Hydrogen Sulfide Inhibits High Glucose-induced Matrix Protein Synthesis by Activating AMP-activated Protein Kinase in Renal Epithelial Cells

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Background: Whether hydrogen sulfide regulates protein synthesis is not known.
Results: In kidney cells, hydrogen sulfide inhibited high glucose-induced synthesis of proteins including matrix proteins by activating AMP-activated protein kinase and inhibiting events in mRNA translation.
Conclusion: Hydrogen sulfide reduces high glucose stimulation of matrix protein synthesis in renal cells.
Significance: Hydrogen sulfide induction may inhibit kidney matrix protein accumulation in diabetes.

Hydrogen sulfide, a signaling gas, affects several cell functions. We hypothesized that hydrogen sulfide modulates high glucose (30 mM) stimulation of matrix protein synthesis in glomerular epithelial cells. High glucose stimulation of global protein synthesis, cellular hypertrophy, and matrix laminin and type IV collagen content was inhibited by sodium hydrosulfide (NaHS), an H₃S donor. High glucose activation of mammalian target of rapamycin (mTOR) complex 1 (mTORC1), shown by phosphorylation of p70S6 kinase and 4E-BP1, was inhibited by NaHS. High glucose stimulated mTORC1 to promote key events in the initiation and elongation phases of mRNA translation: binding of eIF4A to eIF4G, reduction in PDCD4 expression and inhibition of its binding to eIF4A; eEF2 kinase phosphorylation, and dephosphorylation of eEF2; these events were inhibited by NaHS. The role of AMP-activated protein kinase (AMPK), an inhibitor of protein synthesis, was examined. NaHS dose-dependently stimulated AMPK phosphorylation and restored AMPK phosphorylation reduced by high glucose. Compound C, an AMPK inhibitor, abolished NaHS modulation of high glucose effect on events in mRNA transcription as well as global and matrix protein synthesis. NaHS induction of AMPK phosphorylation was inhibited by siRNA for calmodulin kinase kinase β, but not LKB1, upstream kinases for AMPK; STO-609, a calmodulin kinase kinase β inhibitor, had the same effect. Renal cortical content of cystathionine β-synthase and cystathionine γ-lyase, hydrogen sulfide-generating enzymes, was significantly reduced in mice with type 1 diabetes or type 2 diabetes, coinciding with renal hypertrophy and matrix accumulation. Hydrogen sulfide is a newly identified modulator of protein synthesis in the kidney, and reduction in its generation may contribute to kidney injury in diabetes.

Biologically active gases such as nitric oxide and carbon monoxide modulate tissue function. Vessel wall relaxation and vasodilation occur in mice lacking both endothelial nitric-oxide synthase and cyclooxygenase, suggesting the presence of other endothelium-dependent relaxation factors (1). Among the endothelium-dependent relaxation factors, hydrogen sulfide and carbon monoxide have attracted attention. Hydrogen sulfide was proposed as a physiologically active neurotransmitter in the brain (2). Until recently, there was controversy whether endogenous hydrogen sulfide had any physiological significance. A remarkable study reported that when hydrogen sulfide generation was genetically suppressed by deleting cystathionine γ-lyase, an enzyme that generates hydrogen sulfide, mice developed hypertension when compared with the wild type mice despite similar endothelial NOS expression (3). Administration of sodium hydrosulfide (NaHS), a source of hydrogen sulfide, and not ammonia rescued the cystathionine γ-lyase knock-out mice from hypertension (3), demonstrating that hydrogen sulfide regulates hemodynamics in mammals. Hydrogen sulfide is produced in the wall of blood vessels and causes relaxation of smooth muscle cells by opening the K-ATP channels, without recruiting cyclic GMP (4).

There is evidence that in addition to its hemodynamic effects, hydrogen sulfide can affect fundamental cellular responses to injury such as macrophage infiltration, apoptosis, and mitochondrial respiration in the heart (5, 6). The role of hydrogen sulfide in renal physiology and disease is beginning to be explored. Biochemical analysis has shown that the kidney produces hydrogen sulfide catalyzed by three enzymes: cystathionine γ-lyase, cystathionine β-synthase, and 3-mercaptoacetyl-coenzyme A cysteine sulphydryl transferase (7). Hydrogen sulfide is produced in the wall of blood vessels and causes relaxation of smooth muscle cells by opening the K-ATP channels, without recruiting cyclic GMP (4).

The abbreviations used are: NaHS, sodium hydrosulfide; GEC, globerular epithelial cell; mTOR, mammalian target of rapamycin; AMPK, AMP-activated protein kinase; CaMKKβ, Ca/calmodulin-dependent protein kinase kinase β; ANOVA, analysis of variance.
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topyrurate sulfotransferase (7). Hydrogen sulfide content is
decreased in the renal parenchyma in rats with streptozotocin-
duced diabetes, and administration of NaHS inhibited
increases in TGFβ1, reactive oxygen species, and type IV collagen
in diabetic rats (8).
Studies in the heart and kidney have indicated that hydrogen
sulfide inhibits phenotypes that depend on increase in protein
synthesis. NaHS inhibited hypertension-induced cardiac fibrosis,
arteriolar hypertrophy, and oxidative stress in the sponta-
aneously hypertensive rat, increased in left ventricular wall thickness and collagen deposition in the pressure overload
model of cardiac hypertrophy, and reduced the expression of
type IV collagen in the diabetic rat kidney (8–10). However, the
mechanisms underlying hydrogen sulfide modulation of pro-
tein synthesis have not been explored. Both renal hypertrophy and
accumulation of matrix proteins such as type IV collagen and
lamarin are cardinal manifestations of diabetic nephropathy (11).
The goal of the present study was to investigate
whether hydrogen sulfide modulates molecular events involved
in cell hypertrophy and matrix synthesis induced by high glu-
cose employing renal glomerular epithelial cells.

