High-glucose increases NADPH oxidase 4 (NOX4) expression, reactive oxygen species generation, and matrix protein synthesis by inhibiting AMP-activated protein kinase (AMPK) in renal cells. Because hydrogen sulfide (H2S) inhibits high glucose-induced matrix protein increase by activating AMPK in renal cells, we examined whether H2S inhibits high glucose-induced expression of NOX4 and matrix protein and whether H2S and NO pathways are integrated. High glucose increased NOX4 expression and activity at 24 h in renal proximal tubule epithelial cells, which was inhibited by sodium hydrosulfide (NaHS), a source of H2S. High glucose decreased AMPK phosphorylation and activity, which was restored by NaHS. Compound C, an AMPK inhibitor, prevented NaHS inhibition of high glucose-induced NOX4 expression. NaHS inhibition of high glucose-induced NOX4 expression was abrogated by N(ω)-nitro-1-arginine methyl ester, an inhibitor of NOS. NaHS unexpectedly augmented the expression of inducible NOS (iNOS) but not endothelial NOS. iNOS siRNA and 1400W, a selective iNOS inhibitor, abolished the ameliorative effects of NaHS on high glucose-induced NOX4 expression, reactive oxygen species generation, and matrix laminin expression. Thus, H2S recruits iNOS to generate NO to inhibit high glucose-induced NOX4 expression, oxidative stress, and matrix protein accumulation in renal epithelial cells; the two gasotransmitters H2S and NO and their interaction may serve as therapeutic targets in diabetic kidney disease.

Cellular mediators that regulate biological functions include gases (gasotransmitters), i.e. NO, H2S, and carbon monoxide (1). Although the biologic effects of NO are widely known, the role played by H2S in the regulation of cell function is not as well studied. Studies in mice lacking cystathionine γ-lyase (CSE), an H2S-generating enzyme, showed that H2S is a vasodilator that regulates blood pressure (2). In states of ischemic threat, H2S promotes cell survival (3). Our previous studies extended the scope of the H2S regulatory repertoire to protein synthesis; high glucose-induced protein synthesis in kidney epithelial cells was inhibited by H2S (4). In addition, H2S serves as an antioxidant. It inhibits ROS production by the mitochondria, increases GSH production, and scavenges ROS (5–7). Additionally, H2S increases the expression of Nrf-2, a transcription factor that promotes the expression of antioxidant genes, e.g. heme oxygenase 1 and thioredoxin 1 (8–10). ROS-mediated injury is seen in vivo and in vitro models of diabetic kidney disease. NOX4, a member of the NADPH oxidases of the NOX family, is the major source of ROS in the kidney, and its expression is increased in diabetes (11). We have reported that renal NOX4 expression is increased in the diabetic rat and that antisense oligonucleotides against NOX4 protect against diabetic kidney injury (12, 13). Genetic deletion of NOX4 or chemical inhibitors of NOX4 (and NOX1) significantly ameliorate kidney injury induced by diabetes (14–17). Previously, we and others have reported that diabetes inhibits the activity of AMPK in the kidney and that stimulation of AMPK ameliorates kidney injury in rodents with diabetes (13, 18–20). High glucose-induced increase in NOX4 expression also involves reduced activity of AMPK; activation of AMPK by 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside resulted in inhibition of NOX4 and NOX4-dependent kidney hypertrophy, albuminuria, and matrix protein expression (13, 19). We and others have also re-

The abbreviations used are: CSE, cystathionine γ-lyase; ROS, reactive oxygen species; NOX, NADPH oxidase; MCT, mouse kidney cortical proximal tubular epithelial cell; ACC, acetyl-CoA carboxylase; iNOS, inducible NOS; eNOS, endothelial NOS; nNOS, neuronal NOS; ANOVA, analysis of variance; L-NAME, N(ω)-nitro-L-arginine methyl ester; ACD, actinomycin D; AMPK, AMP-activated kinase; DCF, dichlorodihydrofluorescein; CM-H2DCFDA, chloromethyl-2,7’-dichlorodihydrofluorescein diacetate.

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ported that AMPK activation prevents the deleterious effects of glucose in cultured glomerular mesangial cells, podocytes, and renal tubular epithelial cells via inhibition of high glucose-induced NOX4 protein expression and subsequent ROS generation (13, 19, 21). As discussed above, H$_2$S inhibition of high glucose-induced protein synthesis in renal cells involves AMPK activation (4); however, whether H$_2$S regulates NOX4 expression and activity in kidney cells is not known. In this study, we investigated whether H$_2$S exerts antioxidant effects in kidney proximal tubular epithelial cells in a high-glucose environment and whether this effect involves regulation of NOX4 expression. We also explored whether H$_2$S recruits the NO pathway in the regulation of NOX4.

