Production and Actions of Hydrogen Sulfide, a Novel Gaseous Bioactive Substance, in the Kidneys

Min Xia, Li Chen, Rachel W. Muh, Pin-Lan Li, and Ningjun Li

Department of Pharmacology & Toxicology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia

Received December 18, 2008; accepted February 25, 2009

ABSTRACT

Hydrogen sulfide (H_2S), a novel endogenous gaseous bioactive substance, has recently been implicated in the regulation of cardiovascular and neuronal functions. However, its role in the control of renal function is unknown. In the present study, incubation of renal tissue homogenates with L-cysteine (L-Cys) (as a substrate) produced H_2S in a concentration-dependent manner. This H_2S production was completely abolished by inhibition of both cystathionine β -synthetase (CBS) and cystathionine γ -lyase (CGL), two major enzymes for the production of H_2S , using amino-oxyacetic acid (AOAA), an inhibitor of CBS, and propargylglycine (PPG), an inhibitor of CGL. However, inhibition of CBS or CGL alone induced a small decrease in H_2S production. In anesthetized Sprague-Dawley rats, intrarenal arterial infusion of an H_2S donor (NaHS) increased renal

blood flow, glomerular filtration rate (GFR), urinary sodium ($\text{U}_{\text{Na}}\cdot\text{V}$), and potassium ($\text{U}_{\text{K}}\cdot\text{V}$) excretion. Consistently, infusion of both AOAA and PPG to inhibit the endogenous H_2S production decreased GFR, $\text{U}_{\text{Na}}\cdot\text{V}$, and $\text{U}_{\text{K}}\cdot\text{V}$, and either one of these inhibitors alone had no significant effect on renal functions. Infusion of L-Cys into renal artery to increase the endogenous H_2S production also increased GFR, $\text{U}_{\text{Na}}\cdot\text{V}$, and $\text{U}_{\text{K}}\cdot\text{V}$, which was blocked by AOAA plus PPG. It was shown that H_2S had both vascular and tubular effects and that the tubular effect of H_2S might be through inhibition of $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter and $\text{Na}^+/\text{K}^+/\text{ATPase}$ activity. These results suggest that H_2S participates in the control of renal function and increases urinary sodium excretion via both vascular and tubular actions in the kidney.

In addition to NO and CO, hydrogen sulfide (H_2S) has recently been demonstrated to be the third gaseous bioactive substance produced in different mammalian cells (Kimura, 2002; Wang, 2002). Studies have indicated that H_2S plays important role in the regulation of cardiovascular functions. In this regard, both exogenous and endogenous H_2S have been reported to cause vascular smooth muscle relaxation and decrease blood pressure, and inhibition of endogenous H_2S production induces hypertension (Hosoki et al., 1997; Cheng et al., 2004; Yan et al., 2004; Webb et al., 2008). Furthermore, accumulating evidence has shown that H_2S is involved in a variety of physiological and pathological processes in many other organs, such as brain (Eto et al., 2002), heart (Geng et al., 2004), lung (Bhatia et al., 2005; Baskar et al., 2007), liver (Fiorucci et al., 2005), intestine (Teague et al.,

2002), pancreas (Bhatia et al., 2005; Yang et al., 2007), and cavernosum (Srilatha et al., 2007).

It has been reported that mammalian cells generate H_2S from L-cysteine (L-Cys) mainly through two enzymes, cystathionine β -synthetase (CBS) and cystathionine γ -lyase (CGL) (Kimura, 2002; Wang, 2002; Zhao et al., 2003). The enzymatic pathways for H_2S production are tissue-specific. For example, CBS is the predominant enzyme generating H_2S in the nervous system and CGL in the vascular system (Wang, 2002; Zhao et al., 2003). Both CBS and CGL have been reported to be present in the kidneys (Stipanuk and Beck, 1982; House et al., 1997), mainly in renal proximal tubules (House et al., 1997; Ishii et al., 2004; Li et al., 2006). However, the production and actions of H_2S in the kidneys are not clear. The present study determined the enzymatic pathways for the production of H_2S in the renal tissue homogenates and examined the effects of exogenous and endogenous H_2S on renal hemodynamics and excretory functions. The results provide evidence showing that H_2S significantly participates in the control of renal functions, including glomerular and tubular functions.

This work was supported by the National Institutes of Health [Grants HL70726, DK54927]

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.108.149963.

ABBREVIATIONS: H_2S , hydrogen sulfide; L-Cys, L-cysteine; CBS, cystathionine β -synthetase; CGL, cystathionine γ -lyase; AOAA, amino-oxyacetic acid; PPG, propargylglycine; GFR, glomerular filtration rate; U·V, urinary volume; $\text{U}_{\text{Na}}\cdot\text{V}$, urinary sodium; $\text{U}_{\text{K}}\cdot\text{V}$, urinary potassium; FF, fractional filtration; FE_{Na} , fractional excretion of sodium; FE_{K} , fractional excretion of potassium; MAP, mean arterial blood pressure; NKCC, $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter; NCC, Na^+/Cl^- cotransporter; NKA, $\text{Na}^+/\text{K}^+/\text{ATPase}$; RBF, renal blood flow; ANOVA, analysis of variance; G, group; BM, basolateral membrane; HPLC, high-performance liquid chromatography; K_f , glomerular ultrafiltration coefficient.