EXPERIMENTAL PROCEDURES

**Cell Culture**—Glomerular epithelial cells of the rat (GECs)
were grown in DMEM containing 7% FBS, 5 mM glucose, 100
units/ml penicillin, 100 μg/ml streptomycin, and 2 mM gluta-
mine as described previously (12). These cells express nephrin
and podocin, proteins synthesized by the renal glomerular
podocytes in vivo (supplemental Fig. S1). GECs were quiesced
in serum-free medium for 24 h and then incubated with 5 mM
glucose (normal), 30 mM glucose (high glucose), or mannitol (5
mM glucose + 25 mM mannitol, osmotic control) with or with-
out NaHS (50–500 μM, Sigma) for the indicated times.

**Protein Synthesis Measurement**—Protein synthesis was
measured as described previously (13). Serum-starved cells
were labeled with 10 μCi/ml [35S]methionine for the terminal
2 h of incubation. Cells were washed in PBS and lysed in radio-
immunoprecipitation assay buffer followed by centrifugation
at 14,000 rpm for 20 min at 4 °C. Cell protein content was mea-
sured with a Bio-Rad reagent using bovine serum albumin as
standard (Bio-Rad). An equal amount of protein (30 μg) was
spotted onto the 3 MM filter paper (Whatman, Maidstone,
UK). Filters were washed three times by boiling for 1 min in 10%
trichloroacetic acid (TCA) containing 0.1 g/liter methionine
before determining radioactivity.

**Cell Hypertrophy Measurement**—GECs were serum-starved
for 24 h followed by incubation with high glucose with or with-
out NaHS for 48 h. Cells were harvested by trypsinization with
0.05% trypsin/EDTA, and then an equal volume of GECs was
separated into two tubes. Total cell number was calculated
from Cell Signaling (Danvers, MA). Briefly, scrambled RNA (control) or pools of
siRNA for LKB1 or calcium calmodulin-dependent protein
kinase kinase β (CaMKKβ) were diluted into siRNA transfec-
sion medium to a final concentration of 2–20 nM. Diluted
siRNA was incubated with 6 μl of siRNA transfection reagent
for 30 min at room temperature. GECs were washed with PBS
twice and then incubated with the siRNA transfection medium
for 30 min. After 30 min, cells were incubated with the diluted
scrambled RNA or siRNA for LKB1 or CaMKKβ for 8 h, and
then medium was changed to growth medium for 48 h. After
48 h, GECs were quiesced in serum-free medium for 24 h before
performing the experiment.

**Immunoblotting**—Immunoblotting was performed as de-
scribed previously (13–15). Equal amounts of cell lysate protein
(2–20 μg) and tissue homogenates (30 μg) were separated by
SDS-PAGE and transferred to a nitrocellulose membrane. The
membrane was probed with primary antibody overnight at 4 °C.

**Immunohistochemical Studies**—Kidney sections from 3-
month-old NJ controls and type 1 OVE26 diabetic mice were
fixed in 10% formalin and embedded in paraffin. The slides
were heated in a microwave oven in citrate buffer for 15 min,
quenched in 3% hydrogen peroxide for 6 min, and washed in
Tris-buffered saline. The slides were then blocked with Sniper
blocking buffer (Biocare, Concord, CA) for 20 min and incu-
bated with the rabbit polyclonal anti-cystathionine-β-synthetase
(Abgent, San Diego, CA) and cystathionine-γ-lyase (Abcam,
Cambridge, MA).

**Animal Study**—Animal protocols were approved by the
Institutional Animal Care and Use Committee. OVE26 mice
(The Jackson Laboratory, Bar Harbor, ME) develop hypergly-
cemia and type 1 diabetes soon after birth (16, 17); control NJ
and OVE26 mice were studied at the age of 3 months. The
C57BL/KsJ lepr−/− db/db mice, a model of type 2 diabetes, and
its lean littersmates (db/m) (The Jackson Laboratory) were
maintained on regular laboratory chow. Blood glucose concen-
tration was monitored for the emergence of diabetes, which is
usually evident between 6 and 8 weeks of age. In the present
study, lean littersmate control and diabetic mice were studied in
the early phase, after 2 weeks of onset of hyperglycemia. The
db/db mice develop renal hypertrophy at 2 weeks following
onset of diabetes (18, 19). Mice were sacrificed at the end of the
experimental period, and renal cortex was dissected out and
processed for further analysis.