Results

H$_2$S inhibits high Glucose-induced NOX4 expression

Because NOX4 is constitutively active, the amount of ROS released by the enzyme is regulated by control of its protein expression (11, 22). High glucose increased NOX4 expression in mouse kidney proximal tubular epithelial (MCT) cells at 24 h (Fig. 1A). Sodium hydrosulfide (NaHS), a source of H$_2$S, dose-dependently inhibited high glucose-induced NOX4 expression, restoring basal expression at 250 mM (Fig. 1B); we employed this concentration in our experiments. Equimolar mannitol did not regulate NOX4 expression (data not shown), excluding non-osmotic mechanisms. High glucose increased NOX4 mRNA content preceding the increase in protein (Fig. 1C); it was abrogated by NaHS (Fig. 1D). High glucose-induced NOX4 protein expression could be inhibited by actinomycin D, a transcription blocker (Fig. 1E). An NADPH oxidase assay employing lucigenin chemiluminescence showed that NaHS inhibited high glucose-induced NOX4 activation (Fig. 1F). Together, these data show that augmented transcription is involved in the high glucose-induced increase in NOX4 expression and that H$_2$S inhibited high glucose-induced changes in NOX4 expression and activity.

Figure 1. H$_2$S inhibits high glucose-stimulated NOX4 expression. Quiescent MCT cells were incubated with 5 or 30 mM glucose (Glc) for 24 h with or without NaHS. A and B, immunoblotting was done for NOX4 expression, with actin as a loading control. C and D, NOX4 and GAPDH mRNA were measured by quantitative RT-PCR. E, cells were preincubated with actinomycin D (ACD, 2 μg/ml) for 30 min before incubation with high glucose; immunoblotting was done to detect NOX4 and actin. In A–E, composite data from three to six experiments are shown in scatterplots. F, the NADPH oxidase assay was done as described under “Experimental Procedures;” data from three experiments (mean ± S.D.) are shown in a scatterplot. *, p < 0.05; **, p < 0.01 versus 5 mM glucose; #, p < 0.05; ##, p < 0.01 versus 30 mM glucose by ANOVA.
H₂S inhibition of high Glucose-induced NOX4 expression requires AMPK activation

H₂S activates AMPK to inhibit high-glucose stimulation of protein synthesis in kidney glomerular epithelial cells (4). Accordingly, we examined whether AMPK mediated the H₂S inhibition of high glucose-induced NOX4 synthesis. NaHS time-dependently increased AMPK phosphorylation on Thr-172 of the α subunit, with onset at 5 min and return to baseline at 30 min (Fig. 2A), in line with our past observation (4). High glucose, but not equimolar mannitol, reduced AMPK phosphorylation, which was restored by NaHS (Fig. 2B). To confirm that the increase in Thr-172 phosphorylation of AMPK corresponds to the increase in AMPK activity, we examined the status of Ser-79 phosphorylation of acetyl-CoA carboxylase (ACC), a direct substrate of AMPK. NaHS time-dependently stimulated ACC phosphorylation; high glucose could not inhibit ACC phosphorylation in the presence of NaHS (Fig. 2C and D). Preincubation of cells with compound C, a selective AMPK inhibitor, abolished NaHS inhibition of the high glucose-induced increase in NOX4 expression (Fig. 2E). These data show that AMPK activation is required for the inhibitory effect of H₂S on high glucose-induced NOX4 expression. Compound C also increased the basal NOX4 content, suggesting that AMPK constitutively inhibits the expression of that protein.