Materials and Methods

Animals

Experiments were performed on male Sprague-Dawley rats, weighing between 300 and 350 g, purchased from Harlan (Madison, WI). The rats were housed in the Animal Care Facility at the Virginia Commonwealth University with free access to food and water throughout the study, with the exception that they were fasted the night before the renal function experiments. All protocols were approved by the Institutional Animal Care and Use Committee of the Virginia Commonwealth University.

Measurement of H₂S Production in Renal Tissues

The production of H₂S by renal tissue homogenates was measured using spectrophotometry as described previously with slight modifications (Stipanuk and Beck, 1982; Zhao et al., 2001; Cheng et al., 2004). In brief, renal cortical tissues were homogenized in 50 mM ice-cold potassium phosphate buffer (pH 7.4). The tissue homogenates (0.25 ml) were incubated with L-Cys (0.5, 1, and 5 mM, respectively) and pyridoxal 5'-phosphate (2 mM) at 37°C for 90 min after the reaction tubes were flushed with N₂ and sealed. Fifty percent of trichloroacetic acid (0.125 ml) was injected into the reaction tubes to stop the reaction, followed by 0.125 ml of zinc acetate (15 mM) and 0.5 ml of borate buffer (pH 10.01). The tubes were then incubated at 37°C for another 60 min. The reaction solutions were mixed with 0.5 ml of *N,N*-dimethyl-*p*-phenylenediamine sulfate (20 mM, in sulfuric acid, pH 9.0) and 0.02 ml of FeCl₃ (3 M) at 37°C for an additional 30 min and then centrifuged at 5000g for 3 min and filtered with 0.45-μm syringe filters. The absorbance of resulting solution at 670 nm was measured with a spectrophotometer. The H₂S concentration was calculated against the calibration curve of the standard H₂S solutions.

For the measurement of endogenous H₂S levels in the kidneys, renal tissues (50 mg) were homogenized in 0.5 ml of zinc acetate (1%) and mixed with 0.5 ml of borate buffer (pH 10.01). Then, 0.5 ml of *N,N*-dimethyl-*p*-phenylenediamine (20 mM) and 0.5 ml of FeCl₃ (300 mM) were added into tissue homogenates. Reaction tubes were immediately sealed and incubated at 37°C for 30 min with shaking. After incubation, the samples were centrifuged, and the H₂S concentrations were measured as described above.

Zinc acetate can trap H₂S, and the reaction of H₂S with *N,N*-dimethyl-*p*-phenylenediamine produces methylene blue that can be detected at 670 nm with spectrophotometry to represent the levels of H₂S. This method is extensively used for the measurement of H₂S in the tissue (Stipanuk and Beck, 1982; Zhao et al., 2001, 2003; Cheng et al., 2004; Yan et al., 2004).

Effect of Exogenous and Endogenous H₂S on Renal Hemodynamics and Functions

Animal Preparation. Male Sprague-Dawley rats, weighing 300 to 350 g, were anesthetized with ketamine (30 mg/kg i.m.) (Phoenix Pharmaceuticals, St. Joseph, MO) and thiobutabarbital (inaction, 50 mg/kg i.p.) (Sigma-Aldrich, St. Louis, MO) and then surgically prepared for continuously monitoring mean arterial blood pressure (MAP), renal blood flow (RBF), and urine flow rate (U·V) as described previously by us and others (Zou et al., 2001; Zhang et al., 2003). After surgery, the animals received an infusion of 2% albumin saline at a rate of 1 ml/h/100 g body weight throughout the experiment to replace fluid losses and maintain a stable hematocrit of $42.5 \pm 1.2\%$. A catheter was placed into left renal artery for intrarenal arterial infusion (50 μl/min). Glomerular filtration rate (GFR) was measured using fluorescein isothiocyanate-inulin (Sigma-Aldrich). The U·V was determined gravimetrically, and urinary sodium (Na⁺) and potassium (K⁺) concentrations were measured using a flame photometer. RBF, GFR, U·V, urinary Na⁺ excretion (U_{Na}·V), and K⁺ excretion (U_K·V) were factored per gram kidney weight. This surgical preparation has been widely used in the studies of renal physiology.

Experimental Protocols. After a 1.5-h equilibration period and two 20-min control collections of urine and blood samples, different reagents were infused into left renal artery for 30 min (10-min clearance period and 20-min sample collection): group 1, infusion of NaHS, a H₂S donor, at 5, 10, 20, 40, and 80 nmol/min/kg; group 2, infusion of amino-oxyacetic acid (AOAA) (Sigma-Aldrich), a CBS inhibitor, and/or propargylglycine (PPG) (Sigma-Aldrich), a CGL inhibitor, to block the production of endogenous H₂S; group 3, infusion of L-Cys to increase the production of endogenous H₂S; and group 4, infusion of L-Cys + AOAA + PPG. At the end of the experiments, the kidneys were removed, snap-frozen in liquid N₂, and stored in -80°C for measurement of H₂S concentration as described above.

Effect of H₂S on Na⁺/K⁺-2Cl Cotransporter and Na⁺/Cl⁻ Cotransporter in the Kidneys

Animal preparation was as above. The effect of H₂S on Na⁺/K⁺-2Cl cotransporter was determined by intrarenal arterial infusion of NaHS (20 nmol/min/kg) and/or furosemide (0.75 μmol/min/kg) to compare the H₂S-induced increases in U_{Na}·V in the absent and present of furosemide (Furo) (H₂S versus Furo + H₂S). Dose-dependent effects of furosemide on U_{Na}·V were tested first, and the dose that caused maximal diuretic effect was used. Likewise, the effect of H₂S on Na⁺/Cl⁻ cotransporter was determined by comparing the increases in U_{Na}·V induced by NaHS with increases by NaHS plus hydrochlorothiazide (Thia) (1.7 μmol/min/kg) (H₂S versus Thia + H₂S).

