Hydrogen sulfide has recently been found decreased in chronic kidney disease. Here we determined the effect and underlying mechanisms of hydrogen sulfide on a rat model of unilateral ureteral obstruction. Compared with normal rats, obstructive injury decreased the plasma hydrogen sulfide level. Cystathionine-β-synthase, a hydrogen sulfide-producing enzyme, was dramatically reduced in the ureteral obstructed kidney, but another enzyme cystathionine-γ-lyase was increased. A hydrogen sulfide donor (sodium hydrogen sulfide) inhibited renal fibrosis by attenuating the production of collagen, extracellular matrix, and the expression of α-smooth muscle actin. Meanwhile, the infiltration of macrophages and the expression of inflammatory cytokines including interleukin-1β, tumor necrosis factor-α, and monocyte chemoattractant protein-1 in the kidney were also decreased. In cultured kidney fibroblasts, a hydrogen sulfide donor inhibited the cell proliferation by reducing DNA synthesis and downregulating the expressions of proliferation-related proteins including proliferating cell nuclear antigen and c-Myc. Further, the hydrogen sulfide donor blocked the differentiation of quiescent renal fibroblasts to myofibroblasts by inhibiting the transforming growth factor-β1-Smad and mitogen-activated protein kinase signaling pathways. Thus, low doses of hydrogen sulfide or its releasing compounds may have therapeutic potentials in treating chronic kidney disease. 

**KEYWORDS:** cell signaling; chronic inflammation; chronic renal disease

---

**Correspondence:** Chun-Feng Liu, Department of Neurology, Second Affiliated Hospital of Soochow University, 1055 Sansiang Road, Suzhou 215004, China. E-mail: liucf@suda.edu.cn or Li-Fang Hu, Institute of Neuroscience, Soochow University, 199 Ren-Ai Road, Suzhou Industrial Park, Suzhou 215123, China. E-mail: hullifang@suda.edu.cn

Received 26 January 2013; revised 1 August 2013; accepted 29 August 2013; published online 27 November 2013

---

**OPEN**

**see commentary on page 1255**

**Hydrogen sulfide inhibits the renal fibrosis of obstructive nephropathy**

Kai Song1,2,6, Fen Wang1,6, Qian Li1, Yong-Bing Shi2, Hui-Fen Zheng1, Hanjing Peng3, Hua-Ying Shen2, Chun-Feng Liu1,4 and Li-Fang Hu1,4,5

1Institute of Neuroscience, Soochow University, Suzhou, China; 2Department of Nephrology, Second Affiliated Hospital of Soochow University, Suzhou, China; 3Department of Chemistry and Center for Diagnostics and Therapeutics, Georgia State University, Atlanta, Georgia, USA; 4Department of Neurology, Second Affiliated Hospital of Soochow University, Suzhou, China and 5Department of Pharmacology, School of Pharmaceutical Science, Soochow University, Suzhou, China

Kidney International (2013) 85, 1318–1329; doi:10.1038/ki.2013.449; published online 27 November 2013

Tubulointerstitial fibrosis is the final common pathway to chronic kidney diseases (CKD).1 Pathologically, renal fibrosis manifests as the formation of myofibroblasts and the deposition of extracellular matrix proteins in the renal interstitium.2 The mechanisms of renal fibrosis have not been fully elucidated and effective drugs are still scarce. Renin-angiotensin-aldosterone system is one of the main culprits of renal fibrogenesis, and renin-angiotensin–aldosterone system inhibitors remain the first-line drugs in fighting renal fibrosis.3 However, the renin-angiotensin–aldosterone system inhibitors may deteriorate renal function and cause hyperpotassemia when serum creatinine rises above 3 mg/dl.4 New antifibrotic agents are therefore needed to expand therapeutic options and decrease side effects, which is especially important for azotemia patients.

Hydrogen sulfide (H$_2$S) represents the third gasotransmitter along with nitric oxide and carbon monoxide.5 It is generated by cystathionine-γ-lyase (CSE), cystathionine-β-synthase (CBS), and 3-mercaptoppyruvate sulphurtransferase. CBS and CSE are enriched in renal proximal tubules and produce H$_2$S in kidney in a combined way.6 H$_2$S plays various physiological and pathological roles in the kidney. For instance, it exhibits diuretic, natriuretic, and kaliuretic properties by increasing glomerular filtration rate and functions as an oxygen sensor in the renal medulla.6,7 Recently, it was reported that plasma H$_2$S level was decreased in 5/6 nephrectomy rat and uremia patients,8,9 suggesting that uremic toxin of CKD impairs the production of endogenous H$_2$S.

