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Hydrogen Sulfide as an Endogenous Modulator of Biliary Bicarbonate Excretion in the Rat Liver

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ABSTRACT

Cystathionine γ-lyase (CSE) is an enzyme catalyzing cystathionine and cysteine to yield cysteine and hydrogen sulfide (H_2S), respectively. This study aimed to examine if H_2S generated from the enzyme could serve as an endogenous regulator of hepatobiliary function. Gas chromatographic analyses indicated that, among rat organs herein examined, liver constituted one of the greatest components of H_2S generation in the body, at 100 μmol/g of tissue, comparable to that in kidney and 1.5-fold greater than that in brain, where roles of the gas in the regulation of neurotransmission were reported previously. At least half of the gas amount in the liver appeared to be derived from CSE, because blockade of the enzyme by propargylglycine suppressed it by 50%. Immunohistochemistry revealed that CSE occurs not only in hepatocytes, but also in bile duct. In livers *in vivo*, as well as in those perfused *ex vivo*, treatment with the CSE inhibitor induced choleresis by stimulating the basal excretion of bicarbonate in bile samples. Transportal supplementation of NaHS at 30 μmol/L, but not that of *N*-acetylcysteine as a cysteine donor, abolished these changes elicited by the CSE inhibitor in the perfused liver. The changes elicited by the CSE blockade did not coincide with alterations in hepatic vascular resistance, showing little involvement of vasodilatory effects of the gas in these events, if any. These results first provided evidence that H_2S generated through CSE modulates biliary bicarbonate excretion and is thus a determinant of bile salt-independent bile formation in the rat liver. *Antioxid. Redox Signal.* 7, 788–794.

INTRODUCTION

Cysteine metabolism in the liver has been shown to contribute greatly to detoxification processes through multiple mechanisms. Following reduction and decarboxylation processes, this amino acid is converted to taurine, the compound used for conjugation of bile acids. Cysteine serves as a substrate for synthesis of glutathione through reactions of glutamate ligase and glutathione synthase, and is also used to generate sulfate through aspartate transferase and sulfite oxidase; these two compounds have well been shown to play an important role in detoxification of xenobiotics such as acetaminophen. Another important substance generated upon cysteine metabolism *in vivo* is hydrogen sulfide (H_2S). This gaseous compound has recently been shown to account for a signaling molecule in neural and vascular systems. It is produced mainly by two types of pyridoxal 5′-phosphate-dependent enzymes responsible for metabolism of L-cysteine: cystathionine γ-lyase (CSE; EC 4.4.1.1) and cystathionine β-synthase (CBS; EC 4.2.1.22). In other words, although the primary role of the two enzymes is to constitute the transsulfuration pathway that provides cysteine through biotransformation of methionine derived from nutrition, both CSE and CBS are able to use cysteine as the substrate to generate H_2S. The gas synthesized by CBS in brain has been reported to execute neural transduction. On the other hand, CSE-derived H_2S was shown to relax vascular smooth muscle cells through its ability to increase the conductance of potassium channels (22); in this study, H_2S released from the enzyme blocked vasoconstriction of rat aortic rings elicited by glibenclamide, a blocker of the ATP-

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gated K^+ channel. Furthermore, the CSE activities have been reported to be altered under disease conditions; the activity in the liver is reduced in patients with liver cirrhosis and in those exposed to surgical insults or acquired immune deficiency syndrome (8, 10, 20). On the other hand, experimental models of vitamin B_6 deficiency or streptozotocin-induced diabetes revealed alterations in CSE in the liver under these disease conditions (6, 15). Until now, however, effects of such alterations in the activities on organ functions and roles of H_2S under these circumstances have not been fully investigated yet.

This study was designed to focus first on differences in contribution of CSE to tissue H_2S generation; the data indicated that the liver constitutes one of the largest organ components for the gas generation in the body. Based on this result, we further attempted to examine if H_2S derived from the enzyme could play a role in the regulation of hepatobiliary function. The current results first provided evidence that the liver utilizes this gaseous substance as a modulatory determinant of biliary bicarbonate excretion.

