Endogenous carbon monoxide suppression stimulates bile acid-dependent biliary transport in perfused rat liver

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Sano, Tsuyoshi, Masaya Shiomi, Yoshiyuki Wakabayashi, Yuichi Shinoda, Nobuhito Goda, Tokio Yamaguchi, Yuji Nimura, Yuzuru Ishimura, and Makoto Suematsu. Endogenous carbon monoxide suppression stimulates bile acid-dependent biliary transport in perfused rat liver. *Am. J. Physiol.* 272 (*Gastrointest. Liver Physiol.* 35): G1268–G1275, 1997.—This study aimed to investigate whether carbon monoxide (CO), a product of heme oxygenase that degrades protoheme IX, serves as an endogenous modulator for biliary transport. To that end, effects of zinc protoporphyrin IX (ZnPP), a heme oxygenase inhibitor, on the biliary transport were tested in perfused rat liver. Perfusion of 1 μM ZnPP abolished detectable levels of CO in the venous perfusate and increased bile acid-dependent bile output accompanying an increased secretion of bile salts. The ZnPP-induced choleresis coincided with a reduction of tissue guanosine 3′,5′-cyclic monophosphate (cGMP) levels and a decrease in vascular conductance. On administration of 2.5 μM CO, ZnPP-elicited choleresis, decreases in vascular conductance, and cGMP levels were all attenuated. Treatment with 1 μM 8-bromoguanosine 3′,5′-cyclic monophosphate (8-BrcGMP) partly attenuated the ZnPP-induced choleresis in concert with repression of vascular conductance. Furthermore, treatment of the liver with methylene blue, a guanylate cyclase inhibitor, evoked a choleresis similar to that induced by ZnPP. Thus endogenous CO suppression stimulates the biliary transport in part through a cGMP-dependent mechanism.

nitric oxide; guanosine 3′,5′-cyclic monophosphate; heme oxygenase; bile transport; adenosine 5′-triphosphate

CARBON MONOXIDE (CO) has recently attracted the interest of investigators as a novel regulator of cell functions in various tissues and organs, including neuronal cells, vascular smooth muscle cells, platelets, corneal epithelium, and liver (3, 14, 17, 19, 26). It is produced in these cells and tissues by the action of heme oxygenase, which decomposes protoheme IX into CO, free iron, and biliverdin. In the liver, a large portion of the constitutive activity of heme oxygenase is derived from parenchymal cells (2). We recently showed that CO generated by heme oxygenase modulates hepatic sinusoidal tone; zinc protoporphyrin IX (ZnPP), a potent inhibitor of heme oxygenase, eliminated endogenous CO flux and evoked an increase in the vascular resistance concurrent with sinusoidal constriction in the perfused rat liver (19, 20). These results suggest that CO generated in the liver parenchyma also acts as a regulator of bile secretion, either directly or indirectly as a consequence of alterations in the vascular tone. The present study thus aimed to examine the possibility that the heme oxygenase-CO pathway in hepatocytes serves to regulate biliary excretion and its transport. The results disclose that suppression of endogenous CO by ZnPP stimulates bile transport concomitant with an increase in excretion of bile salts and phospholipids.

MATERIALS AND METHODS

Animal preparation. Male Wistar rats (300–320 g) allowed free access to laboratory chow and tap water were fasted 24 h before experiments. After the animals were anesthetized with pentobarbital sodium (40 mg/kg ip), the distal portion of the common bile duct was cannulated with a polyethylene catheter (PE-10) for biliary drainage and the liver was perfused with Krebs-Ringer solution (pH 7.4, 37°C) gassed with carbogen (95% O_2-5% CO_2) as described previously (22, 23). The perfusate was pumped through the liver with a peristaltic pump at a constant flow rate (4.0 ml·min^{-1}·g liver weight^{-1}) in a single-pass mode under monitoring the inlet perfusion pressure (20). Oxygen concentrations in the inlet and outlet of the perfusion circuit were monitored by an oxygen electrode (PO_2-100, Inter-Medical, Tokyo, Japan). The time history of the oxygen tension and the perfusion pressure was introduced continuously into a laboratory computer (Biopac MP100/Quadra 840, Physiotec, Tokyo, Japan). The vascular resistance was estimated as the ratio between the pressure gradient and flow rate, and the vascular conductance was calculated as the reversed value for the resistance as an index of the status of microvascular perfusion (5, 19).

