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Carbon Monoxide Stimulates mrp2-Dependent Excretion of Bilirubin-IXα into Bile in the Perfused Rat Liver

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ABSTRACT

Although carbon monoxide (CO) has been reported to protect against hepatobiliary dysfunction, mechanisms for its actions remain unknown. This study aimed to examine actions of physiologically relevant concentrations of CO on biliary excretion. The effects of transportal administration of CO on bile output and constituents were examined in perfused rat livers. In livers of fed rats, CO regulated bile output biphasically in a dose-dependent manner; transportal administration of CO at 4 μmol/L stimulated bile output by 10%. Under these circumstances, CO increased paracellular junctional permeability and consequently decreased biliary excretion of bile salts. Choleresis elicited by 4 μmol/L CO coincided with significant increases in biliary excretion of bilirubin-IXα and glutathione. The CO-induced choleresis occurred independently of cyclic GMP, coincided with elevated excretion of K^+ and HCO_3^-, and was abolished by tetraethylammonium, suggesting stimulatory effects of the gas on potassium channels. CO-mediated choleresis and increased excretion of organic anions appeared to be mediated by mrp2, because Eisai hyperbilirubinemia rats, which genetically lack the transporter, did not exhibit choleresis upon the CO administration. These results suggest that CO stimulates mrp2-dependent excretion of bilirubin-IXα through mechanisms involving potassium channels, serving as a cooperator standing behind the heme oxygenase reaction to facilitate hepatic heme detoxification. *Antioxid. Redox Signal.* 5, 449–456.

INTRODUCTION

LIVER is a major organ responsible for detoxification of biologically active mediators and xenobiotics and for biliary excretion of their metabolites. These two processes occur through distinct metabolic processes. The former involves enzymatic modification of the noxious substrates by oxygenases and conjugation that can increase hydrophilicity of the compounds, whereas the latter involves excretion of their reaction products by specific transporters expressed at the hepatocellular membrane (10). A variety of endogenous organic compounds, such as bilirubin and leukotrienes, undergo such detoxification processes. Bilirubin is produced as an end product of the heme detoxification via the reaction of heme oxygenase (HO) and biliverdin reductase and is conjugated by glucuronyl transferase (14, 22). This bile pigment possesses a potent antioxidant action that ameliorates acute inflammatory processes (5). On the other hand, leukotrienes are produced by 5-lipoxygenase-mediated oxygenation of arachidonic acid and undergo glutathione conjugation prior to their excretion into bile (10). The reduced form of glutathione (GSH), a potent antioxidant that detoxifies organic hydroperoxides, and its oxidized form (GSSG) are also excreted into bile. These organic compounds share the same molecular mechanism for their recognition and excretion into bile, that is, a conjugate-transporting ATPase called mul-

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tidrug resistance protein 2 (mrp2) (2, 3, 10). This transport ATPase is expressed in the hepatocellular membrane and facilitates the excretion of the organic conjugates into bile. The protein is not expressed in human Dubin–Johnson syndrome, which is therefore associated with an inherited hyperbilirubinemia caused by deficiency in the bilirubin excretion (8, 13). mrp2 serves as a determinant for biliary concentrations of the organic constituents and thus contributes to the generation of the osmotic driving force for bile formation, as well as other transporters such as the bile salt export pump, which represents the rate-controlling step for taurocholate excretion (20). However, mechanisms for regulation of the transporter function and their link to detoxification of noxious stressors and/or xenobiotics have not been fully investigated yet.

