

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

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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-oxycetic 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 ($U_{Na}\cdot V$), and potassium ($U_K\cdot V$) excretion. Consistently, infusion of both AOAA and PPG to inhibit the endogenous H_2S production decreased GFR, $U_{Na}\cdot V$, and $U_K\cdot V$, 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, $U_{Na}\cdot V$, and $U_K\cdot V$, 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 $Na^+/K^+/2Cl^-$ cotransporter and $Na^+/K^+/ATPase$ 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.

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ABBREVIATIONS: H_2S , hydrogen sulfide; L-Cys, L-cysteine; CBS, cystathionine β -synthetase; CGL, cystathionine γ -lyase; AOAA, amino-oxycetic acid; PPG, propargylglycine; GFR, glomerular filtration rate; U-V, urinary volume; $U_{Na}\cdot V$, urinary sodium; $U_K\cdot V$, urinary potassium; FF, fractional filtration; FE_{Na} , fractional excretion of sodium; FE_K , fractional excretion of potassium; MAP, mean arterial blood pressure; NKCC, $Na^+/K^+/2Cl^-$ cotransporter; NCC, Na^+/Cl^- cotransporter; NKA, $Na^+/K^+-ATPase$; 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-

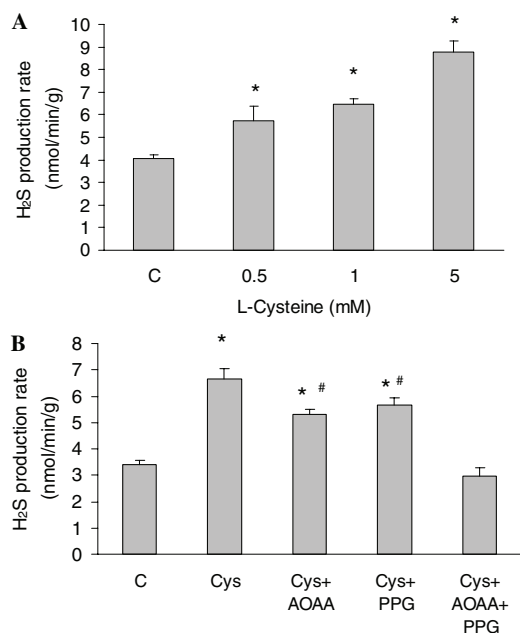


Fig. 1. Enzymatic pathways for the production of H₂S in renal tissue homogenates. A, H₂S production rate by renal tissue homogenates using L-Cys as a substrate at different concentrations. *, $P < 0.05$ versus the values from lower concentration of L-Cys by repeated measures ANOVA with Tukey's post hoc 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*
$U_{\text{Na}}\cdot\text{V}$ ($\mu\text{mol}/\text{min}/\text{g kwt}$)	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*
$U_{\text{K}}\cdot\text{V}$ ($\mu\text{mol}/\text{min}/\text{g kwt}$)	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*

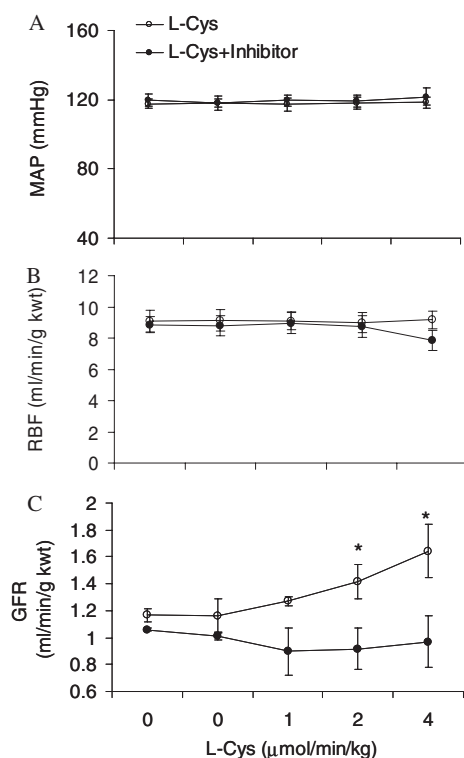
* $P < 0.05$ vs. control (C) by repeated measures ANOVA.