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Tris-buffered saline. The slides were then blocked with Sniper
blocking buffer (Biocare, Concord, CA) for 20 min and incu-
bated with the rabbit polyclonal anti-cystathionine-β-synthetase
(1:500; Abgent, San Diego, CA) overnight at 4 °C in a humidifi-
ced chamber. After rinsing, the slides were incubated with goat
anti-rabbit polymer-horseradish peroxidase (HRP) (Biocare)
for 20 min at room temperature. Immunoreactivity was visualized
with 3’-3’-diaminobenzidine (Biocare). Negative controls
were performed by omitting the incubation with the primary
antibody. Sections were photographed using Olympus AX70
(Melville, NY). Renal cortical lysates were employed in immunoblotting studies employing antibodies against cystathionine β-synthase and cystathionine γ-lyase.

**Statistical Analysis**—Data were obtained from at least three independent experiments or animals and expressed as mean ± S.E. Statistical comparisons between multiple groups were performed by ANOVA single-way analysis, and post hoc analysis was done using Student-Newman-Keuls multiple comparison test employing the GraphPad Prism 4 software. Statistical analyses between two groups were performed by the Student’s t test. A p value of < 0.05 was considered statistically significant.

**RESULTS**

**Hydrogen Sulfide Decreases High Glucose-induced Protein Synthesis, Cellular Hypertrophy, and Extracellular Matrix Protein Accumulation**—Increased protein synthesis contributes to hypertrophy of renal cells and to the accumulation of extracellular matrix proteins in the renal parenchyma in diabetes (11). We examined the effect of hydrogen sulfide on high glucose stimulation of protein synthesis in the GECs. High glucose increased de novo protein synthesis by 30% at 48 h (Fig. 1A, p < 0.01 by ANOVA); equimolar mannitol did not affect protein synthesis for up to 72 h (supplemental Fig. S2A). Co-incubation with NaHS reduced high glucose-stimulated protein synthesis, reaching significance at 250 μM (Fig. 1A, p < 0.05); however, NaHS did not affect the rate of protein synthesis in cells incubated with 5 mM glucose. Similar inhibition of de novo protein synthesis was also seen with mouse podocytes (supplemental Fig. S2B), suggesting that hydrogen sulfide evokes similar responses in GECs from two distinct species. Cellular hypertrophy, measured as protein content per unit cell number, was increased by 25% by high glucose at 48 h (Fig. 1B, p < 0.001). NaHS significantly inhibited high glucose-stimulated cellular hypertrophy (Fig. 1B, p < 0.001), the cell content of protein reaching levels seen in cells incubated with 5 mM glucose; NaHS alone did not affect cellular protein content.

Of relevance to diabetic nephropathy, high glucose promotes synthesis of extracellular matrix proteins in the GECs (20). We examined whether NaHS inhibits high glucose-induced expression of laminin γ1 and collagen IV α5 in the GECs. Laminin γ1 and collagen IV α5 are parts of laminin trimer (laminin α5, β2, γ1, LM-521) and type IV collagen trimer (α3, α4, α5), respectively, deposited in the mature glomerular basement membrane following synthesis by the GECs in vivo (21). High glucose increased the expression of laminin γ1 and collagen IV α5 at 48 h in the GECs (Fig. 1C, p < 0.001, Fig. 1D, p < 0.05); these changes were significantly reduced by co-incubation with NaHS (Fig. 1C, p < 0.001, Fig. 1D, p < 0.05). Similar inhibition of high glucose-stimulation of laminin γ1 synthesis was also observed in mouse podocytes and proximal tubular epithelial cells (supplemental Fig. S2, C and D); laminin γ1 is a component of the basement membrane of tubules. We also examined whether preincubation with NaHS for 30 min followed by removal would still result in inhibition of matrix protein synthesis in the GECs. Thus, GECs were exposed to NaHS or the vehicle for 30 min in a medium containing either 5 mM glucose or 30 mM glucose. Then, the media were changed with fresh media without NaHS with the respective glucose concentrations; laminin γ1 expression was examined 48 h later. Brief exposure to NaHS was sufficient to inhibit high glucose-stimulated laminin γ1 expression 48 h later, suggesting that NaHS sets metabolic reactions in motion in a rapid manner that result
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**FIGURE 2. Hydrogen sulfide inhibits high glucose-stimulated initiation phase of mRNA translation.** Quiescent cells were incubated with 30 mM Glu with or without 250 μM NaHS for 30 and 60 min. Equal amounts of cell lysate protein were immunoblotted with specific antibodies. *A*, antibody against phosphorylated Thr-36/47 on 4E-BP1 (P-4E-BP1) or 4E-BP1, B, antibody against phosphorylated Thr-389 on p70S6 kinase (P-S6K) or p70S6 kinase (S6K). Composite data from three experiments are shown as a histogram (*, p < 0.05; **, p < 0.01 versus control; #, p < 0.05 versus Glu by ANOVA). *B*, antibody against PDCD4 or actin. Composite data from eight experiments are shown as a histogram (*, p < 0.05 versus control; ###, p < 0.001 versus control; #, p < 0.001 versus Glu by ANOVA). *C*, antibody against PDCD4 or phosphorylated p70S6 kinase or p70S6 kinase or hemagglutinin (HA). Composite data from four experiments are shown as a histogram (*, p < 0.05; **, p < 0.001 versus control; #, p < 0.001 versus Glu by ANOVA). *D*, antibody against PDCD4 or 4E-BP1 or 4E-BP1. Composite data from four experiments are shown as a histogram (*, p < 0.05 versus control; #, p < 0.05 versus Glu by ANOVA). *E*, experiments were done as described above with or without incubation for 1 h with 10 μM MG-132, a proteasomal inhibitor. Equal amounts of cell lysates were immunoblotted with antibody against PDCD4 and actin. A representative blot and composite graph from four experiments is shown, *, p < 0.05 versus control; #, p < 0.05 versus Glu by ANOVA).