This study attempted to examine if such a cooperative link between the oxygenase-dependent enzymes and the transporter systems could function to regulate bile formation. Among by-products of the detoxification-associated oxygenase reaction, carbon monoxide (CO) has recently attracted great interest as an important protectant against hepatobiliary dysfunction (4, 25). We have shown that livers use CO derived from the HO reaction to relax sinusoids and thereby to guarantee ample blood supply under varied disease conditions, as well as physiologic circumstances (4, 16, 22, 25). The gas could also have the ability to alter bile formation through multiple mechanisms. Elimination of endogenous CO induces sinusoidal contraction through mechanisms involving soluble guanylate cyclase in hepatic stellate cells and induces bile acid-dependent choleresis (4, 9, 19). On the other hand, we have herein investigated effects of physiologically relevant concentrations of CO on bile formation and biliary constituents in perfused rat livers. To reveal the functional link between the gas and the ATP-dependent transporter, biliary responses upon CO exposure were compared between livers from normal rats and those from mrp2-lacking mutant rats. Results of the current study provided evidence that exogenously administered CO at inducible concentrations stimulates mrp2-dependent excretion of bilirubin-IXα and glutathione into bile, suggesting an important role of the HO-derived gas in regulation of the metabolism of the organic compounds.

MATERIALS AND METHODS

Animal preparation

Male Wistar and Sprague–Dawley rats and Eisai hyperbilirubinemia rats (EHBR) (280–300 g) were obtained from Nippon Biosupply Center Inc. (Tokyo, Japan) (2). All animals were allowed free access to laboratory chow and tap water, and were fasted 24 h when necessary. Protocols for the current experiments were approved according to the Institutional Guidelines for Animal Care and Experiments in Keio University School of Medicine. Rats were anesthetized intraperitoneally with pentobarbital sodium at 50 mg/kg. The livers underwent cannulation of the common bile duct with a PE-10 tube and were perfused *ex vivo* with hemoglobin-free and albumin-free Krebs–Ringer solution (pH 7.4, 37°C) gassed with carbogen according to our previous methods (19, 28). The perfusate contained 30 μmol/L sodium taurocholate and was pumped from the portal vein through the liver at a constant flow rate of 4.0 ml/min/g of liver weight under monitoring of the whole organ vascular resistance (25, 28). Bile was collected every 5 min throughout the experiments.

Bile constituents such as bile salts, phospholipids, glutathione, and potassium (K^+) and bicarbonate (HCO_3^-) ions were determined as described elsewhere (25, 28). We also determined biliary flux of bilirubin-IXα, the end product of HO-mediated heme degradation using enzyme-linked immunosorbent assay as described previously (28, 29). The primary antibody for this assay was monoclonal antibody 24G7, which recognizes both conjugated and unconjugated forms of bilirubin-IXα (4, 28).

Experimental protocols

After the 20-min perfusion for the stabilization, the Krebs–Ringer buffer containing the desired concentrations of CO (2.0–20 μmol/L) was perfused for 40 min transportally as described previously (11, 19). The concentrations of CO were measured in advance in samples collected from the portal inlet using myoglobin-assisted spectrophotometry according to our previous methods (4, 11). When necessary, the desired concentrations of tetraethylammonium (TEA), a potassium channel blocker, were perfused together with CO. In these experiments, the TEA perfusion was started simultaneously with the CO perfusion. After the 40-min observation period of the perfusion, the isolated perfused livers were snap-frozen by liquid nitrogen to determine tissue contents of cyclic GMP (cGMP) and glutathione according to previous methods (11, 21, 28).

Measurements of horseradish peroxidase (HRP) excretion into bile

The effects of CO on biliary excretion of HRP were examined to determine alterations in the paracellular and transcellular fractions of bile transport in the identical single-pass perfused liver described in the previous section. At 20 min after the initial perfusion with a desired concentration of CO, a bolus of 25 mg of HRP was injected over 1 min. Bile samples were collected every 2 min for 10 min, and then every 5 min until the end of experiments. The concentrations of HRP in the collected bile samples were determined spectrophotometrically by measuring the rate of oxidation of 4-aminoantipyrine at 510 nm using a 96-well microplate reader as described previously (14, 19).