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 $U_{\text{Na}}\cdot\text{V}$ compared with furosemide alone. However, NaHS-induced increases in $U_{\text{Na}}\cdot\text{V}$ were significantly reduced in the presence 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 $U_{\text{Na}}\cdot\text{V}$ 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, $U_{\text{Na}}\cdot\text{V}$, and $U_{\text{K}}\cdot\text{V}$ 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.

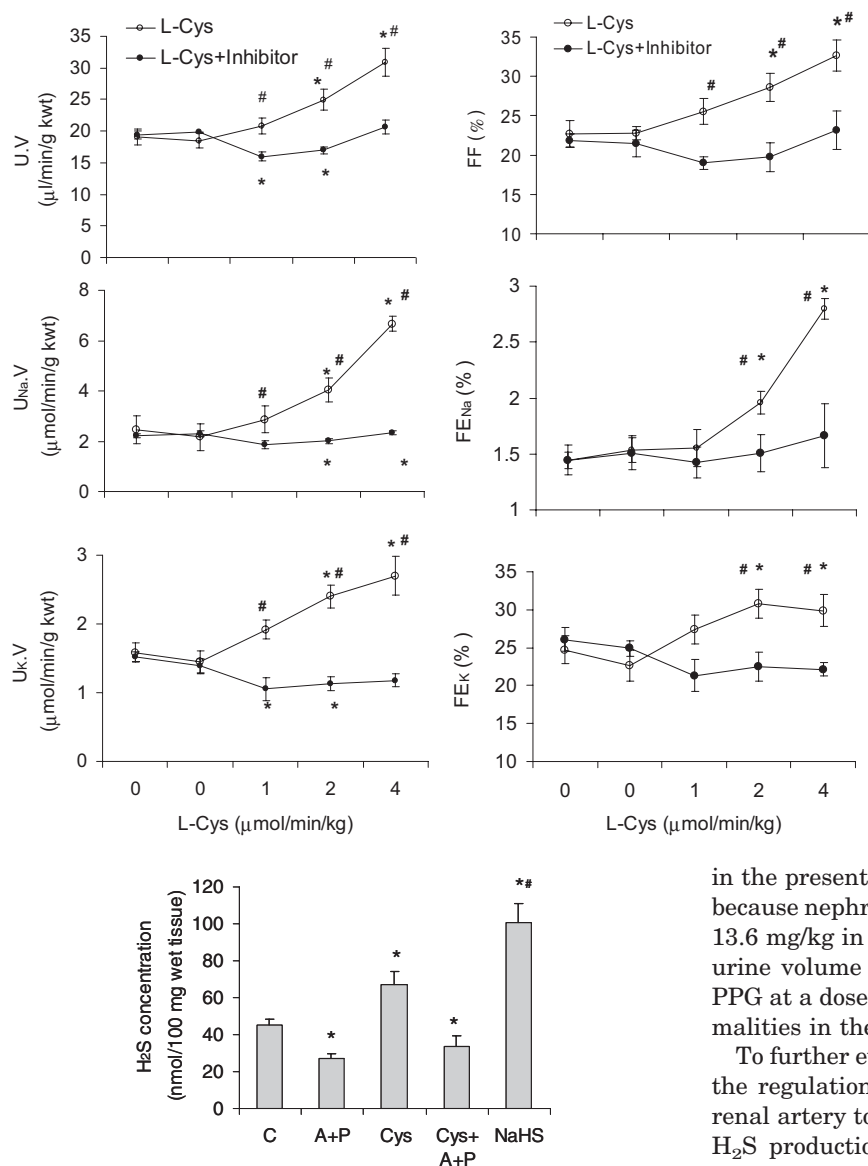


Fig. 4. H₂S levels in the kidney tissues after infusion of different enzyme inhibitors, substrate, or H₂S donor into renal arteries. C, control; A, AOAA; P, PPG; Cys, L-cysteine; *, $P < 0.05$ versus control; and #, $P < 0.05$ versus Cys by ANOVA with Tukey's post hoc test. $n = 5$.

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, $U_{\text{Na}}\cdot\text{V}$, and $U_{\text{K}}\cdot\text{V}$, 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, $U_{\text{Na}}\cdot\text{V}$, and $U_{\text{K}}\cdot\text{V}$. 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 ($n = 5$).