Experimental groups studied. To maintain physiological levels of bile output, we perfused all livers with Krebs solution containing sodium taurocholate, a major conjugated bile acid in rats, at 30 μM, which is a concentration comparable with that determined in the portal circulation (16), unless stated otherwise. We recently showed that this bile acid facilitates directional transport of bilirubin into the biliary compartment (28). Six main protocols were employed in the perfusion experiments. Livers in the first group were perfused with the buffer containing taurocholate for 90 min. In the second group, varied concentrations of ZnPP (0.1–5 μM) were added to the perfusion buffer at 20 min after the addition of taurocholate. The other four groups were prepared to evaluate the actions of exogenous supplement with the following interventions: 2.5 μM CO, 10 μM sodium nitroprusside (SNP; Maruishi Pharmaceutical, Osaka, Japan), a lipophilic guanosine 3′,5′-cyclic monophosphate (cGMP) analog 8-bromoguanosine 3′,5′-cyclic monophosphate (8-BrcGMP; 1 μM; Sigma Chemical), or unconjugated bilirubin (500 nM, Sigma Chemical). As described in RESULTS, these experiments were performed to examine the effects of these reagents on alterations in bile excretion elicited by ZnPP at 1 μM, the lowest concentration that induced a maximum choleretic response. 8-BrcGMP was added to the perfusate 5 min before the start of ZnPP administration. In the CO-treated livers, 20 ml of the CO-saturated Krebs-Ringer solution were prepared

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in a gas-tight syringe and were infused continuously into the main perfusion circuit at a rate of 15 ml/h. Because the perfusion rate in the main circuit ranged ~35 ml/min, the original CO solution was diluted to 0.8%. Under these conditions, the CO concentration in the buffer sampled in the portal inlet was 2.48 ± 0.20 μM (n = 5), which was determined by the method described elsewhere (20). In separate sets of experiments, effects of different concentrations of CO on bile transport were examined. In addition, N^G-nitro-L-arginine methyl ester (L-NAME; 1 mM; Sigma Chemical), a nitric oxide synthase inhibitor, or methylene blue (250 nM; Sigma Chemical) was administered to test the regulatory role of endogenously generated NO and cGMP in bile formation, respectively.

Measurements of bile output, bile acids, phospholipids, and bilirubin in bile. Bile samples were collected at 5-min intervals from the start of control perfusion to the end of experiments. Bile flow rate was expressed in microliters per minute per gram liver, as described previously (25, 28). Concentrations of total bile acids and phospholipids in the bile samples were determined by enzyme assay kits (Enzabile-2, Dai-ichi Chemicals, Tokyo, Japan; Phospholipid C-Test, Wako, Tokyo, Japan) (25). In separate sets of experiments, we examined the time history of bilirubin flux in bile samples during ZnPP administration. Bilirubin concentrations in bile samples were determined by an enzyme-linked immunosorbent assay using a monoclonal antibody 24G7, which recognizes both conjugated and unconjugated bilirubin fractions as described previously (28, 29).

Measurements of horseradish peroxidase excretion into bile. The effects of ZnPP on biliary excretion of horseradish peroxidase (HRP) were examined to determine the paracellular and transcellular fractions of the bile transport in the single-pass perfused liver as described elsewhere (6, 25). Briefly, 20 min after the start of perfusion, 1 μM ZnPP was infused, followed 1 min later by pulse loading with 25 mg HRP 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 bile samples were determined spectrophotometrically by measuring the rate of oxidation of 4-aminoantipyrine at 510 nm as described previously (25). Separately, effects of colchcine on the ZnPP-induced alterations in the HRP excretion were examined to confirm the role of vesicular transport across the hepatocytes in the bile formation (6). In these experiments, colchicine was administered intravenously at 2 mg/kg, 2 h before the start of the isolation procedure for the liver.

Measurements of CO and NO_2^- in hepatic venous perfusate. Concentrations of CO in the effluent of the perfused liver were determined by detecting ferrous CO complex of myoglobin spectrophotometrically as described previously (19). As an index of NO generation in the liver, concentrations of NO_2^- in the hepatic venous perfusate were determined by a fluorescence spectrophotometry based on the reaction of nitrite with 2,3-diaminonaphthalene to form the fluorescent product 1-(H)-naphthotriazole, as described elsewhere (13). Calibration curves were established by examining the fluorescence intensity in the presence of known concentrations of $NaNO_2$. Fluorescence measurements at 450 nm were made under an excitation wavelength of 365 nm using a fluorescence spectrophotometer (F-3000, Hitachi). This method allowed us to obtain the linearity between the concentrations of $NaNO_2$ and the fluorescence intensity in a range between 10 and 500 nM. We confirmed that the presence of 1 μM ZnPP in the perfusate samples did not influence the fluorometry of 1-(H)-naphthotriazole.