Notably, the biological functions of H$_2$S in CKD are not fully understood. Heterozygous cbs$^{-/}$ mice with unilateral nephrectomy, an animal model of CKD, developed proteinuria and collagen deposition, and increased the expressions of matrix metalloproteinase-2 and -9.10 Emerging evidence also demonstrates that H$_2$S exhibits antifibrotic effects in the lung, heart, and liver.11-13 Furthermore, H$_2$S bears some similarities to the other two gaseous molecules, nitric oxide and carbon monoxide, both of which protect the kidney from renal fibrosis.14,15 Taken together, we hypothesize that H$_2$S may attenuate renal fibrosis.
In this study, we examined the effect of H2S on unilateral ureteral obstructive (UUO) animal model and defined its safe and effective dosage range. Furthermore, we investigated the roles of H2S in renal fibroblast proliferation and differentiation. The potential mechanisms were also explored.

RESULTS
CBS expression and plasma H2S levels are decreased in the obstructed kidney
To investigate whether endogenous H2S was involved in the pathogenesis of renal fibrosis, we examined the expression and activity of H2S-producing enzymes CBS and CSE, as well as plasma H2S levels. The CBS expression was nearly completely ablated by obstructive injury at day 14, whereas CSE was increased. In contrast, the expressions of CBS and CSE in the contralateral kidneys were not affected (Figure 1a–c). Moreover, H2S generation in the obstructed kidneys was dramatically reduced compared with sham-operated rats (Figure 1d). Plasma H2S levels were reduced by ∼30% in UUO rats compared with the sham counterparts (27.5 ± 4.3 μmol/l vs. 39.4 ± 6.3 μmol/l, P = 0.021, Figure 1e).

Immunohistochemistry staining indicated that CBS was predominantly expressed in proximal renal tubules. UUO injury time-dependently reduced CBS expression in the obstructed kidneys without affecting that in the contralateral and sham-operated kidneys (Figure 2a and b). In contrast, CSE was mainly located in renal glomeruli, interstitium, and interlobular arteries of normal rats. UUO injury markedly increased the CSE expression in the interstitium of obstructed kidney and increased the renal cortical thickness in UUO rats. However, these effects were not observed in the rats treated with NaHS at a higher dose of 560 μg/kg/day or PAG (Figure 3). Compared with UUO rats, NaHS (56 μg/kg/day) decreased serum creatinine levels, whereas PAG increased serum urea nitrogen concentration. There were no differences of serum electrolytes among each group (Supplementary Data and Supplementary Table S1 online).

NaHS attenuates renal tubulointerstitial injury and collagen deposition in renal interstitium
Hematoxylin and eosin staining results indicated that UUO rats exhibited dilated renal tubules, epithelial cells atrophy, interstitial expansion, and increased infiltration of inflammatory cells.
cells at 14 days after operation. The tubulointerstitial injury of UUO rats was ameliorated when treated with NaHS (5.6 and 56 μg/kg/day) and enalapril. NaHS at 560 μg/kg/day and PAG failed to ameliorate the tubulointerstitial injury compared with UUO group (Supplementary Data and Supplementary Figure S1 online).

Masson trichrome staining demonstrated that UUO injury resulted in a significant accumulation of collagen fibrils (blue area) compared with the sham group. Enalapril and NaHS (5.6 and 56 μg/kg/day) attenuated the fibrotic lesions with less collagen deposited in the renal interstitium. In contrast, NaHS at 560 μg/kg/day and PAG increased the collagen production compared with that of the UUO group (Figure 4a and b).

NaHS inhibits the expression of SMA and fibronectin in the obstructed kidney
To evaluate whether H2S reduced the production of myofibroblasts and extracellular matrix, the renal expression of α-smooth muscle actin (α-SMA) and fibronectin were detected on day 14 after UUO operation. Immunohistochemistry staining and semiquantitative analysis showed that enalapril and NaHS (5.6 and 56 μg/kg/day) decreased the expressions of α-SMA and fibronectin of the UUO rats. In contrast, NaHS 560 μg/kg/day and PAG increased the production of these proteins, but statistical difference was not obtained in the PAG group (Figure 5a–d). These observations were verified by western blot analysis, in which NaHS, especially at the dose of 56 μg/kg/day, markedly reduced the expressions of α-SMA, fibronectin, phospho-Smad3, and transforming growth factor-β1 (TGF-β1) (Figure 6a–d).

NaHS inhibits inflammation in the kidney after obstructive injury
The effect of H2S on the infiltration of inflammatory cells in obstructed kidneys was also determined at 7 days after UUO. CD68 (a macrophage marker) staining in renal cortex revealed an increased macrophage infiltration in the renal interstitium of UUO rats. NaHS (5.6 and 56 μg/kg/day) and enalapril markedly reduced the number of macrophage in the interstitium against the UUO group. This effect was most pronounced in the NaHS (56 μg/kg/d)-treated group. Compared with the UUO group, NaHS at 560 μg/kg/day increased the number of macrophage in renal cortex, whereas PAG tended but failed to significantly increase the CD68-positive cell number (Figure 7a and b).