Effect of H₂S on Na⁺/K⁺-ATPase Activity in Basolateral Membranes from the Kidneys by HPLC Analysis

Basolateral membrane was prepared from the kidney tissue as described previously (Sheikh et al., 1982). HPLC analysis of renal Na⁺/K⁺-ATPase activity in the basolateral membranes was performed by detecting the conversion of ATP into ADP as reported previously (Sudo et al., 2000). ATP as substrate was incubated with renal basolateral membranes at 37°C for 20 min, and then the samples were subjected to HPLC analysis of ADP concentration after being centrifuged and filtered. Effect of H₂S on ouabain-sensitive ATPase activity was used to present the effect of H₂S on Na⁺/K⁺-ATPase activity. The assays included the following groups (G): G1, ATP + basolateral membrane (BM); G2, ATP + BM + ouabain (10 mM); G3, ATP + BM + ouabain + H₂S; and G4, ATP + BM + H₂S. The inhibition on ATPase activity was obtained as the difference in ADP production between G1 and the other groups. The effect of H₂S on Na⁺/K⁺-ATPase activity was calculated using inhibition rate in each group according to the following formula: (G4 - (G3 - G2))/G2. For example, if the productions of ADP in all groups are 300 (inhibition = 0) for G1, 200 (inhibition = 100) for G2, 180 (inhibition = 120) for G3, and 250 (inhibition = 50) for G4, respectively, the effect of H₂S on Na⁺/K⁺-ATPase activity will be (50 - (120 - 100))/100 = 30% ouabain. If H₂S has no effect on Na⁺/K⁺-ATPase activity, G3 = G2 + G4. If H₂S only inhibits Na⁺/K⁺-ATPase activity with no effect on other ATPase activity, G3 = G2.

Statistical Analysis

Data are presented as means ± S.E. The significance of differences in mean values within and between multiple groups was evaluated using analysis of variance (ANOVA), and any significant differences revealed by this procedure were further investigated using the Tukey multiple-range test. Student's *t* test was used to evaluate statistical significance of differences between two groups. *P* < 0.05 was considered statistically significant.

Results

Enzymatic Pathways for the Production of H₂S in Renal Tissue Homogenates. As shown in Fig. 1A, incuba-

Fig. 1. Enzymatic pathways for the production of H₂S in renal tissue homogenates. A, H₂S production rate by renal tissue homogenates using L-Cys as a substrate at different concentrations. *, $P < 0.05$ versus the values from lower concentration of L-Cys by repeated measures ANOVA with Tukey's post hoc test ($n = 5$). B, H₂S production rate by renal tissue homogenates using L-Cys as a substrate (1 mM) in the presence of AOAA (1 mM) and/or PPG (1 mM). *, $P < 0.05$ versus control (C); and #, $P < 0.05$ versus L-Cys by two-way ANOVA with Tukey's post hoc test ($n = 8$).

tion of renal tissue homogenates with L-Cys produced H₂S in a concentration-dependent manner. To determine which enzyme was responsible for the production of H₂S by the renal tissue, the inhibitors of CBS and CGL were added into the reaction systems either separately or in combination. These data were presented in Fig. 1B. H₂S production rates were slightly but significantly inhibited by AOAA (20%) or PPG (15%) alone. However, incubation of the renal tissue homogenates with a combination of AOAA and PPG abolished the H₂S production by renal tissue homogenates incubated with L-Cys as a substrate (Cys + AOAA + PPG).

Effects of Intrarenal Arterial Infusion of an H₂S Donor on Renal Hemodynamics and Excretory Functions. To examine whether H₂S has effects on renal hemodynamics and excretory functions, different doses of NaHS, an H₂S donor, were infused into the renal artery. The doses of NaHS were chosen from preliminary experiments that did not show any systemic effects of H₂S. The data were summarized in Table 1. There was no change in MAP after infusion of NaHS. However, infusion of NaHS significantly increased

U·V, U_{Na}·V, and U_K·V, as well as GFR at a low dose. Higher doses of NaHS induced significant increases in RBF as well. Renal fractional filtration (FF), fractional excretion of sodium (FE_{Na}) and potassium (FE_K) were also increased by infusion of NaHS into the renal artery.

Effects of Inhibition of Endogenous Renal H₂S Production on Renal Hemodynamics and Excretory Functions. To evaluate the role of endogenous H₂S in the regulation of renal hemodynamics and excretory functions, inhibitors of CBS and CGL were infused into the renal artery to block the production of H₂S within the kidney. As summarized in Table 2, infusion of AOAA (0.25, 0.5, and 1 μmol/min/kg) plus PPG (4 μmol/min/kg) significantly reduced GFR, U·V, U_{Na}·V, and U_K·V in a dose-dependent manner, whereas AOAA or PPG alone did not induce significant changes in the renal hemodynamics and excretory functions (data not shown).

Effect of L-Cys, a Substrate for Endogenous H₂S Production, on Renal Hemodynamics and Excretory Functions. As summarized in Figs. 2 and 3, intrarenal arterial infusions of L-Cys had no effect on MAP and RBF but dose-dependently increased GFR, U·V, U_{Na}·V, and U_K·V. To confirm that L-Cys increased these parameters due to the induction of H₂S, inhibitors of CBS and CGL were infused with L-Cys together. As shown in Figs. 2 and 3, inhibition of CBS and CGL considerably blocked the effects of L-Cys on GFR, U·V, U_{Na}·V, and U_K·V. Infusion of L-Cys also significantly increased FF, FE_{Na}, and FE_K, which was blocked by AOAA plus PPG (Fig. 3).