MATERIALS AND METHODS

In vivo *and* ex vivo *determination of bile constituents*

The experimental protocols herein described were approved by our institutional guidelines provided by the Animal Care Committee of Keio University School of Medicine. Male Wistar rats weighing 220–260 g (CLEA Japan, Tokyo, Japan) were allowed free access to laboratory chow and tap water, and were fasted for 24 h prior to experiments. As described elsewhere, rats were anesthetized with an intramuscular injection of pentobarbital sodium at 50 mg/kg, and their common bile ducts were cannulated to collect bile samples. Bile output was monitored *in vivo* according to our previous method (7). When necessary, livers of these rats were perfused *ex vivo* with the oxygenated Krebs–Henseleit buffer at a constant flow rate of 4 ml/min/g of liver in a single-pass mode (14). Bile samples collected through a cannulation were used to determine concentrations of total bile salts, phospholipids, pH values, and bicarbonate (HCO_3^-) according to previous methods described elsewhere (7, 14).

Experimental protocols

Propargylglycine (PPG) was used as a potent inhibitor of CSE. PPG was dissolved in physiological saline as a vehicle and administered intraperitoneally at a dose of 1.5 mmol/kg of body weight at 4 h prior to the preparation for bile duct cannulation. Bile was collected every 10 min until the end of experiments according to our previous method (7). In the case of experiments using the *ex vivo* perfused preparation, livers were excised from the PPG-treated rats and perfused with the Krebs–Henseleit buffer containing 300 μmol/L PPG to avoid a possible reduction of the enzyme blockade due to elimination of the reagent from the system. To examine effects of the intraperitoneal injection of the CSE inhibitor on endogenous H_2S generation, we determined tissue contents of the gas *in vivo*. Livers were excised and snap-frozen at 4 h after the treatment with PPG or vehicle, and the samples were minced with 0.1 *N* NaOH to remove proteins. Amounts of H_2S in the liver tissues were determined by gas chromatography according to previous methods described elsewhere (4). In separate sets of experiments, bile output was monitored every 10 min after establishment of the bile duct cannulation, and concentrations and fluxes of bile constituents were compared between the control and PPG-treated groups. To examine if effects of PPG are attributable to a reduction of the reaction product of CSE such as H_2S, we examined effects of supplementation of NaHS, a soluble donor of the gas at desired concentrations, in the buffer for the *ex vivo* perfusion system. As a control set of the experiments, we compared effects of the same concentrations of *N*-acetylcysteine (NAC), a cysteine donor. In experiments using isolated *ex vivo* perfused livers, sodium taurocholate was added to the buffer at desired concentrations in a range between 0 and 30 μmol/L. Using data collected from these experiments, the bile acid-independent fraction of bile output was determined by plotting bile output as a function of biliary output of bile salts in the samples: the value of the output at the *y*-intercept (zero concentration of bile salts) was regarded as the bile acid-independent fraction (2).

Immunohistochemistry

Liver tissues also served as samples for immunohistochemistry. An anti-CSE antibody was prepared by immunization to rabbit of the C-terminal peptide CYGGTNRYFRRVASE, the sequence of which is identical to that of the rat enzyme. The antibody was purified from the antiserum using affinity chromatography as described elsewhere (3). The specificity of the antibody was confirmed by western blot analyses. For immunohistochemistry, rat livers were removed to prepare OCT compound-embedded frozen sections (7 μm). The sections were immunostained with the anti-CSE antibody using the Vectastain ABC kit (Vector Laboratories), as previously described (5). Semiserial sections were stained with the anti-CSE antibody or with the anti-rat keratin 19 monoclonal antibody (MAB1675; Chemicon, Temecula, CA, U.S.A.) to examine colocalization of the enzyme with biliary epithelium and hepatocellular bile canaliculi, when necessary.

Statistical analyses

The statistical significance of data among different experimental groups was determined by one-way ANOVA and Fisher's multiple comparison test. $p < 0.05$ was considered significant.