Analyses of mrp2-dependent excretion rates of 5-carboxyfluorescein (CF) into bile

To examine the effects of CO on the function of mrp2 to excrete exogenously loaded organic anions, CF diacetate at 2 μmol/L was added to the perfusate containing 1.5 mmol/L probenecid, a potent blocker of the transporter (1, 13). This reagent can enter hepatocytes (23, 24) and is hydrolyzed by esterase into CF to be excreted into bile. After the 10-min loading of CF diacetate, the liver was perfused with the probenecid-free buffer to trigger the excretion of CF into bile. The bile was sampled through the PE-10 tube cannulated into

the common bile duct. The bile samples were deep-frozen until the fluorescence measurements were carried out using a 96-well multichannel fluorescence spectrophotometer. The measurements were performed under epi-illumination at 430 nm, the isosbestic wavelength of the dye that yields fluorescence at 510 nm without interference with pH values of the samples. The concentrations of CF in samples were calibrated with known concentrations of CF dissolved in phosphate-buffered saline. As seen later in Results, the decay of the dye concentrations appeared to fit a single exponential by the use of the conventional least-squares method; thus, biliary CF lifetimes were determined as the time constant (τ) of the exponential curve. This method also allowed us to determine the decay of the dye exclusion independently of the initial amounts of CF loaded into the perfused liver. When necessary, the buffer containing CO at varied concentrations was perfused from 20 min prior to the CF diacetate loading to the end of experiments. The values of τ were compared between the livers treated with or without CO.

RESULTS

CO increases the baseline bile output without altering the vascular resistance

Figure 1 illustrates temporal alterations in the baseline bile output of the isolated perfused livers of fed and fasted rats. As seen in Fig. 1A, in response to the perfusion with 4 μmol/L CO, livers of the fed rats exhibited a significant elevation of the basal bile output without showing any notable changes in the vascular resistance. Figure 1B illustrates net increases in the bile flux measured at 30 min (%ΔBile flux) as a function of the concentrations of CO in the perfusate. The peak of the choleretic response was attenuated by further increases in the CO concentrations, disappearing at >10 μmol/L almost completely. On the other hand, the choleretic response elicited by 4 μmol/L CO was not evident when rats were fasted overnight, consistent with our previous results obtained from livers of fasted rats (14). As choleretic responses by CO in livers of fed rats suggest an increase in bile constituents responsible for the osmotic driving force, concentrations and fluxes of bile salts and phospholipids were examined. As seen in Fig. 1C, concentrations of these constituents were modestly decreased, whereas fluxes of them were hardly changed as a result of choleresis upon the CO perfusion. These results indicated that the CO-elicited choleresis is not accompanied by elevated excretion of bile salts and phospholipids, suggesting the involvement of another compound to yield the osmotic driving force in this event.

CO stimulates biliary excretion of bilirubin-IXα and glutathione

In an attempt to determine biliary constituents responsible for CO-elicited choleresis, we determined concentrations and fluxes of bilirubin-IXα and glutathione, two major organic anions producing the osmotic driving force for bile formation. As seen in Fig. 2A, the biliary concentration and the flux of bilirubin-IXα increased by 1.5- and 2.0-fold, respectively, upon the administration of 4 μmol/L CO. Such an increase in the bilirubin excretion was reduced when the greater concentrations of CO were perfused, exhibiting a profile of the dose responses similar to that of bile output. Biliary excretion of glutathione to CO also exhibited a dose-response curve similar to that of bilirubin-IXα. These results suggested that these organic anions undergo significant condensation in the hepatobiliary compartment and serve as major bile constituents providing osmotic driving force for CO-dependent choleresis.