Figure 2. H₂S inhibition of high glucose-induced NOX4 expression is AMPK-dependent. A and B, immunoblotting showed that NaHS promoted Thr-172 phosphorylation of AMPK and rectified its reduction induced by high glucose (Glc). Man, mannitol. C and D, NaHS increased Ser-79 phosphorylation of ACC, an AMPK substrate, and corrected its reduction induced by high glucose. E, compound C, an AMPK inhibitor, abolished NaHS inhibition of high glucose-induced NOX4 expression. A–E, composite data from three to four experiments (mean ± S.D.) are shown in scatterplots. A and C, *p < 0.5; **p < 0.01 versus no NaHS. B, D, and E, *p < 0.05 versus 5 mM glucose; #p < 0.05; ##p < 0.01 versus 30 mM glucose; §§p < 0.01 versus 30 mM glucose + NaHS by ANOVA.
H₂S promotes the expression of inducible NOS (iNOS)
There are similarities in some of the biological effects of NO and H₂S (23, 24). We examined whether the H₂S effect on high glucose-induced NOX4 expression involves NO. NaHS inhibition of high glucose-induced NOX4 expression was abolished by L-NAME, a general NOS inhibitor. B, NaHS decreased Ser-1177 phosphorylation of eNOS but did not affect its expression. C, MCT cells did not express nNOS; however, nNOS was expressed by the brain (Br), employed as a positive control. D, NaHS increased iNOS expression in a time-dependent manner. E, NaHS increased the mRNA expression of iNOS as measured by quantitative RT-PCR. F, preincubation with ACD (2 μg/ml) for 30 min abolished the NaHS-induced increase in iNOS expression at 4 h. G, NaHS-induced iNOS expression could be inhibited by compound C, indicating that AMPK activation is required for NaHS stimulation of iNOS. H, cells were transfected with siRNA against iNOS (si-iNOS) or control siRNA (si-con) and incubated with or without high glucose and with or without NaHS. Expression of si-iNOS abolished NaHS inhibition of the high glucose-induced NOX4 increase at 24 h. In A, B, and D–H, composite data from three to four experiments (mean ± S.D.) are shown in scatterplots. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus respective control; #, p < 0.05; ###, p < 0.001 versus 30 mM glucose; †, p < 0.05 versus NaHS; §§§, p < 0.001 versus 30 mM glucose + NaHS by ANOVA.
NOS inhibitor (Fig. 3A), suggesting a role for NO in mediating the H$_2$S effect. L-NAME augmented basal NOX4 expression in control 5 mM glucose-treated cells, suggesting that NO exerts a tonal inhibition on NOX4 in the basal state. Next, we explored the type of NOS involved in NO generation. Ser-1177 phosphorylation is required for endothelial NOS (eNOS) activation. NaHS inhibited eNOS phosphorylation for up to 8 h and did not regulate its protein expression (Fig. 3B). MCT cells did not express neuronal NOS (nNOS) (Fig. 3C). These data excluded a role of eNOS or nNOS in mediating the effect of H$_2$S. NaHS augmented the expression of iNOS in a time-dependent manner, peaking at 4 h (Fig. 3D). NaHS increased mRNA expression of iNOS at 2 h (Fig. 3E), and the NaHS-induced iNOS protein expression at 4 h could be blocked by actinomycin D (Fig. 3F). These data indicate that the mechanism of increase in iNOS expression by H$_2$S involves stimulation of its transcription. Additionally, compound C blocked the NaHS-induced increase in iNOS protein (Fig. 3G), showing that AMPK mediated the increase. We tested the requirement of iNOS to mediate NaHS inhibition of the high glucose-induced NOX4 increase. Expression of si-iNOS abrogated the inhibitory effect of NaHS on the high glucose-induced increase in NOX4 (Fig. 3H). Together, these data demonstrate that H$_2$S stimulates iNOS expression in an AMPK-dependent manner to inhibit the high glucose-induced increase in NOX4.

**H$_2$S inhibition of high Glucose-induced ros depends on no production by iNOS**

NOX4 is a major source of ROS produced by the kidney cells on exposure to high glucose (11, 12, 14, 16, 22). We examined whether NO generated by iNOS was involved in H$_2$S inhibition of high glucose-induced ROS generation. DCF fluorescence revealed that high glucose promoted intracellular ROS generation at 24 h (Fig. 4B), which was abolished by NaHS (Fig. 4D). Both L-NAME, a general NOS inhibitor, and 1400W, a selective inhibitor of iNOS, abrogated NaHS inhibition of the high glucose-induced ROS increase (Fig. 4, F and H). These data show that H$_2$S inhibition of high glucose-induced ROS generation requires iNOS activity.