H₂S Levels in the Kidney Tissues after Different Treatments. After each experiment, the kidney was removed and saved, and the H₂S levels within the kidney were measured. The data are summarized in Fig. 4. Intrarenal arterial infusion of AOAA plus PPG substantially reduced the H₂S levels in the kidneys. Infusion of L-Cys significantly increased the H₂S levels. The increases in H₂S levels induced by L-Cys were blocked by AOAA + PPG. In addition, infusion of NaHS into the renal artery also markedly increased the levels of H₂S in the kidneys.

Effect of H₂S on Specific Sodium Transporters in the Kidneys. The major renal sodium transporters include Na⁺/H⁺ exchanger, Na⁺/K⁺/2Cl⁻ cotransporter (NKCC), Na⁺/Cl⁻ cotransporter (NCC), and epithelial Na⁺ channel on the apical side and Na⁺/K⁺-ATPase (NKA) on the basolateral side of the tubule. Carbonic anhydrase is also an important contributor to the Na⁺ reabsorption. In the current study, a substantial increase in U_K·V indicated that epithelial Na⁺ channel might not play an important role in H₂S-induced tubular effects. There was no significant change in urine pH

TABLE 1

Effect of intrarenal infusion of NaHS on renal hemodynamics and urinary excretory functions

NaHS (nmol/min/kg)	C	C	5	10	20	40	80
MAP (mm Hg)	107.8 ± 1.74	107.7 ± 1.72	107.5 ± 1.80	107.8 ± 1.79	107.2 ± 1.89	105.5 ± 1.58	106.1 ± 1.63
RBF (ml/min/g kwt)	8.99 ± 0.91	9.12 ± 0.94	9.12 ± 0.93	9.25 ± 0.93	9.73 ± 0.90*	10.59 ± 1.03*	10.55 ± 1.03*
GFR (ml/min/g kwt)	1.02 ± 0.10	1.04 ± 0.07	1.09 ± 0.08	1.26 ± 0.02*	1.32 ± 0.04*	1.57 ± 0.06*	1.79 ± 0.04*
U·V (μl/min/g kwt)	17.7 ± 1.70	17.2 ± 0.89	19.8 ± 2.07	26.0 ± 1.31*	39.1 ± 4.94*	53.7 ± 5.87*	81.3 ± 3.57*
U _{Na} ·V (μmol/min/g kwt)	2.72 ± 0.35	2.71 ± 0.53	2.96 ± 0.22	3.69 ± 0.21*	5.27 ± 0.70*	12.28 ± 1.31*	19.53 ± 1.40*
U _K ·V (μmol/min/g kwt)	1.69 ± 0.16	1.73 ± 0.26	1.82 ± 0.22	2.14 ± 0.26*	2.37 ± 0.19*	4.46 ± 0.33*	6.36 ± 0.59*
FF (%)	20.39 ± 1.47	20.65 ± 1.36	21.51 ± 1.31	24.71 ± 1.06*	25.77 ± 1.28*	26.95 ± 1.29*	30.86 ± 1.55*
FE _{Na} (%)	1.83 ± 0.25	1.84 ± 0.21	2.04 ± 0.18	2.26 ± 0.08*	3.02 ± 0.11*	5.39 ± 0.15*	7.52 ± 0.24*
FE _K (%)	33.53 ± 7.39	33.65 ± 3.30	34.37 ± 5.19	37.13 ± 2.14*	45.88 ± 4.33*	57.77 ± 6.04*	71.03 ± 7.86*

* $P < 0.05$ vs. control (C) by repeated measures ANOVA.

TABLE 2

Effect of intrarenal infusion of CBS and CGL inhibitors on renal hemodynamics and urinary excretory functions

PPG + AOAA ($\mu\text{mol}/\text{min}/\text{kg}$)	C	C	0.25	0.5	1.0
MAP (mm Hg)	114.75 $\pm$ 2.77	116.50 $\pm$ 1.35	113.63 $\pm$ 3.23	116.38 $\pm$ 1.16	115.00 $\pm$ 1.37
RBF (ml/min)	8.97 $\pm$ 0.36	8.93 $\pm$ 0.35	8.81 $\pm$ 0.39	8.53 $\pm$ 0.32	8.34 $\pm$ 0.37
GFR (ml/min/g kwt)	1.11 $\pm$ 0.05	1.09 $\pm$ 0.06	0.98 $\pm$ 0.05	0.75 $\pm$ 0.03*	0.50 $\pm$ 0.09*
U·V ($\mu\text{l}/\text{min}/\text{g kwt}$)	18.52 $\pm$ 0.58	19.15 $\pm$ 0.93	16.47 $\pm$ 0.31	12.70 $\pm$ 0.86*	11.72 $\pm$ 1.07*
U _{Na} ·V ($\mu\text{mol}/\text{min}/\text{g kwt}$)	2.42 $\pm$ 0.22	2.32 $\pm$ 0.13	1.94 $\pm$ 0.09	1.61 $\pm$ 0.06*	1.55 $\pm$ 0.12*
U _K ·V ($\mu\text{mol}/\text{min}/\text{g kwt}$)	1.80 $\pm$ 0.09	1.85 $\pm$ 0.13	1.51 $\pm$ 0.10	1.26 $\pm$ 0.07*	1.10 $\pm$ 0.07*
FF (%)	21.10 $\pm$ 0.53	19.93 $\pm$ 1.20	18.09 $\pm$ 1.31	14.71 $\pm$ 0.75*	11.81 $\pm$ 1.32*
FE _{Na} (%)	1.50 $\pm$ 0.30	1.47 $\pm$ 0.16	1.37 $\pm$ 0.13	1.48 $\pm$ 0.13	2.15 $\pm$ 0.09*
FE _K (%)	32.39 $\pm$ 1.26	33.98 $\pm$ 1.58	30.94 $\pm$ 1.38	33.41 $\pm$ 1.60	44.06 $\pm$ 0.52*

* $P < 0.05$ vs. control (C) by repeated measures ANOVA.