We compared responses in the livers of Wistar rats and EHBR, which genetically lack mrp2, the major organic anion transporter responsible for excretion of these compounds. As seen in Fig. 3A, livers of EHBR displayed a significant reduction of the basal bile output, being ~60% of the values measured in those of male Wistar rats. Concurrently with such a marked reduction of the basal bile output, the basal excretion of bilirubin-IXα and glutathione in EHBR livers was

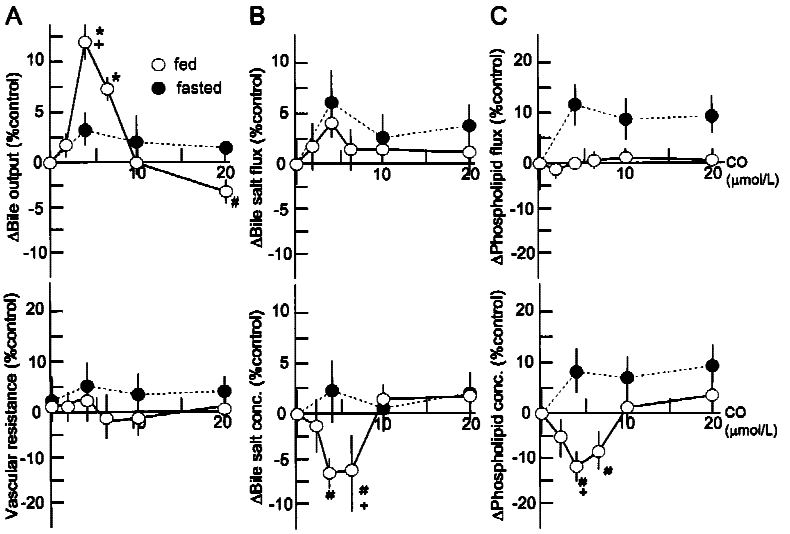

FIG. 1. Concentration-dependent effects of CO on bile output, vascular resistance, and biliary fluxes and concentrations of bile salts and phospholipids in perfused rat livers isolated from fed and fasted rats. Data were collected at 30 min after the start of the CO perfusion and indicate the means ± SE of more than six separate experiments. *$p < 0.05$, significantly increased versus the fed livers perfused without CO; $^{+}p < 0.05$, as compared with the values in fasted livers. #$p < 0.05$, significantly decreased versus the fed livers perfused without CO.

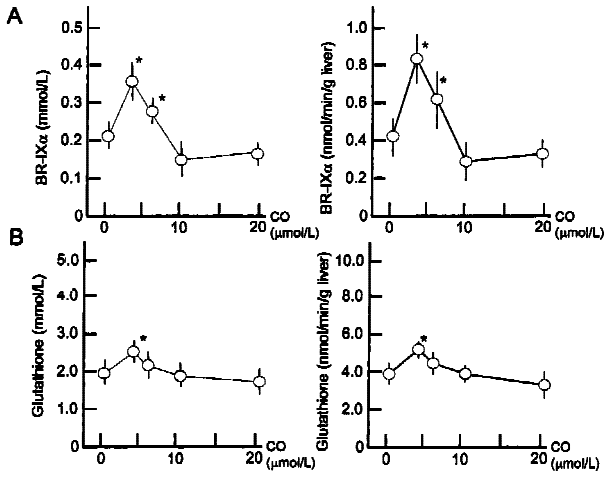

FIG. 2. Alterations in biliary concentrations and fluxes of bilirubin (BR)-IXα and glutathione by different concentrations of CO in perfused rat livers. Data were collected at 30 min after the start of the CO perfusion and indicate the means ± SE of more than six separate experiments. *$p < 0.05$, significantly increased versus the fed livers perfused without CO.

diminished almost completely. When 4 μmol/L CO was perfused, the EHBR livers did not exhibit any notable increase in excretion of these organic anions, being distinct from the responses elicited by the same concentration of CO in male Wistar rats. We also confirmed that the CO-induced choleresis was reproducible in the livers of male Sprague–Dawley rats (data not shown). These results suggest that the choleretic action of CO is operated specifically through mrp2-dependent mechanisms.