in sustained inhibition of matrix proteins for a long period of time. Together, these data show that hydrogen sulfide inhibits high glucose-induced general protein synthesis, cellular hypertrophy, and extracellular matrix protein synthesis in the GECs. We initiated the investigation on the underlying mechanism.

**Hydrogen Sulfide Regulates Translation Initiation and Elongation Phases by Inhibiting mTOR Pathway**—Translation of mRNA is a rate-limiting step in gene expression culminating in protein synthesis (22). High glucose stimulation of protein synthesis and hypertrophy in the GECs involves induction of initiation and elongation phases of mRNA translation (12, 13). Several important events in these phases of translation are under the control of mTOR complex 1 (23, 24). Phosphorylation of 4E-BP1 and p70S6 kinase serves as a readout of mTOR complex 1 activation (22, 25). Under normal conditions, the eukaryotic initiation factor 4E (eIF4E) is held inactive by its binding partner 4E-BP1. Phosphorylation of 4E-BP1 results in the release of eIF4E, permitting it to bind other eIFs to form the eIF4F complex and bind to the cap of the mRNA (26). Mitigation of high glucose-induced protein synthesis by NaHS in the GECs prompted us to test whether NaHS affects the initiation phase of mRNA translation.

High glucose increased 4E-BP1 phosphorylation at 30 and 60 min (*p < 0.05) that was abolished by NaHS (Fig. 2A, p < 0.05). Incubation of GECs with high glucose increased p70S6 kinase phosphorylation on Thr-389 (*p < 0.01) that was significantly reduced by co-incubation with NaHS (Fig. 2B, *p < 0.05). In addition to phosphorylating ribosomal proteins, p70S6 kinase regulates the activation of eIF4F during translation initiation. During the initiation phase, eIF4A, a DEAD box protein, forms eIF4F complex by associating with eIF4G and eIF4E (26). As a part of eIF4F, eIF4A functions as a helicase to resolve complexities in the 5'-untranslated region of the mRNA and assist the 40S ribosomal subunit in the preinitiation complex in locating the AUG start codon (27). This action is facilitated by eIF4B and eIF4H (28). Programmed cell death protein 4 (PDCD4) binds eIF4A at its MA3 domains (29) and keeps it in an inactive complex in the resting cell. When protein synthesis is stimulated, PDCD4 is phosphorylated by p70S6 kinase and undergoes ubiquitination by the E3 ligase Skp-cullin-F-box (SCF)-β-transducin repeat-containing protein (βTRCP) and proteasomal degradation (30). In the GECs, high glucose reduced the expression of PDCD4 (Fig. 2C, *p < 0.05), which was inhibited by NaHS (Fig. 2C, *p < 0.001); NaHS alone tended to augment...
the expression of PDCD4. Expression of dominant negative p70S6 kinase abolished the ability of high glucose to reduce PDCD4 content in the GECs (Fig. 2D). The abundance of PDCD4 in control cells was augmented by dominant negative p70S6 kinase, indicating that the basal expression of the protein was also regulated by the kinase. Rapamycin also abolished high glucose inhibition of PDCD4 expression, confirming the role of mTOR complex 1 (supplemental Fig. S3). Co-immunoprecipitation experiments showed that high glucose promoted dissociation of eIF4A from PDCD4 (p < 0.05) and increased its association with eIF4G (p < 0.05) (Fig. 2E); these data showed that eIF4F formation was stimulated by high glucose. NaHS abrogated these reactions and maintained eIF4A binding to PDCD4 (Fig. 2E, p < 0.05). Due to the greater availability of PDCD4 following NaHS treatment, there was a trend toward greater binding to eIF4A in control cells incubated with 5 mM glucose. The addition of MG-132 inhibited (p < 0.05) high glucose-induced reduction in PDCD4 expression (p < 0.05) (Fig. 2F), confirming that high glucose promoted PDCD4 degradation via the proteasomal pathway; MG-132 alone did not alter the PDCD4 expression.

Activated p70S6 kinase regulates important aspects of the elongation phase of mRNA translation. During elongation phase, movement of aminoacyl tRNA from the A site in the ribosome to the P site is facilitated by the eukaryotic elongation factor 2 (eEF2), which is active when dephosphorylated on Thr-56 (31). Phosphorylation of eEF2 on Thr-56 is under the control of eEF2 kinase, a calcium/calmodulin-dependent kinase III (32). p70S6 kinase phosphorylates eEF2 kinase on Ser-366 and inhibits its activity (33). High glucose increases Ser-366 phosphorylation of eEF2 kinase and decreases eEF2 phosphorylation on Thr-56, resulting in eEF2 activation in the GECs and proximal tubular epithelial cells (12, 13, 19). High glucose promoted Ser-366 phosphorylation of eEF2 kinase (p < 0.05) and dephosphorylation of Thr-56 on eEF2 (p < 0.05); NaHS inhibited these changes (Fig. 3A, p < 0.05, Fig. 3B, p < 0.05). Unlike high glucose, equimolar mannitol did not affect any of the aforementioned phosphorylation events (supplemental Fig. S4). These data demonstrate that hydrogen sulfide abrogates high glucose-stimulated initiation and elongation phases of mRNA translation by inhibiting mTOR complex 1 activity.