Because AMPK phosphorylation was required for NaHS inhibition of high glucose-induced NOX4 expression at 24 h (Fig. 2E) and because an iNOS increase was seen at 4 h following NaHS exposure (Fig. 3D), we tested whether NO generated by iNOS stimulates AMPK phosphorylation, which, in turn, regulates NOX4. NONOate, an NO donor, promptly stimulated AMPK phosphorylation (Fig. 5A). NaHS also stimulated AMPK phosphorylation, which was seen at 8 h, corresponding to the time iNOS would be active (Fig. 5B). Together with the rapid increase in AMPK phosphorylation induced by NaHS, as shown in Fig. 2A, these observations suggest a biphasic regulation where the rapid induction of AMPK phosphorylation (Fig. 2A) may be a direct effect of NaHS, whereas the increase at 8 h is probably mediated by NO generated by iNOS (Fig. 5B). We tested the latter scenario. The expression of si-iNOS abolished AMPK phosphorylation at 8 h (Fig. 5C). We also tested whether NO can mediate NaHS inhibition of high glucose-induced NOX4. NONOate, the NO donor, inhibited high glucose-induced NOX4 expression at 24 h (Fig. 5D). The inhibitory effect of NONOate on high glucose-induced NOX4 was blocked by compound C (Fig. 5E), indicating that it was AMPK-dependent. These data show that NaHS promotes iNOS expression (Fig. 3D) and NO generation, leading to AMPK activation (Fig. 5A) and inhibition of high glucose-induced NOX4 expression (Fig. 2E).

**H$_2$S inhibition of the high Glucose-induced matrix protein increase involves a reduction in NOX4 expression**

Accumulation of extracellular matrix proteins is a hallmark of kidney injury in diabetes; it manifests as thickening of the glomerular and tubular basement membranes, glomerulosclerosis, and tubulointerstitial fibrosis and, importantly, contributes to the loss of kidney function (25). Because we...
employed renal proximal tubular epithelial cells in our study, we examined the status of laminin γ1, an important component of the tubular basement membrane (26). High glucose, but not equimolar mannitol, increased laminin γ1 expression in MCT cells, which was abolished by NaHS (Fig. 6A), confirming our previous report (4). We evaluated the requirement of NOX4 to mediate the high glucose-induced increase in laminin γ1. Reduction in NOX4 expression by siRNA abolished the high glucose-induced increase in laminin γ1 expression (Fig. 6B). These data collectively show that high glucose stimulation of matrix protein requires NOX4 and that H2S inhibition of this process is mediated by inhibiting NOX4.

Figure 5. The NO donor NONOate inhibits high glucose-induced NOX4 expression by stimulating AMPK phosphorylation. A, immunoblotting showed that NONOate, an NO donor, promoted AMPK phosphorylation in a time-dependent manner. B, NaHS induced a second wave of AMPK phosphorylation at 8 h following an increase in iNOS expression seen at 4 h (Fig. 3D). C, expression of si-iNOS abolished NaHS induced AMPK phosphorylation at 8 h. D, NONOate inhibited high glucose (Glc)-induced NOX4 expression at 24 h. E, NONOate inhibition of high glucose-induced NOX4 expression was abolished by compound C (CC), indicating the requirement for AMPK activation. In A–E, composite data from three to six experiments (mean ± S.D.) are shown in scatterplots. *, p < 0.05; **, p < 0.01 versus respective control; #, p < 0.05; ##, p < 0.01 versus 30 mM glucose; †, p < 0.05 versus NaHS; ##, p < 0.001 versus NONOate + 30 mM glucose by ANOVA.

**H2S inhibition of the high Glucose-induced matrix protein increase depends on iNOS activity**

We investigated whether H2S amelioration of the high glucose-induced matrix laminin γ1 expression involved iNOS activation. Preincubation with l-NAME, a general NOS inhibitor, or 1400W, a selective iNOS inhibitor, abolished NaHS inhibition of the high glucose-induced increase in laminin γ1 (Fig.
Hydrogen sulfide regulates NOX4 in kidney epithelial cells

The role of iNOS was directly confirmed by directly employing NONOate, the NO donor, and compound C, the AMPK inhibitor (Fig. 7, D and E). Together, these observations lead to the conclusion that H2S inhibition of high-glucose stimulation of laminin γ1 is mediated by NO generation by iNOS in MCT cells.