CO does not alter excretion of exogenous CF in perfused livers

The observation that CO stimulates mrp2-mediated excretion of organic anions in perfused livers led us to examine if mechanisms for this event involve the direct action of the gas on the function of the transporter. To address this possibility, rat livers were loaded with CF diacetate in the presence of 1.5 m*M* probenecid, an mrp2 inhibitor, and the biliary CF excretion after removal of the inhibitor was traced as a function of time for perfusion. As seen in Fig. 4, concentrations and fluxes of CF in bile samples were low at basal levels when the livers were perfused with the buffer containing probenecid. Upon its removal, the concentrations and fluxes of CF peaked at 10 min, and decreased with time, following a single exponential decay. Such a profile of the biliary CF excretion was unchanged when the livers were perfused with 4 μmol/L CO. The values of τ of the exponential decay of biliary CF concentrations were almost identical between the two groups, where those in the CO-untreated and -treated groups were 7.3 ± 0.4 min and 7.4 ± 0.4 min, respectively. These results suggest that CO does not directly stimulate function of mrp2 at least under the current experimental conditions.

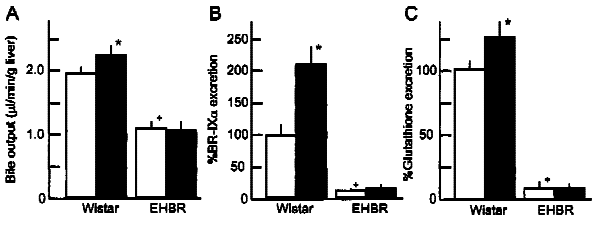

FIG. 3. Disappearance of CO-elicited increases in biliary excretion of bilirubin (BR)-IXα and glutathione and bile output in perfused livers isolated from EHBR. Data were collected at 30 min after the start of the CO perfusion at 4 μmol/L and indicate the means ± SE of more than six separate experiments. Open and filled columns indicate fed and fasted rats, respectively.

Blockade of potassium channels represses CO-elicited choleresis

To further determine mechanisms by which CO stimulates excretion of bilirubin and glutathione, we examined the effects of TEA, a blocker for a wide spectrum of potassium channels, on bile output and constituents such as K^+ and HCO_3^-, because the gas has been known to increase the opening probability of the channels to hyperpolarize cells (17). As seen in Fig. 5, perfusion of CO at 4 μmol/L significantly increased the fluxes of HCO_3^-. These changes were attenuated by coperfusion of TEA in a dose-dependent manner. In these experiments, the whole organ vascular resistance among the groups did not differ, suggesting that the biliary responses modified by TEA do not result from its action on

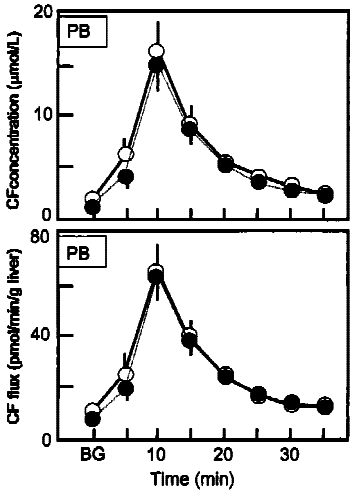

FIG. 4. Temporal alterations in biliary excretion of CF in the presence or absence of 4 μmol/L CO in the perfused rat livers. PB, 1.5 mmol/L probenecid, an inhibitor of mrp2. Note that time courses of the dye exclusion in the control and CO-treated groups are almost identical to each other and those after reaching the peaks follow a single exponential. The time constants (τ) were not statistically different between the two groups. Values are means ± SE of more than six separate experiments. Open and filled circles indicate the control and CO-treated groups, respectively.