Next, we sought to identify the kinases upstream of mTOR complex 1 that may be regulated by hydrogen sulfide. In the GECs, AMP-activated protein kinase (AMPK) inhibits mTOR complex 1 activation induced by high glucose (12, 13). Therefore, we explored whether AMPK is activated by hydrogen sulfide in inhibiting mTOR complex 1 in the high glucose-treated GECs.

**Hydrogen Sulfide Activates AMPK by Inhibiting High Glucose-induced AMPK Dephosphorylation**—AMPK is a heterotrimer consisting of a catalytic subunit (α) and two regulatory subunits (β and γ). Phosphorylation of Thr-172 of the α subunit is essential for AMPK activity (34). NaHS significantly increased AMPK phosphorylation at 5 and 15 min in the GECs and returned to baseline by 30 min (Fig. 4A, p < 0.05). NaHS dose-dependently increased AMPK phosphorylation at 5 min, which reached significance at 250 and 500 μM (Fig. 4B, p < 0.05, p < 0.01). NaHS also induced AMPK phosphorylation in mouse podocytes and proximal tubular epithelial cells with approximately the same temporal profile (supplemental Fig. 5, A and B). We next examined whether NaHS affects high glucose-induced change in AMPK phosphorylation. High glucose reduced AMPK phosphorylation at 5–60 min (p < 0.05 or p < 0.01), but equimolar mannitol, serving as osmotic control, had no effect on AMPK phosphorylation (Fig. 4C). NaHS restored high glucose-induced reduction in AMPK phosphorylation to normal at 30 (p < 0.05) and 60 min (p < 0.01) (Fig. 4D).
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FIGURE 4. Hydrogen sulfide restores high glucose-induced reduction in AMPK phosphorylation. A, quiescent cells were treated with 250 μM NaHS for the indicated times. Equal amounts of cell lysate protein were immunoblotted with antibody against α subunit of AMPK phosphorylated on Thr-172 (P-AMPK) or AMPK antibody. A representative blot and composite graph from four experiments are shown (*, p < 0.05; **, p < 0.01 versus control by ANOVA). B, quiescent cells were treated with the indicated concentration of NaHS for 5 min. A representative blot and composite graph from three experiments are shown (*, p < 0.05 versus control by ANOVA). C, quiescent cells were incubated with 30 mM Glu or 5 mM glucose + 25 mM mannitol for the indicated times. A representative blot and composite graph from five experiments are shown (*, p < 0.05 versus control by ANOVA). D, quiescent cells were incubated with 30 mM glucose with or without 250 μM NaHS for 30 and 60 min. Equal amounts of cell lysate protein were immunoblotted with antibody against phosphorylated Thr-172 on the α subunit of AMPK or AMPK antibody. A representative blot and composite graph from four experiments are shown (*, p < 0.05 versus control, #, p < 0.05; ##, p < 0.01 versus Glu by ANOVA).

synthesis (Fig. 5B, p < 0.05 versus Glu+NaHS). In a similar manner, Compound C blocked the reduction of laminin γ1 content induced by NaHS in GECs treated with high glucose (Fig. 5C, p < 0.05 versus Glu+NaHS). Although incubation with Compound C alone did not affect basal protein synthesis, there was a trend toward increasing laminin γ1 expression that did not reach statistical significance. These data show that AMPK activation is required for hydrogen sulfide mitigation of high glucose-induced protein synthesis and laminin γ1 expression in the GECs.

Hydrogen Sulfide Inhibits High Glucose-induced mRNA Translation via Activation of AMPK—NaHS blocked signaling events necessary for high glucose-induced stimulation of mRNA translation (Fig. 2). We examined whether AMPK activation is required for NaHS-induced inhibition of mRNA translation in high glucose-treated cells. High glucose significantly increased phosphorylation of 4E-BP1 (Fig. 6A, p < 0.01) and p70S6 kinase (Fig. 6B, p < 0.05). High glucose promoted dephosphorylation of eEF2 at Thr-56 (Fig. 6C, p < 0.01); NaHS significantly reversed high glucose-induced changes in phosphorylation of 4E-BP1, p70S6 kinase, and eEF2, respectively (Fig. 6, A–C, p < 0.05–p < 0.001). Compound C significantly abrogated the effect of NaHS on high glucose-induced changes in phosphorylation of 4E-BP1, p70S6 kinase (Fig. 6A, p < 0.05, Fig. 6B, p < 0.001 versus Glu+NaHS), and eEF2 (Fig. 6C, p < 0.01 versus Glu+NaHS). Compound C alone increased phosphorylation of 4E-BP1 (p < 0.01) and p70S6 kinase (p < 0.001) and decreased phosphorylation of eEF2 (p < 0.001) (Fig. 6D, B and C). Furthermore, Compound C abolished the effect of hydrogen sulfide on high glucose-induced reduction in PDCD4 expression (Fig. 6D). These data show that the ameliorative effect of NaHS on mRNA translation and protein synthesis induced by high glucose occurs through the activation of AMPK. Activated AMPK in turn blocks mTOR complex 1-reg-