To confirm the anti-inflammatory effect of NaHS, the mRNA levels of interleukin-1β, tumor necrosis factor-α, and monocyte chemoattractant protein-1 were determined by quantitative PCR. All these inflammatory molecules were
Figure 3 | Sodium hydrosulfide (NaHS) treatment decreases the size and increases the cortical thickness of unilateral ureteral obstructive (UUO) kidney. (a) General appearance of four representative left kidneys of rats subjected to various treatments. UUO rats received vehicle (saline) treatment. NaHS (5.6, 56, and 560 μg/kg/day) and DL-propargylglycine (PAG; 25 mg/kg/day) were intraperitoneally given once daily 3 days before and continued for 2 weeks after operation, whereas enalapril (10 mg/kg/day) was given via intragastric. (b) Relative lengths of the left kidney calibrated by the right counterpart of the same rat in different groups. (c) Renal cortex thickness in the mid-portion of left kidney in coronal section. Data are mean ± s.e.m. (n = 7), ###P < 0.001 versus sham group; *P < 0.05, ***P < 0.001 versus UUO group.

Figure 4 | Sodium hydrosulfide (NaHS) treatment attenuates the accumulation of collagen fibrils in renal interstitium in the obstructed kidneys at 14 days after unilateral ureteral obstructive (UUO) operation. Representative pictures of (a) Masson trichrome staining and (b) semiquantitative analysis of the proportion with the blue color area over the whole field area in all groups are indicated. PAG, DL-propargylglycine. Scale bar = 200 μm. Mean ± s.e.m. (n = 4), ###P < 0.001 versus sham group; *P < 0.05, ***P < 0.001 versus UUO group.
increased by UUO injury. NaHS (5.6 and 56 μg/kg/day) and enalapril suppressed the expression of these cytokines, whereas NaHS at 560 μg/kg/day and PAG were unable to exhibit anti-inflammatory effects (Figure 7c).

**NaHS suppresses the NRK-49F cell proliferation induced by fetal bovine serum (FBS)**

We further performed an *in vitro* study to investigate the antiproliferation effect of H$_2$S on renal fibroblast using NRK-49F cells. Cells were exposed to various concentrations (1–500 μmol/l) of NaHS for 30 min, followed by stimulation with 10% FBS for 24 h. Cells with serum-free medium were set as controls. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay showed the cell number was significantly increased after incubation with 10% FBS for 24 h ($P<0.05$, Figure 8a). Preincubation with NaHS (10, 50, 100, and 500 μmol/l) reduced the cell number by 17.6%, 14.8%, 21.4%, and 16.2%, respectively. However, statistical difference was only reached in 100 μmol/l NaHS group and the dose-dependent effect was not observed.

Furthermore, we continued to test whether H$_2$S inhibited the DNA synthesis of renal fibroblast cells using bromodeoxurytidine (BrdU) incorporation assay. The DNA synthesis triggered by 10% FBS was significantly decreased by NaHS (100 μmol/l) before treatment (Figure 8b and c). To delineate the mechanism beneath the antiproliferation effect of H$_2$S, the expression of proliferation-related genes, including proliferating cell nuclear antigen (PCNA) and c-Myc, was also assessed. Preincubation with 100 μmol/l NaHS consistently attenuated the expressions of PCNA and c-Myc proteins induced by 10% FBS stimulation for 24 h (Figure 8d–f).

**NaHS blocks the differentiation of quiescent renal fibroblasts to myofibroblasts**

Because TGF-β1 is the most potent cytokine controlling the phenotype switch from renal fibroblasts to myofibroblasts, we investigated the effect of H$_2$S on renal fibroblast activation induced by TGF-β1. Reverse transcription-PCR demonstrated that NaHS pretreatment inhibited the mRNA expressions of collagen I, α-SMA, and fibronectin induced by TGF-β1 (Figure 9a–d). Western blot analysis also revealed a significant elevation of α-SMA and fibronectin expression in NRK-49F cells after TGF-β1 stimulation. These effects were essentially abolished by NaHS (Figure 9e and f).

---

**Figure 5** Sodium hydrosulfide (NaHS) inhibits the expressions of fibronectin and α-smooth muscle actin (α-SMA) in the obstructed kidneys at 14 days after unilateral ureteral obstructive (UUO) operation. Representative immunohistochemistry images for the expression of (a) fibronectin and (b) α-SMA, and semiquantitative analysis of these two proteins are presented. PAG, DL-propargylglycine. Scale bar = 200 μm. Data are mean ± s.e.m. of 10 non-overlapping fields from four animals per group, ***$P<0.001$ versus sham group; *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus UUO group.
NaHS abolishes the phosphorylation of the MAPKs and Smad3

TGF-β1-Smad and mitogen-activated protein kinase (MAPK) pathways are two critical signaling mechanisms implicated in renal fibrosis. We therefore examined the effects of H2S on the TGF-β1-induced phosphorylation of Smad3, p38, c-Jun N-terminal kinase (JNK), and extracellular signal–regulated kinase (ERK) with western blot analysis. TGF-β1 (2 ng/ml) triggered the phosphorylation of these proteins with distinct patterns. The phosphorylation of p38 peaked at 0.5 h after TGF-β1 stimulation, quicker than that of the other molecules. Specifically, phosphorylation of ERK and JNK peaked at 2 h after TGF-β1 exposure whereas Smad3 at 1 h (data not shown). Pretreatment with 100 μmol/l NaHS abolished the increase of Smad3 and MAPKs phosphorylation induced by TGF-β1 stimulation (Figure 10).