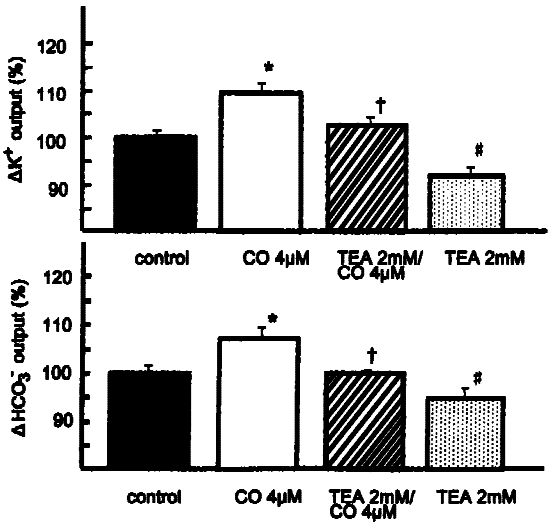

FIG. 5. Effects of TEA on the CO-elicited increases in fluxes of potassium and bicarbonate ions in perfused rat livers. Data were collected from livers perfused with or without 4 μmol/L CO for 30 min, and indicate the means ± SE of more than six experiments. *$p < 0.05$, significantly increased versus the fed control livers; †$p < 0.05$, as compared with the values in fed livers perfused with 4 μmol/L CO; #$p < 0.05$, significantly decreased versus the fed control livers.

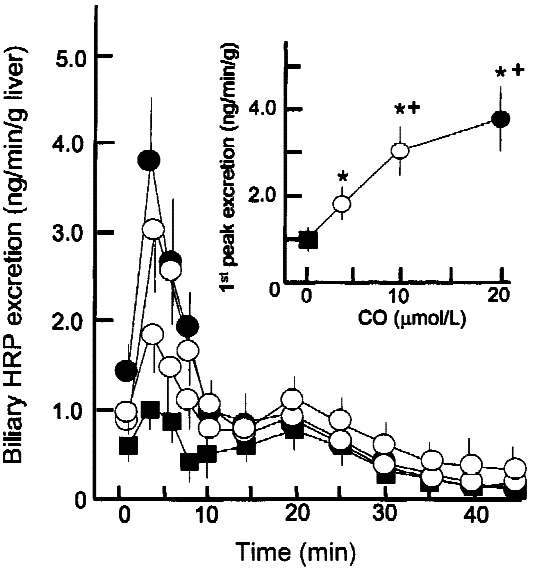

FIG. 6. Differences in time course of biliary HRP excretion in the perfused livers of fed rats. At 20 min after the initial perfusion with desired concentrations of CO, a bolus of HRP (25 mg) was injected. The initial peak at 4 min after injection of HRP reflects paracellular transport of portally injected HRP. Values are means ± SE of more than five experiments. **Inset:** Relationship between CO concentrations and heights of the first peak at 4 min after the injection of HRP. Note that the height of the initial peak increases with elevated concentrations of CO in the perfusate, and reaches the maximum level with 20 mmol/L. Filled square and open, shaded, and filled circles denote CO concentrations of 0, 4, 10, and 20 μmol/L, respectively. *$p < 0.05$, significantly increased versus the CO-untreated group; +$p < 0.05$, significantly increased versus the 4 μmol/L CO-treated group.

the vascular system, but from that on nonvascular components such as hepatocytes and/or the biliary system. Collectively, the stimulatory action of CO on potassium channels appeared to be involved in mechanisms for choleretic responses.

The choleretic response induced by 4 μmol/L CO did not coincide with an increase in tissue contents of cGMP. On the other hand, the perfusion with CO at concentrations of >10 μmol/L induced modest, but significant, elevation of the cGMP contents in the liver (data not shown). As mentioned in the previous section, such concentrations of CO did not induce choleresis and rather elicited a repression of the bile output comparable to the level measured in the absence of the gas. These results suggest that the CO-induced choleretic response is an event occurring independently of its effect to activate soluble guanylate cyclase.