Fig. 2. Effect of infusion of L-Cys into renal artery on systemic and renal hemodynamics. Open circle, infusion of L-Cys alone in different doses. Solid circle, infusion of L-Cys in the same doses as control, plus AOAA (1 $\mu\text{mol}/\text{min}/\text{kg}$) and PPG (4 $\mu\text{mol}/\text{min}/\text{kg}$). *, $P < 0.05$ versus control (0) by repeated measures ANOVA with Tukey's post hoc test; #, $P < 0.05$ versus inhibitor-treated animals by t test ($n = 5$).

after NaHS infusion, even at a large dose (data not shown), suggesting that Na^+/H^+ exchanger and carbonic anhydrase were unlikely the central factors mediating the effect of H_2S . Therefore, we examined the effects of H_2S on NKCC, NCC, and NKA. NaHS + furosemide induced a significant increase in $\text{U}_{\text{Na}}\cdot\text{V}$ compared with furosemide alone. However, NaHS-induced increases in $\text{U}_{\text{Na}}\cdot\text{V}$ were significantly reduced in the present of furosemide compared with NaHS alone, indicating an inhibition of NKCC by H_2S (Fig. 5A). There was no significant difference in NaHS-induced increases in $\text{U}_{\text{Na}}\cdot\text{V}$ between the animals treated with NaHS and NaHS + thiazide, indicating that NCC was not involved in the tubular effects of H_2S (Fig. 5B). We were unable to conduct the similar in vivo experiment to examine the effect of H_2S on NKA, as we did on NKCC and NCC, due to the strong vessel constrictor effect of the NKA inhibitor (Aalkjaer and Mulvany, 1985). Therefore, we detected the effect of H_2S on NKA in vitro using isolated

renal basolateral membrane. Figure 5C shows that the HPLC chromatogram clearly separated ATP and ADP and detected the production of ADP after the incubation of renal basolateral membrane with ATP. NaHS inhibited NKA activity in a concentration-dependent manner with a maximal inhibition of 41% (Fig. 5D). Although H_2S did not inhibit NKA activity by 100%, 1 μM NaHS induced an inhibition of 19%, suggesting that H_2S is relatively a potent Na^+/K^+ -ATPase inhibitor. Partial inhibitions of NKCC and NKA by H_2S may be because the doses/concentrations of NaHS used in these experiments were not the maximal ones.

Discussion

Our results indicated that both CBS and CGL were functioning to produce H_2S in the kidneys and that the inhibition of CBS or CGL alone could be compensated by the increase in the activity of the other enzyme in H_2S production. This is consistent with a previous study, which showed that inhibition of CGL activity by 93% lowered H_2S production by only 30% (Stipanuk and Beck, 1982). A recent report suggested that CGL was an essential enzyme for the synthesis of endogenous H_2S in the kidneys based on the findings that PPG prevented the recovery of renal function after ischemic injury, whereas exogenous H_2S protected the kidney from ischemic injury (Tripatara et al., 2008). The results from this report might not be able to rule out that inhibition of endogenous H_2S production requires blockade of both CBS and CGL in the kidneys, because the enhanced renal injury after PPG administration could be a nephrotoxic effect of PPG (Konno et al., 2000) under the ischemic conditions.

Given the facts that H_2S plays an important role in the regulation of vascular function (Hosoki et al., 1997; Zhao et al., 2003; Cheng et al., 2004; Yan et al., 2004; Webb et al., 2008) and that the control of renal function is largely associated with the regulation of renal vascular functions, H_2S may significantly participate in the control of renal functions. In the present study, intrarenal arterial infusion of an H_2S donor increased RBF and GFR, which was accompanied by increases in FF, indicating that H_2S may produce greater vasodilation in preglomerular arterioles than in postglomerular arterioles. In addition, the present study demonstrated that H_2S increased U·V, $\text{U}_{\text{Na}}\cdot\text{V}$, and $\text{U}_{\text{K}}\cdot\text{V}$ much more than RBF and GFR, implying that H_2S has direct effects on renal tubular functions. Indeed, both FE_{Na} and FE_{K} were increased by NaHS, suggesting an inhibitory effect of H_2S on ion transport in the renal tubules. It was also shown that H_2S exhibited more potent effects on tubular activity than vascular activity.

Fig. 4. H₂S levels in the kidney tissues after infusion of different enzyme inhibitors, substrate, or H₂S donor into renal arteries. C, control; A, AOAA; P, PPG; Cys, L-cysteine; *, $P < 0.05$ versus control; and #, $P < 0.05$ versus Cys by ANOVA with Tukey's post hoc test. $n = 5$.