Discussion

Our data show that H2S inhibits high glucose-induced NOX4 expression, subsequent ROS generation, and matrix protein laminin increase in MCT cells by harnessing iNOS activation. The serial events are as follows: H2S stimulates an increase in iNOS expression (Fig. 3D), and the increased iNOS expression and NO generation mediate H2S inhibition of high glucose-induced NOX4 expression (Fig. 3H), ROS generation (Fig. 4H), and increased matrix laminin content (Fig. 7C). AMPK activity is required for both H2S-stimulated iNOS expression (Fig. 3G) and NO inhibition of high glucose-induced NOX4 expression (Fig. 5E). These data show that H2S serves as an antioxidant in high glucose-treated kidney epithelial cells by recruiting NO to inhibit NOX4, ROS generation, and matrix protein increase (Fig. 8).

Previous reports describe a complex picture of regulatory interactions between NOX4 and NOX5s. NOX4-derived ROS promote eNOS dysfunction and decrease NO bioavailability in mesangial cells exposed to high glucose and angiotensin II (13, 29). Although it has been suggested that renal NOX4 may be either protective or not involved in certain models of kidney injury (30, 31), the injurious action of the oxidase has been established by recent reports showing that NOX4 genetic deletion in mice attenuates diabetes-mediated renal cell injury, including loss of podocyte function (14, 16, 32). The role of podocyte NOX4 in kidney pathology has been confirmed by the observation that overexpression of NOX4 in podocytes causes renal injury (33). Homocysteine-induced mitochondrial toxicity in brain endothelial cells and in the heart is associated with reduced CSE expression, increased NOX4 expression, and suppression of eNOS; these abnormalities are corrected by H2S (34, 35). Our findings in kidney epithelial cells are in agreement with these findings in that high glucose-induced NOX4 expression could be corrected by H2S. The mechanism by which H2S inhibited high glucose-induced NOX4 expression revealed a novel insight in our studies: the protective effect of H2S required the activation of iNOS, which is usually implicated in tissue injury. Previous studies have reported recruitment of eNOS by H2S in promoting angiogenesis, vasorelaxation, and wound healing (23). In the kidney, eNOS-derived NO exerts a protective effect, as shown by the occurrence of more severe diabetic kidney injury in eNOS knock-out mice (36, 37). Although we find a protective role for iNOS in high glucose-induced kidney cell injury in vitro, the data on iNOS in diabetic kidney disease in vivo are conflicting. Because diabetes-induced kidney damage assessed as matrix increase was worse in iNOS knock-out mice, iNOS has been suggested to have a protective
role (38). However, diabetic kidney injury in rats was associated with an increase in iNOS (39); it is unclear whether the iNOS increase in this model represents a failed attempt at protection or whether the variance from the mouse model is related to different rodent species being examined. A protective role for iNOS is also suggested by the observation that it facilitates the ameliorative effect of ischemic preconditioning on subsequent ischemic injury in the kidney (40). In ischemia reperfusion injury of the heart, the healing effect of astragaloside is dependent on iNOS (41). Studies focusing on individual cell types also show a complex picture of iNOS. In the endotoxemic model, activation of iNOS in leukocytes infiltrating the myocardium reduces the contraction of cardiac myocytes, whereas iNOS is facilitative in augmenting contraction in response to adrenergic agents (42). In rat aortic smooth muscle cells, high glucose and methyloglyoxal cause oxidative stress by increased NO production by iNOS and increase in NOX4 expression (43); in contrast, high glucose-induced NOX4 increase, ROS generation, and matrix protein expression are inhibited by increased production of NO by iNOS in H2S-treated MCT cells in our study. In the mouse podocytes, another type of kidney epithelial cell, amelioration of high glucose-induced cell injury by tadalafil also requires stimulation of iNOS expression (44). Taken together, these data suggest that whether iNOS

Figure 7. H2S inhibition of high glucose-induced laminin γ1 and NOX4 is iNOS-dependent. A and B, immunoblotting showed that NaHS inhibited the high glucose (Glc)-induced increase in laminin γ1 (Lamγ1) expression, and the increase was abolished by L-NAME, a general NOS inhibitor, or by 1400W, a selective inhibitor of iNOS. C, the immunoblot of lysates from cells transfected with si-iNOS or control si-RNA and treated with or without high glucose or NaHS from Fig. 3H was probed with antibody against laminin γ1; thus, the iNOS and actin bands are the same as in Fig. 3H. NaHS inhibition of high glucose-induced laminin γ1 at 24 h could be abrogated by si-iNOS. D, NONOate abolished high glucose-induced laminin γ1 expression. E, compound C (CC) inhibited NONOate reduction of high glucose-induced laminin γ1 expression. Composite data from three to five experiments (mean ± S.D.) are shown in scatterplots. *, p < 0.05; **, p < 0.01 versus 5 mM glucose; #, p < 0.05; ##, p < 0.01 versus 30 mM glucose; ◊, p < 0.05 versus NONOate + 30 mM glucose; §, p < 0.05; §§, p < 0.01 versus 30 mM glucose + NaHS by ANOVA.