**DISCUSSION**

UUO is a well-established animal model featured by progressive renal fibrosis and tubulointerstitial injury. In this study, we demonstrated that exogenous H2S suppressed collagen and extracellular matrix deposition in the UUO rats. We also identified that H2S blocked renal fibrosis via multiple mechanisms. It inhibited renal fibroblast proliferation by reducing DNA synthesis and downregulating the expressions of proliferation-related proteins. H2S also blocked cell differentiation by suppressing the TGF-β1-Smad and MAPK signal pathways. Besides, H2S inhibited the inflammatory process induced by UUO injury.

The association of H2S and renal fibrosis is not well defined. Our study demonstrated that plasma H2S levels and endogenous H2S production in the obstructed kidneys were decreased in UUO rats. As UUO model is devoid of any exogenous toxin, or a ‘uremic’ environment, our finding implies that renal fibrosis per se could reduce the H2S generation. Our study further demonstrated that CBS was predominantly expressed in renal tubules whereas CSE was mainly located in glomeruli and interstitial vessels, consistent with a recent report. UUO injury markedly decreased the expression of CBS but increased that of CSE in the renal
interstitium. This finding is consistent with previous studies that CBS is more predisposed to decrease in kidney disease compared with CSE. For instance, CBS was decreased 6 weeks earlier than CSE in rat remnant kidney.8 In renal obstructive (UUO) operation.

Figure 7 | Sodium hydrosulfide (NaHS) inhibits the macrophage infiltration in the obstructed kidneys at 7 days after unilateral ureteral obstructive (UUO) operation. (a) Representative pictures of kidney sections stained with anti-CD68. A total of 10 fields from 4 animals were included in analyzing the number of CD68-positive cells in the interstitium and (b) the group data are shown correspondingly. (c) Quantitative PCR results of the mRNA levels of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and monocyte chemoattractant protein-1 (MCP-1) in the kidneys among different treatment groups. PAG, DL-propargylglycine. Scale bar = 100 μm. Data are presented as mean ± s.e.m., ***P < 0.001 versus sham group; *P < 0.05, **P < 0.01, ***P < 0.001 versus UUO group.

pulmonary fibrosis induced by bleomycin.11 Previous studies demonstrated that H2S level in vertebrate blood varied from 10–300 μmol/l by the methylene blue assay.22,23 Using a sensitive fluorescent probe,24 we found the plasma H2S level in normal rat was ~30–40 μmol/l, whereas its counterpart in the UUO rat was 20–30 μmol/l. Nevertheless, the precise determination of H2S in biological samples is still controversial. More sensitive techniques such as monobromobimane-based assay and quantum dot/nanoparticle methods recently indicated that blood and tissue H2S concentrations were as low as 0.15–0.9 μmol/l.25,26 These findings may justify the application of H2S at relatively low doses in treating renal fibrosis.

Although H2S is beneficial in most animal models of renal diseases, endogenous H2S has been reported detrimental in acute kidney injury caused by nephrotoxic drugs. For instance, the CSE inhibitor PAG (50 mg/kg/day) reduced the kidney damage of Wistar rats induced by cisplatin and adriamycin.27,28 This is inconsistent with our data in which PAG was unable to relieve renal fibrosis. In fact, 50 mg/kg/day PAG treatment resulted in a great weight loss of normal Sprague–Dawley rats in our study (data not shown), and the dose had to be lowered to 25 mg/kg/day. Such conflicting
aspects of H2S implicate that diverse mechanisms of H2S may exist in different animal models and species. Despite this, caution must be taken in interpreting the effect of PAG because of its nonselective inhibitory action of CSE. It has been reported that D-amino acid oxidase and L-alanine transaminase can also be inhibited by PAG.29,30 Possibilities cannot be ruled out that the protective effect of PAG in renal disease may be mediated by inactivating other enzymes rather than CSE.