Although the above results from exogenous H₂S indicate that H₂S may play an important role in the regulation of renal function, the conclusion cannot be drawn until the role of endogenous H₂S is confirmed. Our results showed that combined inhibition of CBS and CGL significantly attenuated GFR, U·V, $U_{Na}\cdot V$, and $U_K\cdot V$, revealing that endogenous H₂S is important in the maintenance of basal renal filtration and tubular functions. Inhibition of either CBS or CGL alone did not have a marked effect on renal hemodynamics and excretory functions. This is probably due to the observation that inhibition of either one of these two enzymes only reduced a small portion of H₂S production; the effect of this small change in H₂S production could not be detected in the complicated in vivo environment.

It should be noted that although PPG, an inhibitor of CGL used in the present study, was shown to be toxic to renal proximal tubular cells (Konno et al., 2000), the effects of PPG

in the present study were unlikely due to its nephrotoxicity, because nephrotoxicity of PPG needs a large dose (300 versus 13.6 mg/kg in the present study) and induces an increase in urine volume (Konno et al., 2000). It has been shown that PPG at a dose of 50 mg/kg does not cause histological abnormalities in the kidneys (Triguero et al., 1997).

To further evaluate the physiological significance of H₂S in the regulation of renal function, we infused L-Cys into the renal artery to determine the effect of increased endogenous H₂S production on renal functions. Previous studies have documented that incubation of isolated arteries with L-Cys increases H₂S production and induces vasodilation (Cheng et al., 2004). Our results showed that L-Cys dose-dependently increased GFR, U·V, $U_{Na}\cdot V$, and $U_K\cdot V$. Because there was no significant increase in RBF after infusion of L-Cys, the increase in GFR was probably due to the increases in FF and glomerular ultrafiltration coefficient (K_f). It has been documented that glomerular mesangial cells and podocytes contain contractile proteins and respond to vasoactive hormones, thereby modulating the K_f (Stockand and Sansom, 1998; Pavenstädt, 2000). It is possible that H₂S has a direct effect on mesangial cells or through an unknown mechanism increases K_f and consequently GFR. Our results also confirmed that L-Cys-induced changes in renal functions were attributed to increased production of H₂S, because inhibition of CBS and CGL in combination abolished the L-Cys-induced effects on renal hemodynamics and excretions. This was consistent with the results in our biochemical analyses. These data further demonstrate that endogenous H₂S participates in the regulation of renal functions. The results from the measurement of renal H₂S concentrations showed that renal H₂S concentrations were well associated with the changes in

Fig. 3. Effects of infusion of L-Cys into renal artery on renal excretory function. *, $P < 0.05$ versus control (0) by repeated measures ANOVA with Tukey's post hoc test; #, $P < 0.05$ versus inhibitor-treated animals by t test ($n = 5$).

Fig. 5. Effect of NaHS on specific renal sodium transporters. A, $U_{Na·V}$ after intrarenal arterial infusion of NaHS, a H₂S donor, and/or furosemide. C, control infused with vehicle; H, H₂S donor; P, post-control; F, furosemide; HF, H + F; H - C, difference in $U_{Na·V}$ between H and C; HF - F, difference in $U_{Na·V}$ between HF and F; *, $P < 0.05$ versus C; #, $P < 0.05$ versus HF - F by paired t test ($n = 5$). B, $U_{Na·V}$ after intrarenal arterial infusion of H₂S donor and/or thiazide. T, thiazide, the rest of the labels are similar to those in A; *, $P < 0.05$ versus C; #, $P < 0.05$ versus T ($n = 5$). C, representative HPLC chromatograms of ATP and ADP standards and ADP produced after incubation of renal BM with ATP. D, calculated inhibition rates in Na⁺/K⁺-ATPase activity by NaHS in renal BM. *, $P < 0.05$ versus 1 μM NaHS; #, $P < 0.05$ versus 10 μM NaHS ($n = 6$).

renal functions, which additionally indicates an important role of H₂S in the regulation of renal functions.

There was a concern that infusion of L-Cys, unlike NaHS, did not increase RBF despite the fact that it increased H₂S in the kidney. This discrepancy indicates that L-Cys may produce other effects in addition to the enhancement of H₂S production. L-Cys has been reported to be a weak inhibitor of S-adenosylhomocysteine hydrolase (Knudsen and Yall, 1972), an enzyme that produces adenosine (Kloor et al., 2003). Adenosine is well known for increasing afferent arteriolar resistance and decreasing efferent arteriolar resistance, which is accompanied by little change in total RBF (Spielman and Thompson, 1982). Infusion of L-Cys may decrease the production of adenosine within the kidneys, resulting in a preglomerular vessel relaxation and postglomerular vessel constriction. This postglomerular vessel constriction may then counteract the effect of H₂S on RBF.

As a result, L-Cys induced little change in RBF in the current study. Slightly higher FF in L-Cys-infused rats may be taken in support of the notion that there are adenosine-mediated effects of L-Cys. However, this adenosine-mediated effect may not be a major portion of L-Cys-induced action because inhibition of CBS and CGL abolished the effect of L-Cys on renal function, suggesting that H₂S-mediated action of L-Cys is predominant. The speculation concerning the effect of L-Cys on RBF needs to be proven in future studies. In addition, there are possibly other mechanisms that may explain the differences between L-Cys- and NaHS-induced effects on RBF, such as the possibility that L-Cys or NaHS may be metabolized in a different manner that produces other unknown renal active metabolites.