RESULTS

Liver constitutes the largest organ component for CSE-derived H_2S generation

Figure 1 illustrates tissue contents of H_2S in different organs. The control liver treated with vehicle contained ~80 nmol/g of tissue of the gas (Fig. 1A). Livers from rats pretreated with 1.5 mmol/kg PPG, an inhibitor of CSE, suppressed the constitutive levels of the gas by 50%. The dose of PPG used in this experiment appeared to be sufficient enough to block the enzyme, as indicated by dose responses of the H_2S contents as a function of doses of the inhibitor (Fig. 1B). When the tissue gas

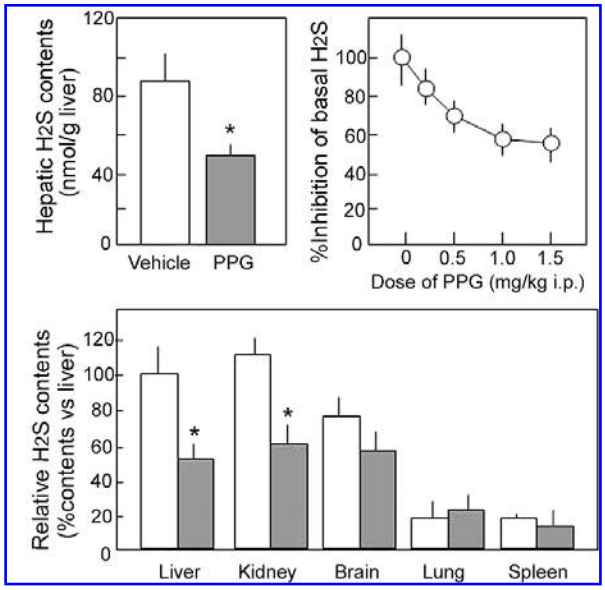

FIG. 1. Effects of administration of PPG, an inhibitor of CSE, on tissue contents of H_2S *in vivo*. (**A**) The effects of the PPG administration on hepatic H_2S contents. PPG was intraperitoneally injected at 1.5 mmol/kg at 4 h prior to the experiments. Data indicate means ± SE of more than eight separate experiments. *$p < 0.05$ as compared with the vehicle-treated control group. (**B**) Dose-dependent effects of PPG on the basal H_2S contents in rat livers. (**C**) Differences in the sensitivity to PPG administration among organs. Open and filled bars represent the tissue H_2S contents in the vehicle- and PPG-treated groups, respectively. Data indicate means ± SE of four separate experiments. *$p < 0.05$ as compared with the vehicle-treated control group.

contents were compared among different organs (Fig. 1C), liver appeared to constitute the largest organ component for endogenous H_2S production; the level was comparable to that measured in the kidney and 1.5-fold greater than that in the brain. So far as judged by sensitivity to PPG, the gas generation in the liver and kidney depended largely on CSE, whereas that in other organs, such as brain, lung, and spleen, seemed CSE-independent; the finding is consistent with previous observations in mouse brain tissues where CBS constitutes a major source for the gas generation (1).

CSE-derived H_2S is a determinant of the basal bile output and biliary HCO_3^- excretion

Figure 2 demonstrates protein expression of CSE in rat liver tissues. Western blot analyses indicated that the purified polyclonal antibody used in this study specifically recognized the enzyme at 40 kDa (Fig. 2A). Immunohistochemistry using the same antibody revealed that the most intense reactivities were seen in periductal regions of portal triads, whereas walls of hepatic arterial walls and terminal portal veins displayed little reactivities, if any. In addition, a modest expression of CSE was notable in hepatocytes, indicating intralobular homogeneity in its expression (Fig. 2B), whereas nonspecific IgG did not stain the slice (Fig. 2C). Figure 2D and E illustrates semiserial sections stained with the anti-CSE and anti-keratin 19 antibodies, respectively. As seen, cytokelatin-positive ductular structures connecting to bile canalicular networks near the portal triad exhibited notable CSE expression, whereas an artery adjacent to the portal vessel did not display evident immunoreactivities. The staining disappeared when the anti-CSE antibody was absorbed by adding the antigen peptide (data not shown).