**Figure 6.** AMPK mediates hydrogen sulfide inhibition of high glucose-stimulated mTOR complex 1 activation. Quiescent cells were preincubated with 10 μM Compound C (CC) for 30 min. Cells were incubated with high glucose with or without 250 μM NaHS for 30 and 60 min. Equal amounts of cell lysate protein were immunoblotted with antibody against the indicated proteins. A, antibody against phospho-4E-BP1 (Thr-47) or 4E-BP1 (**, p < 0.01 versus control, #, p < 0.05 versus Glu; §§, p < 0.001 versus Glu + NaHS by ANOVA). Representative blots and histograms on composite data from five experiments are shown. B, antibody against phospho-p70S6 kinase (P-S6K) (Thr-389) and p70S6 kinase (S6K) (**, p < 0.05; ***, p < 0.001 versus control, #, p < 0.05 versus Glu; §§, p < 0.001 versus Glu + NaHS by ANOVA). Representative blots and histograms on composite data from five experiments are shown. C, antibody against phospho-eEF2 (Thr-56) or eEF2 (**, p < 0.01; ***, p < 0.001 versus control, #, p < 0.05 versus Glu; §§, p < 0.01 versus Glu + NaHS by ANOVA). Representative blots and histograms on composite data from five experiments are shown. D, antibody against PDCD4 or actin (**, p < 0.05; **, p < 0.01 versus control, #, p < 0.01 versus Glu; §§, p < 0.001 versus Glu + NaHS by ANOVA). Representative blots and histograms on composite data from four experiments are shown.

**Figure 7.** Hydrogen sulfide increases AMPK phosphorylation through the CaMKKβ pathway. A, serum-starved GECs were preincubated with 10 μM STO-609 (STO), a selective inhibitor of CaMKKβ, for 1 h followed by treatment with or without 250 μM NaHS for 5 min. Equal amounts of cell lysate protein were immunoblotted with antibody against phosphorylated Thr-172 on the α subunit of AMPK (p-AMPK) or AMPK antibody. A representative blot and composite graph from four experiments are shown (*, p < 0.05 versus control, #, p < 0.05 versus NaHS by ANOVA). B, control (CON) scrambled RNA and siRNA for LKB1 and/or CaMKKβ (CKKβ) was diluted into the siRNA transfection medium to a final concentration of 2–20 nM. Diluted siRNA was incubated with 6 μM of siRNA transfection reagent for 30 min at room temperature. GECs were washed with PBS twice and then incubated with the siRNA transfection medium for 30 min. After 30 min, cells were incubated with the diluted siRNA of control and LKB1 or CaMKKβ for 8 h. and then medium was changed to growth medium for 48 h. After 48 h, GECs were quiesced in serum-free medium for 24 h. GECs were treated with 250 μM NaHS for 5 min. Equal amounts of cell lysates were immunoblotted with the indicated antibodies. The histogram represents the composite data from five experiments (*, p < 0.05; **, p < 0.01 versus control, ###, p < 0.001 versus NaHS alone by ANOVA).

ulated pathways of initiation and elongation phases of mRNA translation, in agreement with a previous study (13).

**Role of CaMKKβ in Hydrogen Sulfide Induction of AMPK Phosphorylation—**LKB1 and CaMKKβ are two major kinases for AMPK phosphorylation (38). LKB1 is a tumor suppressor, and several human tumor cell lines, including HeLa cells, lack LKB1 expression (39). To identify the kinase upstream of AMPK, we first examined the effect of NaHS on AMPK phosphorylation in HeLa cells. NaHS-stimulated AMPK phosphorylation in HeLa cells was abrogated by STO-609, a CaMKKβ specific inhibitor (40) (supplemental Fig. S6A, p < 0.01). These data suggested that CaMKKβ may be activated by NaHS to induce AMPK phosphorylation.

Incubation of GECs with NaHS led to increased AMPK phosphorylation (Fig. 7A, p < 0.05). STO-609 abolished NaHS induction of AMPK phosphorylation (p < 0.05 versus NaHS); composite data from several experiments showed that it did not affect basal AMPK phosphorylation. These data suggested that CaMKKβ serves as the upstream kinase induced by NaHS to stimulate AMPK phosphorylation in the GECs. To further explore upstream kinases, we employed pools of specific siRNA against LKB1 and CaMKKβ. Immunoblotting with the respec-
tive antibodies showed that the GECs express both LKB1 and CaMKKβ (Fig. 7B), the former being more abundant. Expression of LKB1 and CaMKKβ proteins was significantly reduced in cells transfected with the respective siRNA but not in the cells transfected with control scrambled RNA (Fig. 7B, supplemental Fig. S6B). Treatment of GECs with NaHS increased AMPK phosphorylation in the control RNA-transfected cells (p < 0.01 versus control by ANOVA) and in LKB1 siRNA-transfected cells (p < 0.05 versus control by ANOVA). NaHS could not significantly increase AMPK phosphorylation in CaMKKβ siRNA-transfected cells when compared with control RNA-transfected cells treated with NaHS (p < 0.001). In cells transfected with siRNA for both CaMKKβ and LKB1, NaHS was not able to induce phosphorylation of AMPK (Fig. 7B, top panel), suggesting that CaMKKβ accounts for most of the phosphorylation of AMPK following exposure to NaHS, with a possible additional contribution from LKB1.