Excess CO increases paracellular junctional permeability and cancels choleresis

The observation that CO at >4 μmol/L blunts the choleretic responses led us to examine if such concentrations of the gas could reduce concentrations of biliary constituents and result in a decrease in the osmotic driving force for bile formation. Figure 6 illustrates differences in the time course of biliary HRP excretion in the perfused livers of fed rats. Previous studies demonstrated that the initial peak at 4 min and the second one at 20 min after completion of the transportal HRP injection reflect paracellular and transcellular transport of portally injected HRP (14, 19). As seen, the height of the initial peak increased with elevated concentrations of CO in the perfusate, and reached the maximum level with 20 μmol/L by fourfold versus the control values. These results suggest that the paracellular permeability increased in response to the gas administration even in fed rats, consistent with our previous data collected from fasted rats.

DISCUSSION

The current study demonstrated that CO at micromolar concentrations has the ability to induce choleresis and to stimulate biliary excretion of glutathione and bilirubin-IXα in perfused livers of fed rats. Such an action of CO appears to result from cGMP-independent mechanisms and to depend on TEA-sensitive potassium channels. Quantitative analyses of bile constituents revealed that glutathione and/or bilirubin-IXα appear to be major components generating osmotic driving force for bile formation. A range of CO concentrations eliciting choleresis is 4–6 μmol/L in the perfusate; such concentrations could occur under disease conditions such as endotoxemia as a consequence of stress-induced HO-1 induction and subsequent degradation of heme in the liver (11). As the biliary excretion of the aforementioned organic anions is an important process of xeno-

biotic detoxification under varied stress conditions, such an ability of stress-inducible levels of CO could serve as an important fail-safe mechanism to guarantee bile formation for excretion of detoxified metabolites.

The effect of gaseous mediators on biliary excretion was previously reported by Trauner *et al.* (26), where a stimulatory effect of nitric oxide (NO) on biliary excretion of glutathione was suggested in a similar model using isolated rat livers perfused with the taurocholate-free oxygenated buffer. In this report, the liver perfused with high concentrations of NO donors exhibited a dose-dependent choleresis in parallel with an elevation of glutathione. The mechanisms of this event are unlikely to involve cGMP-mediated processes because administration of a membrane-permeable cGMP analogue did not increase biliary excretion of glutathione. Although such effects of NO appear to be similar to those of CO, the current finding provides several unique features of this CO-mediated event. First, a novel role of CO in regulating biliary excretion of bilirubin is revealed for the first time. As we reported previously, physiologic concentrations of bile salts (*e.g.,* 30 μmol/L taurocholate) are necessary to guarantee directional excretion of bilirubin-IXα from hepatocytes into bile canaliculi (29). As livers were not perfused with bile salts in the previous study, the excretion of bilirubin was not able to be assessed. Second, the current results demonstrated that CO-mediated choleresis is notable only in livers of fed rats, but not in those of fasted ones, where the osmotic driving force for bile formation by glutathione is not sufficient because of its reduced contents in the liver. Although it has been known that hepatic glutathione concentration of fed rats is higher than that of fasted ones (6, 18, 26, 27), feeding effects on CO-mediated choleresis have not been assessed in these studies. Third, effects of CO on biliary excretion of organic anions occur in a concentration-specific manner, and excess concentrations of the gas cause a repression of the responses. The mechanisms for repression of bile output by excess CO appear to involve reduced concentrations of the organic anions in bile due to the increase in paracellular junctional permeability, suggested in Fig. 5 of the current study, as well as in our previous report (14).

These lines of evidence led us to further examine mechanisms by which CO regulates excretion of bilirubin and glutathione into bile. As these two organic anions were present at millimolar levels in bile and constitute a major osmotic driving force for bile formation, we examined if the function of mrp2, the anion transporter for these compounds, could be regulated by CO. The current study confirmed the requirement of mrp2 in the CO-mediated choleresis using EHBR. However, results collected from analyses of biliary exclusion of exogenously applied CF, a fluorescent organic anion excreted by mrp2, did not support a notion that CO directly stimulates function of the organic anion transporter. On the other hand, the data showing repressive effects of TEA suggest that potassium channels could be a potentially important target of CO as also documented in previous observations (7). Thus, the functional link between the ion channels and mrp2 should be examined further to reveal the entire mechanisms for the CO-mediated choleresis and enhanced excretion of the organic anions.