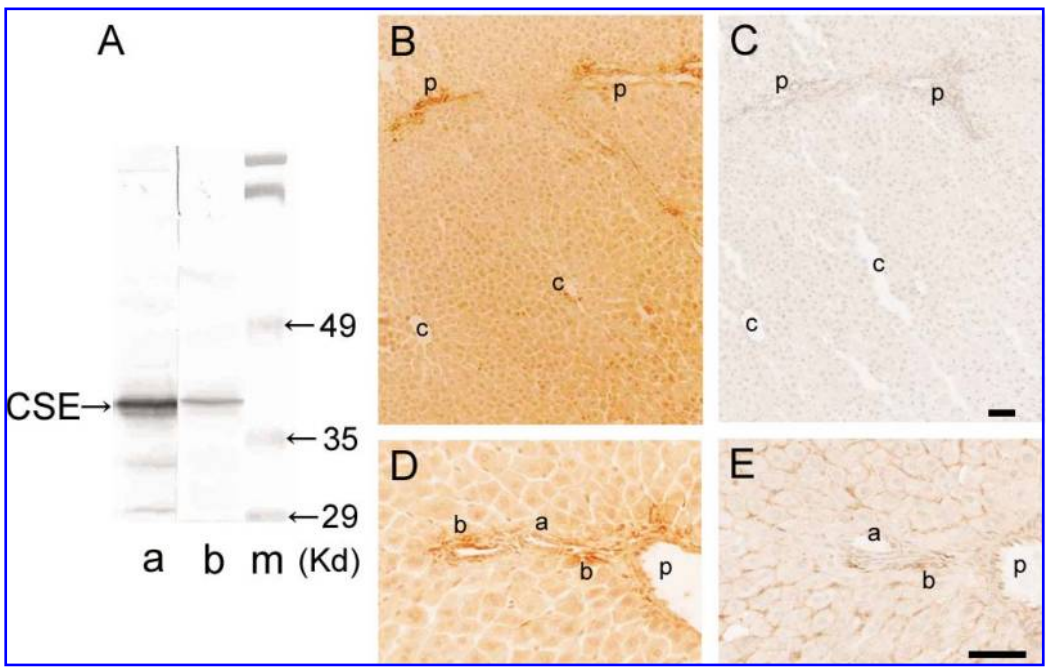

FIG. 2. Expression of CSE in the rat liver. (**A**) western blot analyses using the anti-rat CSE antiserum (lane a) and the affinity column-purified antibody (lane b). m: molecular markers. Note a single band in lane b. (**B** and **C**) Intralobular distribution of CSE in the rat liver stained with the purified anti-CSE antibody and with nonspecific chicken IgG, respectively. p and c: portal and central venules. (**D** and **E**) High magnification of a representative slice stained with the anti-CSE antibody and with the anti-keratin 19 antibody, respectively. a and b: artery and biliary duct. Bars = 50 μm.

TABLE 1. EFFECTS OF BLOCKADE OF CSE BY PPG ON BASAL BILE OUTPUT AND BILIARY HCO_3^- EXCRETION

Groups	*Basal bile output (μl/min/g of liver)*	*Biliary HCO_3^- concentration (mmol/L)*
Vehicle ($n = 6$)	1.73 ± 0.09	27.9 ± 1.2
PPG ($n = 6$)	2.11 ± 0.05*	33.0 ± 0.7*

*$p < 0.05$ as compared with the vehicle-treated control group.

We determined the effects of systemic administration of PPG on bile output and biliary constituents *in vivo* according to the identical protocol used in Fig. 1. As shown in Table 1, the PPG administration significantly stimulated basal bile output by 15%. The biliary concentration of HCO_3^- was also significantly elevated in the PPG-treated group. As PPG inhibits CSE and could not only reduce endogenous H_2S, but also modify cysteine metabolism, it is necessary to examine the direct effects of exogenous H_2S administration on hepatobiliary function. However, such experiments were difficult, because the administration of NaHS, the H_2S-donating reagent, is known to change systemic blood pressure *in vivo* through its vasorelaxing action (22). We thus used livers perfused *ex vivo* with the taurocholate-free Krebs solution to prove roles of CSE-derived H_2S in the basal bile excretion.

As illustrated in Fig. 3, the hepatic vascular resistance was comparable among four groups tested (*e.g.*, vehicle, PPG, PPG + NaHS, and PPG + NAC). Under these circumstances, the basal bile output was significantly elevated by 20% in perfused livers of the PPG-treated rats as compared with those treated with vehicle. This response was slightly greater than that observed in the experiments *in vivo* (Table 1), presumably because the perfusion of the organ was carried out under cholate-free conditions, as discussed later in Results. The choleretic response elicited by the PPG treatment was repressed by coperfusion of NaHS at 30 μmol/L, the concentration being comparable to the PPG-sensitive fraction of the gas generation. On the other hand, coperfusion of the same concentration of NAC, a reagent entering cells to yield cysteine, did not alter the CSE-elicited choleretic response. Like the aforementioned observations *in vivo* (Table 1), the PPG treatment significantly enhanced biliary HCO_3^- concentrations, and coperfusion of 30 μmol/L NaHS completely attenuated the changes in the perfused rat livers. On the other hand, the NAC coperfusion did not repress the PPG-induced elevation of the HCO_3^- concentration (Fig. 3B).