Fibroblast proliferation and phenotypic transition to myofibroblast are two major cellular events of renal fibrosis.31 FBS contains numerous growth factors that promote the proliferation and differentiation in many types of cells. The effect of H2S on cell proliferation seems to be cell specific. Some studies reported that H2S promoted the proliferation in rat intestinal epithelial cells and human colon cancer cells, whereas others demonstrated that it inhibited the proliferation of lung fibroblasts and pancreatic stellate cells.32,33 Our data revealed that NaHS at 100 μmol/l not only decreased the cell number, but also inhibited the DNA synthesis stimulated by FBS. These findings were further verified by the fact that NaHS downregulated the expressions of proliferation-related genes including PCNA and c-Myc.34 Previous studies also indicated that H2S induced DNA damage in many cell types.34 The proliferation and cell cycle proteins such as p53, p21, and cyclin D1 were downregulated by H2S.35,36

Figure 8 | Sodium hydrosulfide (NaHS) suppresses the proliferation of NRK-49F cells. Serum-starved cells were preincubated with NaHS at concentrations as indicated for 30 min, followed by stimulation with 10% fetal bovine serum (FBS) for 24 h. Serum-starved cells without FBS treatment served as controls (CON). (a) MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay shows the optical density (OD) value of various treatment groups. (b, c) Representative micrographs (original magnification ×200, b) and (c) group data display the number of bromodeoxyuridine (BrdU)-positive cells. Cells were stained with anti-BrdU antibody (red) and 4',6-diamidino-2-phenylindole (DAPI; blue) was used to visualize the nuclei. N = 6. (d–f) Representative pictures (d) and group data analysis (e, f) for proliferating cell nuclear antigen (PCNA) and c-Myc expressions in NRK-49F cells subjected to various treatments. Relative abundance was determined by normalizing to β-actin. Data are mean ± s.e.m. of three independent experiments. ##P < 0.01, ###P < 0.001 versus control group; *P < 0.05, **P < 0.01, ***P < 0.001 versus 10% FBS group.
and protein expressions of α-SMA and fibronectin induced by TGF-β1. In addition, H2S attenuated the phosphorylation of Smad3, a major pathway in the signal transduction of TGF-β1. These data suggested that H2S inhibited the fibroblast differentiation and extracellular matrix production partially by blocking the TGF-β1-Smad signaling. In addition to Smad-dependent pathway, MAPK kinases also engage in the effect of TGF-β1. ERK, p38, and JNK have been found to be activated in UUO model and regulate the proliferation, migration, and differentiation of fibroblast.42 Our data indicated that H2S reversed the phosphorylation increase of p38, ERK, and JNK stimulated by TGF-β1 in renal interstitial fibroblasts, implying that the blockade of MAPK kinases activation may be another mechanism responsible for the antifibrotic effect of H2S.

Inflammation plays an important role in the priming and progression of renal fibrosis.43 One of the pathological features of the UUO rat model is the marked infiltration of inflammatory cells in the renal interstitium. Substantial data support the anti-inflammation action of H2S in multiple organs and systems.44 Our study echoed with these studies that H2S exhibited an anti-inflammatory property by reducing macrophage recruitment in renal interstitium. The inflammatory cytokines such as interleukin-1β, tumor necrosis factor-α, and monocyte chemoattractant protein-1 were also downregulated by NaHS. MAPKs are involved in modulating inflammatory processes. For example, JNK has been identified with the activation of macrophages and upregulation of proinflammatory mediators, and JNK inhibition resolves the inflammation.45 Thus, the anti-inflammatory effect of H2S may be mediated by inhibiting the MAPK signal pathway.

In summary, this study demonstrates for the first time that H2S exhibited antifibrotic effects on obstructed nephropathy and inhibited the proliferation and differentiation of renal fibroblasts both in vitro and in vivo. The antifibrotic mechanisms of H2S may involve its anti-inflammation as well as its blockade on TGF-β1 and MAPK signaling. This may provide a therapeutic option for renal fibrosis based on H2S modulation. Low dosage of H2S or its releasing compounds may have therapeutic potentials in treating CKD.

MATERIALS AND METHODS
Reagents and antibodies
BrdU, PAG, enalapril, and NaHS, as well as antibodies against PCNA, c-Myc, and BrdU were purchased from Sigma-Aldrich (St Louis, MO). Recombinant human TGF-β1 and Trizol reagent were obtained from Invitrogen (Carlsbad, CA). Antibodies against α-SMA, fibronectin, CBS, and ERK/phosphorylated-ERK were from Santa Cruz Biotechnology (Santa Cruz, CA). CSE antibody was purchased from Abnova Chemical (Taiwan, China). Other antibodies unless specified were from Cell Signaling Technology (Danvers, MA).

Animal surgery and experimental protocols
Male Sprague-Dawley rats (180–200 g) were purchased from Shanghai Laboratory Animal Commission (Shanghai, China).
UUO operation was performed as described previously. Rats were randomly divided into the following seven groups: sham, UUO, UUO with enalapril (10 mg/kg/day), UUO with three doses of NaHS (5.6, 56, and 560 μg/kg/day), and UUO with PAG (25 mg/kg/day). NaHS and PAG were given intraperitoneally once daily 3 days before surgery and continued for 1 to 2 weeks after operation. Enalapril was given via intragastric. UUO rats received vehicle (saline) treatment. All rats were killed at day 7 or 14 days after surgery. The kidneys were then harvested and plasma was collected accordingly. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Soochow University.