H2S regulation of NOX4 in kidney epithelial cells
Hydrogen sulfide regulates NOX4 in kidney epithelial cells

![Diagram](image_url)

Figure 8. A schematic showing pathways of interaction between NO and H₂S signaling pathways in high glucose-treated MCT cells that were explored in this study.

H₂S acts as an inhibitor or promoter of injury is cell type- and context-dependent.

Data from this study indicate that AMPK mediates iNOS inhibition of high glucose-induced NOX4 transcription. Previous studies have shown that H₂S stimulates AMPK phosphorylation by the activation of calcium calmodulin kinase kinase β in podocytes (4). In this study, H₂S activated AMPK in a biphasic manner. The first burst occurred within minutes, subsided by 1 h, and was involved in increasing the expression of iNOS, as the latter could be blocked by the AMPK inhibitor (Fig. 3G). The second round of AMPK activation in our study occurred after iNOS activation and led to the inhibition of NOX4 expression induced by high glucose. Another AMPK activator, adiponectin, also inhibits NOX4 expression in the kidney (20).

The increase in NOX4 by high glucose appears to involve transcription, as it was blocked by actinomycin D (Fig. 1E). This is consistent with previous reports showing that the NOX4 promoter contains binding sites for transcription factors such as Smad2/3 and NF-κB that are regulated by glucose or transforming growth factor β, a major mediator of glucose actions in renal and vascular cells (45–47). Future studies are needed to identify the mechanism by which AMPK activation abrogates high glucose-induced NOX4 transcription. It would also be interesting to examine the mechanisms by which iNOS-derived NO reduces high glucose-induced NOX4 expression. It is tempting to speculate that the protective effect of NO and iNOS may be through the blockade of factors involved in NOX4 transcription. NF-κB would be a candidate of choice because it has been shown to control NOX4 mRNA expression, and it can be S-nitrosylated and inhibited by NO (46). It should also be mentioned that NO has been documented to suppresses NOX-dependent ROS production by S-nitrosylation (48, 49).

H₂S exerts an ameliorative effect on ROS generation, proteinuria, and matrix protein accumulation in the glomeruli and tubulointerstitium in rodents with diabetes (50–52), and our data provide a mechanistic basis for these salutary effects. The aforementioned indices of kidney injury occur as a composite result of injury to many cell types. Because podocytes regulate permeability of the glomerular basement membrane to albumin, and proximal tubular epithelial cells reabsorb significant amounts of the filtered albumin, proteinuria is likely to be a composite result of injury to both cell types. Similarly, thickening of the glomerular basement membrane involves injury to the podocytes, glomerular mesangial expansion occurs because of mesangial cell injury, and tubulointerstitial fibrosis is due to dysregulation of matrix protein metabolism in proximal and other tubular epithelial cells and renal fibroblasts. Thus, the beneficial effects of H₂S in ameliorating albuminuria and glomerular matrix expansion and tubulointerstitial fibrosis are likely to involve both glomerular and tubular mechanisms.

The phosphodiesterase-5 inhibitor tadalafil augments generation of H₂S by CSE and abolishes high glucose-induced kidney cell injury by recruiting iNOS in kidney podocytes (44). Administration of phosphodiesterase-5 inhibitors ameliorates kidney injury in diabetic rodents and humans (39, 53, 54). The limitation of this study is that the beneficial role of iNOS was studied in an in vitro model of high glucose-induced kidney cell injury. Future plans include critically testing the role of iNOS directly in animal models of diabetic kidney disease and examining whether the beneficial effects of H₂S and phosphodiesterase-5 inhibitors involve iNOS. The combination of these agents with NOX4 small-molecule inhibitors (currently in a phase II clinical trial in diabetic patients) could represent a novel therapeutic intervention to treat diabetic kidney disease.