As HCO_3^- serves as a putative constituent yielding the driving force for bile formation, we determined if the bile acid-independent bile formation is elevated in livers of the PPG-treated groups. As seen in Fig. 4, where the output was plotted as a function of biliary fluxes of bile salts, the *y*-intercept of the line for the PPG-pretreated groups became markedly decreased and dissociated from that for the control groups. The difference between the two groups became smaller with increasing fluxes of bile salts, but the difference was still evident when the flux of bile salts reached the physiologic levels (70 nmol/min/g of liver). Such a dependency of the PPG effect on bile salts was consistent with the current data indicating differences in the choleretic responses between *in vivo*

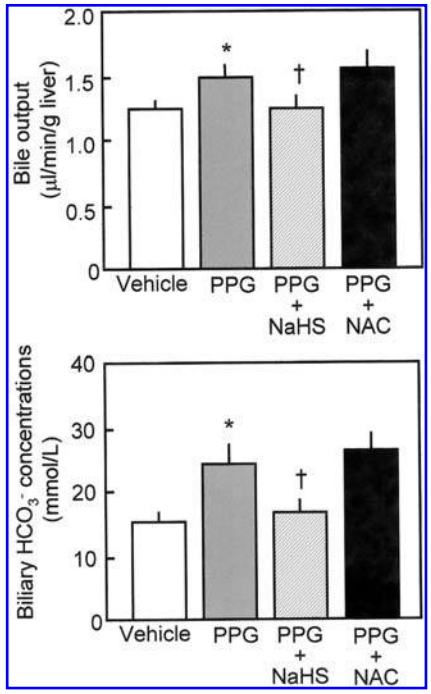

FIG. 3. Effects of the CSE blockade by PPG and supplementation of NaHS on the basal bile output and biliary HCO_3^- concentration in *ex vivo* perfused rat livers. PPG at 1.5 mmol/kg was administered *in vivo* intraperitoneally at 4 h prior to the isolation of the perfused liver. Either NaHS or NAC was perfused *ex vivo* into the liver at a concentration of 30 μmol/L, when necessary. Data indicate means ± SE of seven to nine separate experiments in each group. *$p < 0.05$ as compared with the vehicle-treated group; †$p < 0.05$ versus the PPG-treated group.

(Table 1) and *ex vivo* (Fig. 3) perfused livers. We further investigated whether biliary output of glutathione, another major constituent for bile acid-independent bile formation, could also be elevated under the blockade of CSE. As seen in Fig. 5, total amounts of glutathione excreted into bile was comparable irrespective of the PPG treatment, suggesting that this constituent plays little role in generation of the osmotic driving force, if any. Interestingly, the ratio between reduced and oxidized forms of glutathione (GSH/GSSG) was significantly elevated by the CSE blockade with PPG. Moreover, the PPG-induced elevation of GSH/GSSG in bile was further elevated with coperfusion with 30 μmol/L NaHS. As one might expect, the PPG pretreatment significantly caused a reduction of total glutathione presumably through inhibition of the transsulfuration pathway. The PPG-elicited decrease in hepatic glutathione contents was unchanged upon administration of NaHS, suggesting that the event is not mediated by endogenous H_2S. Among the three groups, >90% of glutathione was present as the reduced form (data not shown). These results suggest that suppression of CSE-derived H_2S accelerates biliary excretion of GSH, whereas its hepatic contents are reduced. Moreover, exogenous supplementation of the gas under the CSE blockade further increases its excretion into bile. Physiologic implications of this phenomenon will be mentioned later in the Discussion. Collectively, the present results suggest that H_2S endogenously generated by