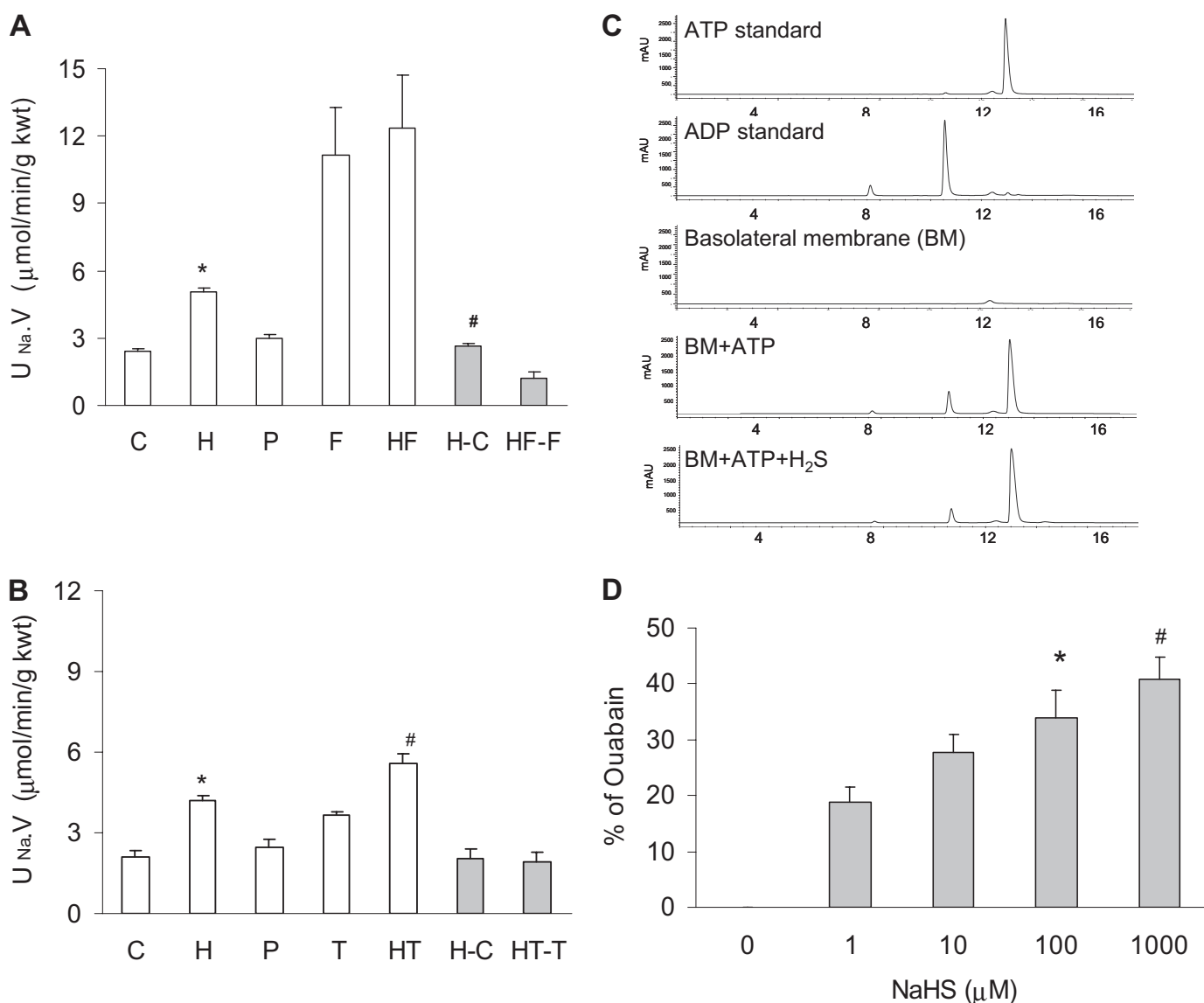


Fig. 5. Effect of NaHS on specific renal sodium transporters. A, $U_{Na·V}$ after intrarenal arterial infusion of NaHS, a H_2S donor, and/or furosemide. C, control infused with vehicle; H, H_2S donor; P, post-control; F, furosemide; HF, H + F; H - C, difference in $U_{Na·V}$ between H and C; HF - F, 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 H_2S 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 H_2S 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 H_2S in the kidney. This discrepancy indicates that L-Cys may produce other effects in addition to the enhancement of H_2S 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 H_2S 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 H_2S -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 H_2S on sodium reabsorption, we performed experiments to dissect the effects of H_2S 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.

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