHS1/Nox5β fragment was excised using a HindIII/ NsiI digest, and the resulting band was gel-purified. The purified DNA was provided to the University of Ottawa Core Transgenic Facility for pronuclear injection. Subsequent Nox5βodT founders were identified by PCR genotyping.

STZ Injection and 24-Hour Urine Collection
Mice ages 8–10 weeks were intraperitoneally injected with low-dose STZ (5 mg/kg) or sodium citrate control one time per day for 5 days as per the Animal Models of Diabetic Complications Consortium. For 24-hour urine collection, mice were individually housed in metabolic cages for 24 hours, and urine was collected. Urine volume was measured and recorded for normalization of 24-hour albumin excretion.

Immunohistochemistry and Renal Pathology
Nox5βodT mice were anesthetized with isofluorane and perfused with 20 ml PBS through the left ventricle. The kidneys were decapsulated, and the poles were removed. Portions of each kidney were placed in either 4% paraformaldehyde/PBS for paraffin embedding or 2.7% gluteraldehyde for electron microscopy, or they were embedded in Spurr resin. Samples were sectioned at 70 nm, placed on copper for transmission electron microscopy, and stained with uranyl acetate and lead citrate. Percent foot process effacement was calculated as previously described. Samples were screened on a Hitachi H-7100 Transmission Electron Microscope. Analytical measurements were performed using Axiovision software.

Statistical Analyses
GraphPad PRISM software was used to analyze all experimental data. Values are reported as the mean±SEM. Statistical significance was determined using either t test or one-way ANOVA followed by Newman–Keuls multiple comparison test.

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DISCLOSURES
None.

REFERENCES
41. Thallas-Bonke V, Thorpe SR, Coughlan MT, Fukami K, Yap FY, Sourris KC, Penfold SA, Bach LA, Cooper ME, Forbes JM: Inhibition of NADPH...


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Supplementary Figure 1
Supplementary Figure 2
Supplementary Figure 3
Supplementary Figure 4
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**SUPPLEMENTARY FIGURE LEGENDS**

Supplementary Figure 1. Nox1 (red) and Nox2 (green) staining on non-diabetic (a) and diabetic (b) human renal biopsies. Nox4 (red) staining on non-diabetic (c) and diabetic (d) human renal biopsies (Scale bar = 50µm).

Supplementary Figure 2. (a) QPCR demonstrating relative fold change in Nox1 mRNA expression in vehicle and AngII stimulated human podocytes. Values are expressed as relative fold change compared to untreated control. (b) QPCR demonstrating relative fold change in Nox4 mRNA expression in vehicle and AngII stimulated human podocytes. Values are expressed as relative fold change compared to untreated control. (c) RT-PCR demonstrating no upregulation of Nox5 splice variants α, δ, and γ in hPODs following stimulation with 500nM AngII. (d) QPCR verifying that Nox5β is the only splice variant that is significantly induced in hPODs in response to 500nM AngII stimulation.

Supplementary Figure 3. Western blot demonstrating relatively small changes in Nox4 protein expression in hPODs in response to 500nM AngII stimulation at the time points assayed.

Supplementary Figure 4. Lysates of human and mouse podocyte cell lines infected with HA-tagged adNox5 and western blotted with HA (a) and Nox5 (b) antibodies. (c) Renal cortex sections from STZ-injected non-tg and Nox5<sup>pod+</sup> mice stained for Nox4 or Nox5 expression by immunofluorescence.