To determine which sodium transporter is responsible for the inhibitory effect of H₂S on sodium reabsorption, we performed experiments to dissect the effects of H₂S on specific

sodium transporters along the renal tubule. Our results demonstrated that the tubular effects of H₂S might be partially through inhibition of NKCC and NKA activity. The inhibitory effects of H₂S on both NKCC and NKA are similar to those of NO (Ortiz and Garvin, 2002). The interaction between H₂S and NO in this regard may need further attention. It should be noted that H₂S exhibits both vascular and tubular effects and that the inhibitors of NKCC, NCC, and NKA also have effects on renal hemodynamics (Langård et al., 1984; Okusa et al., 1989; Sawaya et al., 1991; Dobrowolski et al., 2000; Dobrowolski and Sadowski, 2005; Lorenz et al., 2006). Therefore, there is a possibility that there are interactions/counteractions between the vascular effects of H₂S and those of Na⁺ transport inhibitors. These interactions/counteractions can also change U_{Na}·V and may be responsible for the further increases or no further effect in U_{Na}·V when NaHS is infused in combination with different Na⁺ transport inhibitors. Moreover, it cannot be ruled out that H₂S affects other tubular transporters simultaneously in addition to its effects on NKCC and NKA, because such effects, if there are any, may be covered in the complicated signal interactions/counteractions in the in vivo experiments. Thus, the results from the current study are not conclusive regarding the effects of H₂S on specific sodium transporters. A study using isolated and perfused different tubular segments will clarify the effects of H₂S on specific tubular transporters in the future.

In summary, the present study demonstrated that the kidney tissues were capable of producing H₂S from L-Cys via CBS and CGL; exogenous H₂S produced dose-related increases in RBF, GFR, and urinary excretion; inhibition of endogenous H₂S reduced GFR, U·V, U_{Na}·V, and U_K·V; induction of endogenous H₂S production increased GFR, U·V, U_{Na}·V, and U_K·V; FE_{Na} and FE_K were also increased by H₂S; and the inhibitory effect of H₂S on tubular reabsorption involved NKCC and NKA. These results suggest that H₂S participates in the control of renal function via both vascular and tubular actions in the kidney.