Histological analysis and immunohistochemical staining
Kidney samples were fixed in 10% formalin, embedded in paraffin, and sectioned 4 μm thick. Sections were deparaffinized and stained with Masson trichrome reagents and observed under a light microscope. Renal collagen (blue area) was semiquantitatively measured at ×200 magnification in 10 randomly selected fields from 4 animals using Image Pro-Plus 6.0 software (Bethesda, MD).

Figure 10 | Sodium hydrosulfide (NaHS) abolishes the phosphorylation increase of mitogen-activated protein kinase (MAPK) and Smad3. Serum-starved cells were incubated with NaHS (100 μmol/l) for 30 min, followed by transforming growth factor-β1 (TGF-β1; 2 ng/ml) treatment for 1 h in the presence or absence of NaHS. Cell lysates were subjected to western blotting with antibodies against (a) phospho-Smad3, (b) phospho-p38/p38, (c) phospho-JNK/JNK, and (d) phospho-ERK/ERK. Relative abundance was quantified by densitometry and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), p38, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK), respectively. Data are mean ± s.e.m., n = 3, *P < 0.05, **P < 0.01, ***P < 0.001 versus control group; *P < 0.05, **P < 0.01 versus TGF group.

Plasma H2S and tissue H2S synthesis measurement
Plasma H2S level was detected using Dansyl azide (DNS-Az), a novel fluorescent H2S probe (kindly gifted by Professor Binghe Wang, Georgia State University) as described previously. Briefly, DNS-Az stock solution (2.2 mmol/l) was added at 10 μl per well in a 96-well plate, followed by mixing with 100 μl plasma. Fluorescence was immediately measured by a fluorescent microplate reader (Tecan M200, Grodig, Austria) with an excitation at 360 nm and emission at 528 nm.
Tissue H₂S production measurement was performed as previously described.⁴⁶ In brief, the left kidney homogenates were mixed with 2 mmol/l pyridoxal 5'-phosphate and 10 mmol/l l-cysteine and 250 μl 10% trichloroacetic acid to reach a total volume of 750 μl. After incubation at 37 °C for 30 min, 250 μl 1% zinc acetate was added to trap H₂S. Then, 130 μl of 20 mmol/l N,N,N-trimethyl-p-phenylenediamine sulfate in 7.2 mol/l HCl and 133 μl of 30 mmol/l FeCl₃ in 1.2 mol/l HCl were added. The absorbance of the dimethylphenylenediamine fluorescence was measured at 530 nm by a microplate reader (Tecan M200). Absorbance at 490 nm was measured by a microplate reader (0.1–500 μl) for 30 min, followed by incubation with or without 10% FBS for 30 min. MTT (0.5 mg/ml) was added at 4 h before treatment termination. At the end of treatment, medium was removed and dimethylsulfoxide at 150 μl per well was then added. Absorbance at 490 nm was measured by a microplate reader (Tecan M200).

For the BrdU incorporation, cells were seeded onto coverslips. After being pretreated with 100 μmol/l NaHS for 30 min in Dulbecco’s modified Eagle’s medium (4.5 g/l glucose) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY). After preincubation with NaHS at various concentrations (1–500 μmol/l) for 30 min, recombinant human TGF-β1 (2 ng/ml) was added for various periods of time. In the control group, cells were treated with vehicle only. In proliferation studies, 10% FBS served as stimulators and the cells were subjected to serum-free medium for 24 h before experimentation.

Cell proliferation assay
Cell proliferation was assessed by MTT assay and BrdU incorporation. After being pretreated with 100 μmol/l NaHS for 30 min, cells were incubated in medium with or without 10% FBS for 5 h, followed by incubation with or without 10% FBS for 24 h. MTT (0.5 mg/ml) was added at 4 h before treatment termination. At the end of treatment, medium was removed and dimethylsulfoxide at 150 μl per well was then added. Absorbance at 490 nm was measured by a microplate reader (Tecan M200).

For the BrdU incorporation, cells were seeded onto coverslips. After being pretreated with 100 μmol/l NaHS for 30 min, cells were incubated in medium with or without 10% FBS for 5 h, pulsed with BrdU (10 μmol/l) for another 5 h. Next, cells were fixed in 4% paraformaldehyde for 20 min and DNA was denatured with 2 N HCl at 37 °C for 10 min. After blocking, the incorporated BrdU was detected with a mouse anti-BrdU antibody (1:100, Sigma) at 4 °C overnight. Visualization was carried out using an advanced chemiluminescence kit (GE Healthcare, Buckinghamshire, UK). The band density was quantified by ImageJ software (Bethesda, MD). GAPDH or β-actin was used as an internal control.