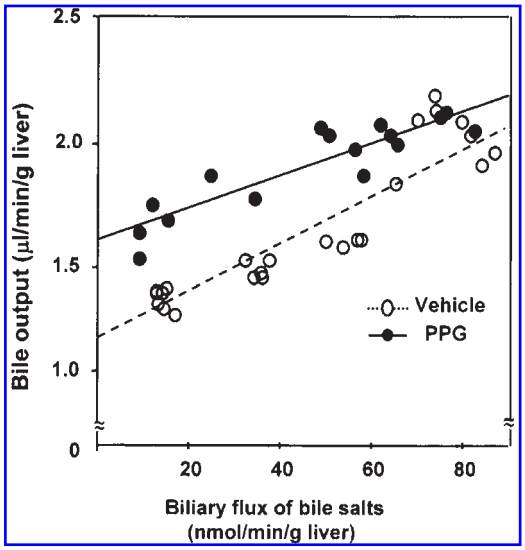

FIG. 4. Alterations in bile salt-independent fraction of bile output by the blockade of CSE by PPG. PPG at 1.5 mmol/kg was administered *in vivo* intraperitoneally at 4 h prior to the isolation of the perfused liver. Note the significant elevation ($p < 0.05$) of the *y*-intercept by the PPG treatment, and the difference in the basal bile output between the two groups becomes smaller with increased excretion of bile salts in bile.

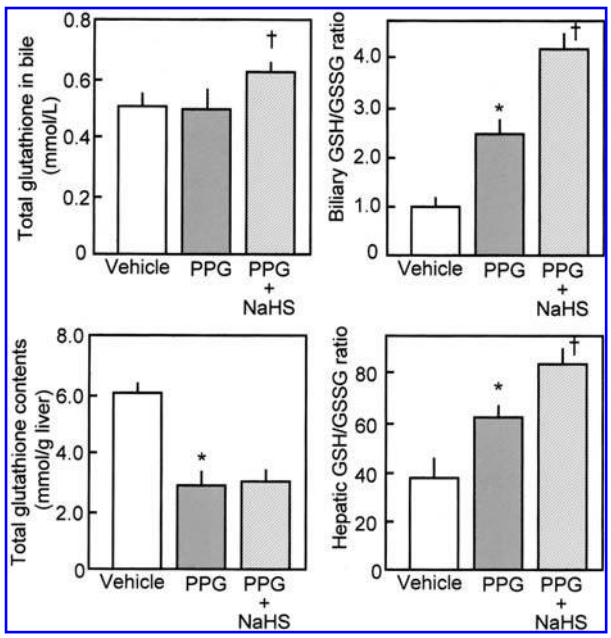

FIG. 5. Effects of the CSE blockade by PPG and supplementation of 30 μmol/L NaHS on biliary excretion and hepatic contents of glutathione in perfused rat livers. PPG at 1.5 mmol/kg was administered *in vivo* intraperitoneally at 4 h prior to the isolation of the perfused liver. Data indicate means ± SE of seven to nine separate experiments in each group. *$p <$ 0.05 as compared with the vehicle-treated group; †$p < 0.05$ versus the PPG-treated group.

CSE modulates the basal excretion of HCO_3^- in bile, playing a role in the regulation of the basal bile output through mechanisms dependent on bile acid-independent choleresis.

DISCUSSION

The present study first provided evidence for the presence of considerable amounts of H_2S in the liver. Furthermore, the gas appeared to serve as an endogenous modulator of the basal bile formation in the liver. Mechanisms for regulation of the basal bile formation involve the bile acid-independent process rather than bile acid-dependent one. Several lines of the current data support this concept: First, the effect of blockade of CSE, the enzyme producing ~50% of the basal H_2S generation, causes an increase in the bile acid-independent bile output *ex vivo* and *in vivo*. Second, as judged by data from *ex vivo* perfused livers, the difference in the excretion between PPG-treated and -untreated groups becomes increased as the biliary excretion of bile salts is reduced (Fig. 4), suggesting that the bile acid-independent fraction plays a major role. Thirdly and most importantly, between the two major biliary constituents for this fraction, HCO_3^-, but not glutathione, is elevated upon the CSE blockade and repressed by supplementation with H_2S, indicating that the former is attributed to generating the driving force for the bile formation. These results collectively suggest that stimulation of HCO_3^- plays an important role in the bile acid-independent choleresis elicited by suppression of CSE-derived H_2S generation.