Collectively with previous reports (11, 12, 16), the current results suggest the whole picture of HO-mediated protective mechanisms against noxious stimuli to the liver that is schematized in Fig. 7. Using the rat experimental model of endotoxemia, we have shown that the stimulated liver increases degradation of heme through at least two mechanisms: an increase in the substrate and an induction of HO-1. When such livers are exposed to methemoglobin, the heme derived from senescent erythrocytes or from hemolysis in circulation, the heme serves as a substrate for both HO-1 and HO-2 in hepatocytes and facilitates endogenous CO generation (11). Furthermore, cell damages could cause denaturation of heme proteins and provide free heme to be degraded by the HO (15). As a result, the livers are exposed to sufficient amounts of CO that could stimulate sinusoidal flow and bile formation. Under these circumstances, our results showed that overproduced CO keeps sinusoids in relaxing states through mechanisms involving its inhibitory actions on cytochrome P450 monooxygenases (11). In addition to such HO-mediated protective mechanisms, the current study raised the possibility that stress-inducible levels of CO could directly stimulate bile formation by facilitating excretion of glutathione and bilirubin through mechanisms independent of its cGMP-independent vasorelaxing actions. In this context, the current study shed light on a novel action of CO on biliary excretion of the heme-degrading pigment, serving as a cooperator standing behind the HO reaction to facilitate hepatic heme detoxification. At present, it is unknown whether the direct choleretic action of CO operated by cGMP-independent pathways could involve alterations in cytochrome P450-derived eicosanoids in hepatocytes. However, such a possibility could be supported by our previous studies suggesting that micromolar levels of CO contribute to maintain stroke volume of bile canalicular contraction in cultured hepatocyte couplets, where the gas appears to inhibit the monooxygenase activities and thereby modulates calcium-dependent contrac-

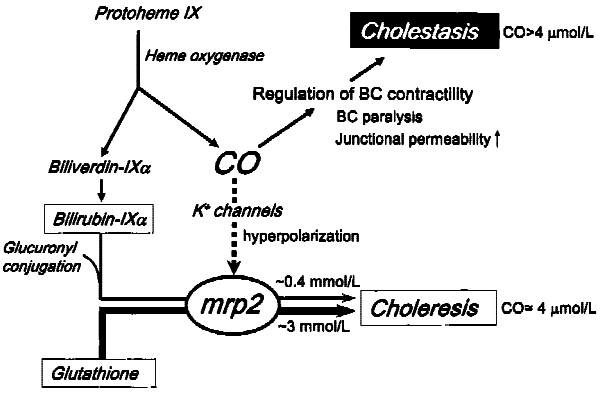

FIG. 7. Possible mechanisms involving CO-mediated choleresis. Stress-inducible CO (~4 μmol/L) could stimulate bile formation by facilitating mrp2-dependent excretion of glutathione, a major component for yielding osmotic driving force for bile formation, and bilirubin-IXα, the end product of heme degradation. CO increases conductance of potassium channels and hyperpolarizes membrane that could help efflux of organic anions through mrp2. Greater concentrations of CO could reduce this choleretic response through multiple mechanisms involving impaired bile canalicular (BC) contraction and increased paracellular junctional permeability.

tion processes (21). Further investigation is necessary to examine whether such a mechanism is involved in the regulation of mrp2 function in hepatocytes.

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ABBREVIATIONS

CF, 5-carboxyfluorescein; cGMP, cyclic GMP; CO, carbon monoxide; EHBR, Eisai hyperbilirubinemia rat(s); HO, heme oxygenase; HRP, horseradish peroxidase; mrp2, multidrug resistance protein 2; NO, nitric oxide; TEA, tetraethylammonium.

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