Kidney Cortex Expression of Cystathionine β-Synthase and Cystathionine γ-Lyase Is Decreased in Diabetes—We examined the expression of cystathionine β-synthase and cystathionine γ-lyase, the enzymes that generate hydrogen sulfide, in the kidney cortex from type 1 and type 2 diabetic mice. Immunoblotting showed that the expression of cystathionine β-synthase and cystathionine γ-lyase was reduced in the kidney cortex of OVE26 mice with type 1 diabetes (Fig. 8A, p < 0.05 versus control) and in db/db mice with type 2 diabetes (Fig. 8B, p < 0.01 versus control). Immunoperoxidase staining of the kidney showed that normal mice expressed cystathionine β-synthase mainly in the tubules; the expression of the enzyme was significantly reduced in diabetic mice (Fig. 8C). Reduction in expression of cystathionine β-synthase and cystathionine γ-lyase occurred at a time when renal hypertrophy and onset of matrix laminin and type IV collagen accumulation are evident in these models of diabetes (17–19, 41). These data suggest that conditions for decreased synthesis of hydrogen sulfide exist in the kidney in both type 1 and type 2 diabetes that would facilitate increased protein synthesis required for renal hypertrophy and matrix protein accumulation.

**DISCUSSION**

Our data show that hydrogen sulfide inhibits high glucose induction of synthesis of proteins and matrix protein expression in the GEC. The underlying mechanism involves activation of AMPK by its upstream kinase CaMKKβ. By promoting activity of AMPK, hydrogen sulfide inhibits mTOR complex 1 and events in the initiation and elongation phases of translation that are under its control. Reduction of cystathionine β-synthase and cystathionine γ-lyase, enzymes that generate hydrogen sulfide, in the kidneys of mice with type 1 and type 2 diabetes supports the notion that inhibition of hydrogen sulfide contributes to clinical expression of diabetic nephropathy (Fig. 9).

Hydrogen sulfide is generated by the action of cystathionine γ-lyase and cystathionine β-synthase on L-cysteine in the presence of pyridoxal 5’-phosphate; 3-mercaptoppyruvate sulfotransferase desulfurates mitochondrial 3-mercaptoppyruvate to release hydrogen sulfide (42). Both cystathionine γ-lyase and cystathionine β-synthase are expressed in the kidney (7, 43). Following release, hydrogen sulfide can be stored in cells as bound sulfane sulfur that can be released by reducing conditions (44). Bound sulfane sulfur is the major determinant of physiological functions of hydrogen sulfide (45–47). Recent studies indicate that hydrogen sulfide has diverse effects on cell function including cell survival, hemodynamics, and inflammation (44); however, its role in protein synthesis has not been examined in detail.

Our data suggest that in the context of high glucose exposure, hydrogen sulfide serves as an inhibitor of protein synthesis. The prediction would be that hydrogen sulfide generation is reduced in the kidney in diabetes. Previous studies have shown that plasma hydrogen sulfide level is lower in human subjects with type 2 diabetes (48, 49) and in end stage kidney disease patients on hemodialysis (50). Relevant to diabetic kidney disease, hydrogen sulfide levels in the kidney are reduced in a chemical model of type 1 diabetes in the rat (8). Our finding of reduction in the expression of cystathionine β-synthase and cystathionine γ-lyase in the kidney suggests that synthesis of hydrogen sulfide would be reduced, coinciding with renal hypertrophy and matrix accumulation in mice with either type...
1 or type 2 diabetes. Reduction in hydrogen sulfide would remove an endogenous break on pathways regulating protein synthesis in the kidney and facilitate high glucose to induce renal cell hypertrophy and augment expression of matrix proteins.