Reverse transcription-PCR and quantitative PCR
Total RNA was extracted with Trizol reagent and reverse transcription-PCR was performed using an Advantage RT-for-PCR kit and PCR Master Mix kit (Fermentas, Vilnius, Lithuania) according to the manufacturer’s instructions. The quantitative PCR reactions were performed using a real-time SYBR technology and on ABI Prism 7000 DNA Detection System (Applied Biosystems, Foster, CA). For each sample, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S RNA was also assayed as an internal control and final result were normalized to and expressed as ratios of the target gene/internal controls. The primers used in the PCR reactions are listed in Supplementary Table S2 online.

Western blot
Given the heterogeneous expression of CBS and CSE in the kidney, the whole kidney was halved in the middle. One half was used for histological studies, and the other half was homogenized for western blotting analysis. Protein samples (20–80 μg) were separated by 8–10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking in 5% milk/Tris-buffered saline and Tween-20 buffer, membranes were individually incubated with antibodies against proteins of interest at an appropriate dilution (β-SMA (1:1000), fibronectin (1:500), CBS(1:500), CSE (1:250), ERK/phospho-ERK (1:500), JNK/phospho-JNK (1:500), P38/phospho-P38 (1:500), phospho-Smad3 (1:500), PCNA (1:1000), and c-Myc (1:1000)) at 4 °C overnight. Membranes were then washed and incubated with appropriate horseradish peroxidase-conjugated secondary antibody for 1 h. Visualization was carried out using an advanced chemiluminescence kit (GE Healthcare, Buckinghamshire, UK). The band density was quantified by ImageJ software (Bethesda, MD). GAPDH or β-actin was used as an internal control.

Statistical analysis
All data are presented as mean ± s.e.m. Statistical significance was determined using Student’s t-test or one-way analysis of variance followed by a post hoc analysis (Tukey’s test) where applicable. The significance level was set at P<0.05.

DISCLOSURE
A patent application on the potential therapeutic option of H₂S-releasing compounds in renal fibrosis (201210524309.1) has been filed to China Intellectual Property Office. All the authors declared no competing interests.

ACKNOWLEDGMENTS
We were grateful for the kind gift of Dansyl azide by Professor Binghe Wang from Georgia State University. This work was supported by the research grants from the National Natural Science Foundation of China (81200495/2012 to KS and 81171212 to LF-H) and the start-up funding for imported overseas talents of Soochow University (Q421500210), and was also funded by a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD). HP was supported by the Center for Diagnostics and Therapeutics (CDT) program and Georgia State University Fellowship through the CDT.

SUPPLEMENTARY MATERIAL
Table S1. Renal function and serum electrolytes among different animal groups.
Table S2. The primers used for reverse transcription and quantitative PCR reactions.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

REFERENCES
1. Harris RC, Neilson EG. Toward a unified theory of renal progression. Annu Rev Med 2006; 57: 365–380.
2. Zeisberg M, Neilson EG. Mechanisms of tubulointerstitial fibrosis. J Am Soc Nephrol 2010; 21: 1819–1834.
3. Boor P, Ostendorf T, Floege J. Renal fibrosis: novel insights into mechanisms and therapeutic targets. Nat Rev Nephrol 2010; 6: 643–656.
4. Boor P, Sebekova K, Ostendorf T et al. Treatment targets in renal fibrosis. Nephrol Dial Transplant 2007; 22: 3391–3407.
5. Wang R. Shared signaling pathways among gasotransmitters. Proc Natl Acad Sci USA 2012; 109: 8801–8802.
6. Xia M, Chen L, Muh RW et al. Production and actions of hydrogen sulfide, a novel gaseous bioactive substance, in the kidneys. *J Pharmacol Exp Ther* 2009; **329**: 1056-1062.

7. Beltowski J. Hypoxia in the renal medulla: implications for hydrogen sulfide signaling. *J Pharmacol Exp Ther* 2010; **334**: 358-363.

8. Aminzadeh M, Vaziri N. Down-regulation of the renal and hepatic hydrogen sulfide (H2S) producing enzymes and capacity in chronic kidney disease. *Nephrol Dial Transplant* 2012; **27**: 498-504.

9. Perna AF, Luciano MG, Ingrasso D et al. Hydrogen sulphide-generating pathways in haemodialysis patients: a study on relevant metabolites and transcriptional regulation of genes encoding for key enzymes. *Nephrol Dial Transplant* 2009; **24**: 3756-3763.

10. Fang L, Li H, Tang C et al. Hydrogen sulfide ameliorates hyperhomocysteinemia-associated chronic renal failure. *Nephrol Dial Transplant* 2009; **24**: F410–F419.

11. El-Seweidy MM, Sadik NA, Shaker OG. Role of sulfurous mineral water and hydrogen sulfide in the kidneys. *Can J Physiol Pharmacol* 2009; **87**: 531–538.

12. Be Perna AF, Luciano MG, Ingrosso D et al. Nitric oxide generation ameliorates the pathogenesis of pulmonary fibrosis induced by bleomycin in rats. *J Am Soc Nephrol* 2013; **24**: 1145–1152.