**Experimental procedures**

**Cell culture**

We employed the proximal tubular epithelial cells for the following reasons. First, the proximal tubular epithelial cells constitute the most numerous cell type in the kidney cortex. Observations of this cell type are relevant to our previous studies of the H₂S pathway in the kidney cortex in diabetic mice (4). Second, there is increasing recognition that progressive kidney functional impairment in diabetes strongly correlates with tubulointerstitial disease of the kidney (55); proximal tubules play a major role in the latter. Murine kidney proximal tubular epithelial (MCT) cells (kindly provided by Dr. Eric Neilson, Northwestern University) were grown in DMEM (catalog no. 11885084, Life Technologies) containing 7% fetal bovine serum (catalog no. H9260, Life Technologies) containing 7% fetal bovine serum (catalog no. H9260, Life Technologies) containing 7% fetal bovine serum (catalog no. H9260, Life Technologies), 5 mM glucose, 100 units/ml penicillin, 100 μg/ml streptomycin (catalog no. 15240062, Gibco), and 2 mM glutamine. Cells were incubated with 5 mM or 30 mM glucose (catalog no. G7021, Sigma-Aldrich) with or without NaHS (catalog no. 161527, Sigma-Aldrich) following 24 h of serum starvation; 5 mM glucose plus 25 mM mannitol (catalog no. M1902, Sigma-Aldrich) was employed as an osmotic control. The MCT cells were transfected with siRNA against NOX4 (catalog no. sc-41587, Santa Cruz Biotechnology), iNOS (catalog no. sc-36092, Santa Cruz Biotechnology), or scrambled RNA (catalog no. sc-37007, Santa Cruz Biotechnology) using Lipofectamine RNAiMax (catalog no. 13778150, Invitrogen).

**Immunoblotting**

Equal amounts of cell lysates and tissue homogenates were employed in immunoblotting as described previously (4, 44). Antibodies against the following antigens were employed: NOX4 (catalog no. sc-30141, Santa Cruz Biotechnology),
Hydrogen sulfide regulates NOX4 in kidney epithelial cells

cystathionine β-synthase (CBS) (catalog no. sc-67154, Santa Cruz Biotechnology), CSE (catalog no. sc-135203, Santa Cruz Biotechnology), laminin γ1 (catalog no. sc-5584, Santa Cruz Biotechnology), ACC (catalog no. 3662, Cell Signaling Technology), phospho-ACC (catalog no. 3661, Cell Signaling Technology), phospho-AMPK (catalog no. 2531, Cell Signaling Technology), AMPK (catalog no. 2532, Cell Signaling Technology), phospho-eNOS (catalog no. 9571, Cell Signaling Technology), eNOS (catalog no. 9572, Cell Signaling Technology), nNOS (catalog no. 4231, Cell Signaling Technology), iNOS (catalog no. 610432, BD Transduction Laboratories), and actin (catalog no. A2066, Sigma-Aldrich).

Detection of ROS and NO production

Quiescent MCT cells were preincubated with 100 μM l-NAME (catalog no. N5751, Sigma-Aldrich) or 50 μM 1400W, an iNOS inhibitor (catalog no. W4262, Sigma-Aldrich) for 30 min, followed by incubation with or without 5 or 30 mM glucose for 24 h. Detection of intracellular ROS was performed by confocal microscopy employing an Olympus FV-1000 microscope with a ×20 superapochromatic objective, numerical aperture 0.75 objective following incubation with 10 μM CM-H2DCFDA (catalog no. C6827, Molecular Probes) in Hanks’ balanced salt solution (catalog no. 14025092, Life Technologies) for 30 min as described previously (12). The excitation wavelength was 488 nm from an argon laser. The emission wavelength range was 500–600 nm.

NADPH oxidase assay

This assay was employed to measure NADPH oxidase activity using lucigenin-enhanced (catalog no. M8010, Sigma-Aldrich) chemiluminescence. It was performed as described previously (13, 19), and superoxide production was expressed as relative chemiluminescence (light) units per milligram of protein.

Statistical analysis

Data were expressed as mean ± S.D. Statistical comparisons between multiple groups were performed by ANOVA single-way analysis, and post hoc analysis was done using Student-Newman-Keuls multiple comparisons test employing GraphPad Prism 4 software. p < 0.05 was considered significant.

Author contributions—B. S. K. and Y. G. designed the study, analyzed the data, and wrote the paper. H. J. L. designed and performed most of the experiments and data analyses. D. Y. L. conducted the DCF image analysis and NADPH oxidase activity assay. M. M. M., D. F., G. G. C., and H. E. A. contributed to data analysis. All authors reviewed the data and approved the final version of the manuscript.

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