As seen in alterations in hepatic contents of glutathione, PPG not only suppressed CSE-derived H_2S, but also reduced the glutathione contents. As the decrease in the hepatic glutathione contents was not restored by supplementation of NaHS, this event is not mediated by the gas, but occurs as a consequence of CSE-dependent transsulfuration processes. Of interest is that biliary excretion of total glutathione [reduced (GSH) and oxidized (GSSG) forms of glutathione] was unchanged despite the reduction in their hepatic contents. Furthermore, the relative amounts of GSH in bile were increased with supplementation of NaHS. Considering biochemical properties of the gas as a potent reductant with small molecular weight, this result raised a possibility that exogenously administered H_2S is utilized to increase reducing equivalents for GSH in bile. Several possibilities should be taken into account for mechanisms by which H_2S increases the ratio of GSH/GSSG in bile: First, the blockade of CSE by PPG could inhibit the conversion of cysteine into H_2S and thereby save this amino acid for the glutathione synthesis even when the supply of the substrate from the transsulfuration pathway is inhibited. Secondly, H_2S could be used directly as a reducing equivalent to increase GSH in bile. Thus, the role of CSE-mediated conversion of cysteine into H_2S for a fail-safe mechanism to maintain the reducing equivalent deserves further studies to provide evidence that the gas serves as a novel endogenous reductant.

Among gaseous substances detected in mammalian tissues, H_2S has recently been suggested to account for a novel neurovascular transmitter, although receptor mechanisms for the gas signal transduction remain largely unknown. The current results first suggest that the liver could have the ability to ex-

ecute remodeling of HCO_3^- excretion and increase the basal bile formation when exposed to disease conditions causing a decrease in the enzyme activity; such circumstances involve cirrhosis and surgical insults as previously reported both experimentally and clinically (8, 20). When considering effects of other gaseous mediators on the quality control of bile excretion, which were previously reported from our laboratory and other, it is not unreasonable to hypothesize that the liver could utilize multiple gases to regulate biliary function under physiologic and pathologic conditions. In the rat model of endotoxemia, nitric oxide (NO) suppresses oxidative phosphorylation via blockade of mitochondrial cytochrome *c* oxidase, and thereby down-regulates bile acid-dependent bile formation (17, 19). Although mechanisms for transcriptional regulation of the CSE expression remain largely unknown, previous studies revealed that exposure to excess NO caused up-regulation of the CSE expression in aortic tissues and increased endogenous generation of H_2S to modulate the vascular tone. As shown in the current study, the excess dose of exogenous NaHS supplementation reduced the basal bile output, suggesting that H_2S causes cholestasis with its excess amounts. In this context, quantitative determination of these two gases in the endotoxemic liver deserves further studies provided that the functional link of their overproduction to biliary function can be demonstrated.

On the other hand, the current results together with our previous data collectively suggest that a reduction of H_2S and an increase in carbon monoxide (CO) share common roles in the regulation of bile formation in that both events stimulate excretion of bile constituents besides bile salts. CO at micromolar levels not only modulates sinusoidal tone (18), but also has the ability to induce choleresis and to stimulate biliary excretion of major organic anions such as glutathione and bilirubin-IXα through mechanisms involving multidrug resistance protein 2 (13). Such effects of CO on biliary excretion occur in a concentration-specific manner, and excess concentrations of the gas repress the choleretic response and lead to cholestasis through the increase in paracellular junctional permeability and suppression of bile canalicular contractility (11, 16). In contrast to CSE, heme oxygenase-1 is up-regulated by surgical insults or by liver cirrhosis, and the parenchyma is exposed to high concentrations of CO (9, 12, 21). Thus, under disease conditions, overproduced CO and reduced H_2S could cooperatively increase the bile acid-independent fraction of bile output through increased excretion of organic anions and HCO_3^-, respectively. Although physiologic implication of the current observations remains to be fully understood, such remodeling of a quality of bile could benefit the increasing solubility of organic anions or protect against cholestasis possibly occurring under the aforementioned disease conditions. Further investigation is necessary to examine if alterations of these gases could regulate a quality of bile cooperatively with modulation of H_2S generation under a variety of hepatobiliary disease conditions.

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ABBREVIATIONS

CBS, cystathionine β-synthase; CO, carbon monoxide; CSE, cystathionine γ-lyase; GSH, reduced form of glutathione; GSSG, oxidized form of glutathione; HCO_3^-, bicarbonate; H_2S, hydrogen sulfide; NAC, *N*-acetylcysteine; NO, nitric oxide; PPG, propargylglycine.

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