13. Morrissey JJ, Stoll P, Goebel U et al. Effects of hydrogen sulfide on rat pancreatic stellate cells. *Pancreas* 2012; **41**: 74–83.

14. Tripatara P, Patel NS, Thomhill BA. Ureteral obstruction as a model of renal interstitial fibrosis and obstructive nephropathy. *Kidney Int* 2011; **78**: 9672–9675.

15. Wang L, Lee JY, Kwak JH et al. Protective effects of low-dose carbon monoxide against renal fibrosis induced by unilateral ureteral obstruction. *Am J Physiol Renal Physiol* 2008; **294**: F508-F517.

16. Chevalier RL, Forbes MS, Thomhill BA. Ureteral obstruction as a model of renal interstitial fibrosis and obstructive nephropathy. *Kidney Int* 2009; **75**: 1310–1312.

17. Bos EM, Wang R, Snijder PM et al. Cystathionine γ-lyase protects against renal ischaemia/reperfusion by modulating oxidative stress. *J Am Soc Nephrol* 2013; **24**: 759–770.

18. Wu N, Siow YL, Karmin O. Ischaemia/reperfusion reduces transcription factor P1P1 expression in the kidney. *Nephrol Dial Transplant* 2009; **24**: 1260–1267.

19. Tripatara P, Patel NS, Collino M et al. Mechanistic connection between inflammation and cardioprotection induced by H2S preconditioning and post-infarction reperfusion injury and dysfunction. *J Am Soc Nephrol* 2016; **27**: 487–495.

20. Bottinger EP, Bitzer M. TGF-beta signaling in renal disease. *J Am Soc Nephrol* 2002; **13**: 2690-2690.

21. Freguet M, Almers W, Pendlebury BA. A fluorescent probe for fast and quantitative detection of hydrogen sulfide in blood. *Antioxid Redox Signal* 2010; **12**: 941–957.

22. Deplancke B, Gaskins HR. Hydrogen sulfide induces serum-independent cell cycle entry in nontransformed rat intestinal epithelial cells. *FASEB J* 2003; **17**: 1164–1175.

23. Deplancke B, Gaskins HR. Hydrogen sulfide inducetion of carbon monoxide enzyme in human lung fibroblasts. *Comp Physiol* 2010; **285**: 18225–18233.

24. Deplancke B, Gaskins HR. Hydrogen sulfide induced inactivation of pig heart L-alanine transaminase by L-propargylglycine. Half-site reactivity. *J Biol Chem* 1996; **271**: 1038–1048.

25. Deplancke B, Gaskins HR. Hydrogen sulfide attenuates carbon tetrachloride-induced hepatotoxicity, liver cirrhosis and portal hypertension in rats. *Arch Biochem Biophys* 2011; **506**: 48–57.

26. Deplancke B, Gaskins HR. Protective effects of low-dose carbon monoxide against renal fibrosis induced by unilateral ureteral obstruction. *Am J Physiol Renal Physiol* 2008; **294**: F508-F517.

27. Deplancke B, Gaskins HR. Cystathionine gamma-lyase deficiency and overproliferation of smooth muscle cells. *Cardiovasc Res* 2010; **86**: 487–495.

28. Deplancke B, Gaskins HR. Hydrogen sulfide suppresses migration, proliferation and myofibroblast transdifferentiation of human lung fibroblasts. *Pulm Pharmacol Ther* 2009; **22**: 554-561.

29. Deplancke B, Gaskins HR. Hydrogen sulfide-induced DNA damage and changes in apoptotic gene expression in human lung fibroblast cells. *FASEB J* 2007; **21**: 247–255.

30. Deplancke B, Gaskins HR. Hydrogen sulfide ameliorates renal fibroblasts: role of beta1 integrin/focal adhesion kinase signaling. *Pulm Pharmacol Ther* 2009; **22**: 554-561.

31. Liu Y. Cellular and molecular mechanisms of renal fibrosis. *Nat Rev Nephrol* 2011; **7**: 684-696.

32. Fang LP, Lin Q, Tang CS et al. Hydrogen sulfide induces serum-independent cell cycle entry in nontransformed rat intestinal epithelial cells. *FASEB J* 2003; **17**: 1164–1175.

33. Freguet M, Almers W, Pendlebury BA. A fluorescent probe for fast and quantitative detection of hydrogen sulfide in blood. *Antioxid Redox Signal* 2010; **12**: 941–957.

34. Freguet M, Almers W, Pendlebury BA. A fluorescent probe for fast and quantitative detection of hydrogen sulfide in blood. *Antioxid Redox Signal* 2010; **12**: 941–957.

35. Freguet M, Almers W, Pendlebury BA. A fluorescent probe for fast and quantitative detection of hydrogen sulfide in blood. *Antioxid Redox Signal* 2010; **12**: 941–957.