References

- Aalkjaer C and Mulvany MJ (1985) Effect of ouabain on tone, membrane potential and sodium efflux compared with [³H]ouabain binding in rat resistance vessels. *J Physiol* **362**:215–231.
- Baskar R, Li L, and Moore PK (2007) Hydrogen sulfide induces DNA damage and changes in apoptotic gene expression in human lung fibroblast cells. *FASEB J* **21**:247–255.
- Bhatia M, Wong FL, Fu D, Lau HY, Mochhala SM, and Moore PK (2005) Role of hydrogen sulfide in acute pancreatitis and associated lung injury. *FASEB J* **19**:623–625.
- Cheng Y, Ndisang JF, Tang G, Cao K, and Wang R (2004) Hydrogen sulfide-induced relaxation of resistance mesenteric artery beds of rats. *Am J Physiol Heart Circ Physiol* **287**:H2316–H2323.
- Dobrowolski L, B dzyńska B, and Sadowski J (2000) Differential effect of furosemide on renal medullary and cortical blood flow in the anaesthetized rat. *Exp Physiol* **85**:783–789.
- Dobrowolski L and Sadowski J (2005) Furosemide-induced renal medullary hypoperfusion in the rat: role of tissue tonicity, prostaglandins and angiotensin II. *J Physiol* **567**:613–620.
- Eto K, Asada T, Arima K, Makifuchi T, and Kimura H (2002) Brain hydrogen sulfide is severely decreased in Alzheimer's disease. *Biochem Biophys Res Commun* **293**:1485–1488.
- Fiorucci S, Antonelli E, Mencarelli A, Orlandi S, Renga B, Rizzo G, Distrutti E, Shah V, and Morelli A (2005) The third gas: H₂S regulates perfusion pressure in both the isolated and perfused normal rat liver and in cirrhosis. *Hepatology* **42**:539–548.
- Geng B, Yang J, Qi Y, Zhao J, Pang Y, Du J, and Tang C (2004) H₂S generated by heart in rat and its effects on cardiac function. *Biochem Biophys Res Commun* **313**:362–368.
- Hosoki R, Matsuki N, and Kimura H (1997) The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. *Biochem Biophys Res Commun* **237**:527–531.
- House JD, Brosnan ME, and Brosnan JT (1997) Characterization of homocysteine metabolism in the rat kidney. *Biochem J* **328**:287–292.
- Ishii I, Akahoshi N, Yu XN, Kobayashi Y, Namekata K, Komaki G, and Kimura H (2004) Murine cystathionine gamma-lyase: complete cDNA and genomic sequences, promoter activity, tissue distribution and developmental expression. *Biochem J* **381**:113–123.
- Kimura H (2002) Hydrogen sulfide as a neuromodulator. *Mol Neurobiol* **26**:13–19.
- Kloor D, Lüdtke A, Stoeva S, and Osswald H (2003) Adenosine binding sites at S-adenosylhomocysteine hydrolase are controlled by the NAD⁺/NADH ratio of the enzyme. *Biochem Pharmacol* **66**:2117–2123.
- Knudsen RC and Yall I (1972) Partial purification and characterization of S-adenosylhomocysteine hydrolase isolated from *Saccharomyces cerevisiae*. *J Bacteriol* **112**:569–575.
- Konno R, Ikeda M, Yamaguchi K, Ueda Y, and Niwa A (2000) Nephrotoxicity of D-propargylglycine in mice. *Arch Toxicol* **74**:473–479.
- Langård O, Holdaas H, Vikse A, Sejersted OM, and Eide I (1984) Ouabain inhibits renin release by a direct renal haemodynamic effect. *Scand J Clin Lab Invest* **44**:557–563.
- Li N, Chen L, Muh RW, and Li PL (2006) Hyperhomocysteinemia associated with decreased renal transsulfuration activity in Dahl S rats. *Hypertension* **47**:1094–1100.
- Lorenz JN, Dostanic-Larson I, Shull GE, and Lingrel JB (2006) Ouabain inhibits tubuloglomerular feedback in mutant mice with ouabain-sensitive α1 Na,K-ATPase. *J Am Soc Nephrol* **17**:2457–2463.
- Okusa MD, Persson AE, and Wright FS (1989) Chlorothiazide effect on feedback-mediated control of glomerular filtration rate. *Am J Physiol* **257**:F137–F144.
- Ortiz PA and Garvin JL (2002) Role of nitric oxide in the regulation of nephron transport. *Am J Physiol Renal Physiol* **282**:F777–F784.
- Pavenstädt H (2000) Roles of the podocyte in glomerular function. *Am J Physiol Renal Physiol* **278**:F173–F179.
- Sawaya BP, Weihprecht H, Campbell WR, Lorenz JN, Webb RC, Briggs JP, and Schnermann J (1991) Direct vasoconstriction as a possible cause for amphotericin B-induced nephrotoxicity in rats. *J Clin Invest* **87**:2097–2107.
- Sheikh MI, Kragh-Hansen U, Jørgensen KE, and Røigaard-Petersen H (1982) An efficient method for the isolation and separation of basolateral-membrane and luminal-membrane vesicles from rabbit kidney cortex. *Biochem J* **208**:377–382.
- Spielman WS and Thompson CI (1982) A proposed role for adenosine in the regulation of renal hemodynamics and renin release. *Am J Physiol* **242**:F423–F435.
- Srilatha B, Adaikan PG, Li L, and Moore PK (2007) Hydrogen sulphide: a novel endogenous gasotransmitter facilitates erectile function. *J Sex Med* **4**:1304–1311.
- Stipanuk MH and Beck PW (1982) Characterization of the enzymic capacity for cysteine desulphhydration in liver and kidney of the rat. *Biochem J* **206**:267–277.
- Stockand JD and Sansom SC (1998) Glomerular mesangial cells: electrophysiology and regulation of contraction. *Physiol Rev* **78**:723–744.
- Sudo J, Terui J, Iwase H, and Kakuno K (2000) Assay of ATPase and Na,K-ATPase activity using high-performance liquid chromatographic determination of ADP derived from ATP. *J Chromatogr B Biomed Sci Appl* **744**:19–23.
- Teague B, Asiedu S, and Moore PK (2002) The smooth muscle relaxant effect of hydrogen sulphide in vitro: evidence for a physiological role to control intestinal contractility. *Br J Pharmacol* **137**:139–145.
- Triguero A, Barber T, García C, Puertes IR, Sastre J, and Viña JR (1997) Liver intracellular L-cysteine concentration is maintained after inhibition of the trans-sulfuration pathway by propargylglycine in rats. *Br J Nutr* **78**:823–831.
- Tripatara P, Patel NS, Collino M, Gallicchio M, Kieswich J, Castiglia S, Benetti E, Stewart KN, Brown PA, Yaqoob MM, et al. (2008) Generation of endogenous hydrogen sulfide by cystathionine gamma-lyase limits renal ischemia/reperfusion injury and dysfunction. *Lab Invest* **88**:1038–1048.
- Wang R (2002) Two's company, three's a crowd: can H₂S be the third endogenous gaseous transmitter? *FASEB J* **16**:1792–1798.
- Webb GD, Lim LH, Oh VM, Yeo SB, Cheong YP, Ali MY, El Oakley R, Lee CN, Wong PS, Caleb MG, et al. (2008) Contractile and vasorelaxant effects of hydrogen sulfide and its biosynthesis in the human internal mammary artery. *J Pharmacol Exp Ther* **324**:876–882.
- Yan H, Du J, and Tang C (2004) The possible role of hydrogen sulfide on the pathogenesis of spontaneous hypertension in rats. *Biochem Biophys Res Commun* **313**:22–27.
- Yang G, Yang W, Wu L, and Wang R (2007) H₂S, endoplasmic reticulum stress, and apoptosis of insulin-secreting beta cells. *J Biol Chem* **282**:16567–16576.
- Zhang AY, Chen YF, Zhang DX, Yi FX, Qi J, Andrade-Gordon P, de Garavilla L, Li PL, and Zou AP (2003) Urotensin II is a nitric oxide-dependent vasodilator and natriuretic peptide in the rat kidney. *Am J Physiol Renal Physiol* **285**:F792–F798.
- Zhao W, Ndisang JF, and Wang R (2003) Modulation of endogenous production of H₂S in rat tissues. *Can J Physiol Pharmacol* **81**:848–853.
- Zhao W, Zhang J, Lu Y, and Wang R (2001) The vasorelaxant effect of H₂S as a novel endogenous gaseous KATP channel opener. *EMBO J* **20**:6008–6016.
- Zou AP, Li N, and Cowley AW Jr (2001) Production and actions of superoxide in the renal medulla. *Hypertension* **37**:547–553.

Address correspondence to: Dr. Ningjun Li, Department of Pharmacology and Toxicology, Medical College of Virginia, Virginia Commonwealth University, P.O. Box 980613, Richmond, VA 23298. E-mail: nli@vcu.edu