Kidney hypertrophy and accumulation of extracellular matrix proteins causing renal fibrosis are cardinal manifestations of diabetic kidney disease. Previous studies have linked renal hypertrophy, which appears early in the course of diabetes, with accumulation of matrix proteins that becomes evident later (51). Hyperglycemia induces renal hypertrophy and podocyte apoptosis in type 1 diabetic mice by inhibiting AMPK activity (35). Both hypertrophy and renal fibrosis require stimulation of protein synthesis. Previous studies showed that hydrogen sulfide reduced hypertrophy of intramyocardial arterioles and cardiac ventricular fibrosis (9), supporting the contention that it is an inhibitor of protein synthesis. There is consensus that the initiation phase of mRNA translation is a rate-limiting step in synthesis of proteins (22). Because both the initiation and the elongation phases of mRNA translation are partly under the control of mTOR complex 1, hydrogen sulfide modulation of high glucose induction of mTOR complex 1 activity was explored, using phosphorylation of p70S6 kinase and 4E-BP1 as a readout. High glucose increased the phosphorylation of these two mTOR complex 1 targets that was inhibited by hydrogen sulfide. Our data show that hydrogen sulfide averted high glucose induction of phosphorylation of 4E-BP1 and p70S6 kinase and degradation of PDCD4 induced by high glucose. This would reduce formation of eIF4F by locking up eIF4E and eIF4A in their respective inhibitory complexes with 4E-BP1 and PDCD4, respectively. Hydrogen sulfide showed a trend toward increased PDCD4 expression in cells incubated with 5 mM glucose; these data suggest that the basal expression of PDCD4 may be under the control of AMPK induced by hydrogen sulfide. Because p70S6 kinase 1 governs PDCD4 degradation, these data are consistent with suppression of mTORC1-p70S6 kinase 1 axis by AMPK induced by hydrogen sulfide. Because the increment in PDCD4 was greater than basal levels seen in cells incubated with 5 mM glucose, it is possible that there are additional mechanisms by which hydrogen sulfide regulates PDCD4 expression. In addition to the events in the initiation phase, p70S6 kinase also regulates the elongation phase of translation. In the current study, high glucose induced changes in phosphorylation of p70S6 kinase 1, eEF2 kinase, and eEF2, which would stimulate the elongation phase of translation; these changes were significantly inhibited by hydrogen sulfide. Thus, the inhibitory effect of hydrogen sulfide on protein synthesis is achieved by interference with key events in both the initiation and the elongation phases of mRNA translation. Because hydrogen sulfide did not affect basal protein synthesis, it appears to be effective as an inhibitor in the context of stimulation of protein synthesis by agents such as high glucose in the GEC.

High glucose recruits mTORC1 to promote protein synthesis and hypertrophy in kidney epithelial and mesangial cells (13, 19, 35, 52–54). One mechanism by which mTOR complex 1 is stimulated by high glucose in the GECs involves inhibition of AMPK phosphorylation and activity (12, 13). Given the similarity between AMPK and hydrogen sulfide as inhibitors of protein synthesis in the context of high glucose exposure, possible mediation of the effect of hydrogen sulfide by AMPK was investigated. Hydrogen sulfide dose-dependently stimulated Thr-172 phosphorylation of the catalytic subunit of AMPK. High glucose-induced reduction in AMPK phosphorylation and activity between AMPK and hydrogen sulfide as inhibitors of protein synthesis was restored to normal level by hydrogen sulfide. Reversal of the ameliorative effect of hydrogen sulfide on high glucose-induced stimulation of protein synthesis and inhibition of AMPK phosphorylation by Compound C, an AMPK-selective inhibitor, demonstrated that AMPK was the mediator of the effect of hydrogen sulfide on protein synthesis in cells exposed to high glucose. Thr-172 phosphorylation of the catalytic subunit of AMPK is catalyzed by LKB1 and CaMKKβ. Observations with STO-609 and siRNA showed that in the GEC, hydrogen sulfide stimulation of AMPK is mediated by CaMKKβ. The mechanism by which AMPK inhibits mTOR complex 1 involves the TSC1/TSC2-Rheb pathway (55); recently, AMPK has also been shown to inhibit mTOR complex 1 by phosphorylating raptor, independent of TSC2 (56). Further investigation is needed to study the mechanism by which CaMKKβ is stimulated by hydrogen sulfide. Hydrogen sulfide induction of AMPK phosphorylation has also been described in the brain in a rodent model of cardiac arrest (57).

There are potential clinical implications for the use of agents that generate hydrogen sulfide. As mentioned above, NaHS ameliorated kidney injury in a rat model of chemically induced diabetes; however, the molecular mechanisms by which hydrogen sulfide exerted its protective effect were not explored (8).
NaHS has been shown to attenuate hypertension in an experimental model of renal artery stenosis by reducing the mRNA and protein expression of renin (58). Reduction in ischemia-reperfusion-induced injury to the kidney has been reported by augmenting cystathionine γ-lyase expression in the kidney (59). Sen et al. (60, 61) have also described ameliorative effects of hydrogen sulfide on the progression of kidney disease in rodents with hyperhomocysteinemia. Hydrogen sulfide affords protection to the heart against ischemia-reperfusion injury (5, 62), and clinical trials evaluating its therapeutic role in amelioration of ischemic heart disease are underway (44). The mechanisms of cardiac protection may involve diverse pathways and include inhibition of macrophage infiltration of the myocardium, reduction in mitochondrial respiration and preservation of mitochondrial structure, and inhibition of apoptosis (5, 61). These protective phenotypes were reproduced by myocardium-specific overexpression of cystathionine γ-lyase (5). Other mechanisms contributing to hydrogen sulfide protection of myocardium include opening of the K-ATP channels in mitochondria and/or sarcolemna (63, 64), induction of antioxidant genes by Nrf-2, and resistance to apoptosis by stimulation of survival pathways (65). Recruitment of specific pathways by hydrogen sulfide in diverse tissues is likely to be dependent on the context of injury. Future studies are needed to explore whether some of these pathways are also stimulated by hydrogen sulfide in the kidney in the setting of hyperglycemia-induced injury. The availability of agents that promote hydrogen sulfide release in vivo (42) makes it worthwhile to test it as a therapeutic modality in diabetic kidney disease.

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