Determination of cGMP in taurocholate-treated perfused liver and in hepatocyte suspension. Tissue samples to determine cGMP were prepared from liver pieces excised and snap-frozen in liquid nitrogen after 30 min perfusion (20-min stabilizing perfusion followed by 10 min perfusion with and without 1 μM ZnPP). When added, 2.5 μM CO was infused together with 1 μM ZnPP and the liver was similarly snap-frozen in liquid nitrogen. cGMP was extracted using a solution of 0.1 M perchloric acid at 0°C and was determined by an enzyme-linked immunoassay using assay kits (RPN225, Amersham), as described elsewhere (4). The same samples extracted from the liver tissue were also used to examine tissue ATP contents as an index of the viability of perfused livers. The ATP contents were determined by the luciferin/luciferase method using a multiwell microplate chemiluminescence analyzer (Dia-Iatoron, Tokyo, Japan) as described elsewhere (12).

RESULTS

ZnPP-induced increase in biliary output and its reversal by CO. Figure 1 illustrates the time history of bile outflow in the liver perfused with the taurocholate-containing Krebs buffer. As seen, ZnPP administration at 1 μM evoked a significant increase in the bile output, which reached a maximum level at 10 min, exhibiting ~20% increase as compared with the baseline output.

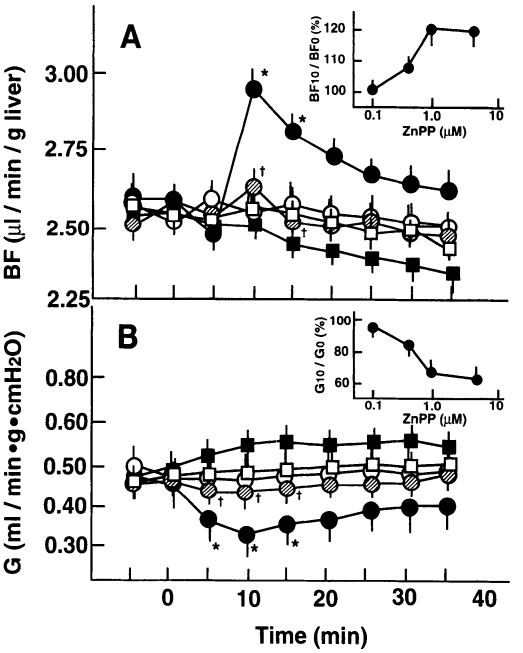

Fig. 1. Elevation in taurocholate-dependent biliary outflow (BF; *A*) and reduction of vascular conductance (*G*; *B*) by zinc protoporphyrin IX (ZnPP) administration in perfused rat liver. Data represent means ± SE of 10–12 experiments. ZnPP was added in the perfusate (*time 0*) at a final concentration of 1 μM. ZnPP elicited a significant increase in bile output (filled circles; $*P < 0.01$) compared with control (open circles). Note that supplement with 2.5 μM CO attenuated ZnPP-induced choleretic response (hatched circles; $\dagger P < 0.05$), whereas the same concentration of CO per se (open squares) did not alter baseline bile output (open circles). Group treated with 4 μM CO alone (filled squares) shows time-dependent decline in bile output, but without statistical significance. *Insets*: dose-response curves showing relationship between ZnPP concentrations (0.1, 0.5, 1, and 5 μM) and relative %changes in bile output (*A*; BF_{10}/BF_0) and vascular conductance (*B*; G_{10}/G_0) measured at 10 min as compared with baseline levels measured at 0 min.

This increase in the bile output was followed by a gradual decrease. ZnPP also evoked a decrease in the vascular conductance in a similar manner to our previous results with the taurocholate-free perfused liver (19). The time history of the ZnPP-induced alterations in the vascular conductance constituted a trace inversely correlated to that of the bile output. We also examined a dose-dependent alteration in the responses of the bile output and the vascular resistance elicited by ZnPP. As shown in the insets in Fig. 1, these responses became greater with increasing concentrations ranging between 0.1 and 1 μM and reached a plateau level at 5 μM. We therefore examined whether CO supplement attenuated the changes elicited by ZnPP at 1 μM, the lowest concentration that induced a maximum choleretic response.

The choleretic response induced by the ZnPP administration was eliminated by supplement of 2.5 μM CO, whereas the same concentration of CO per se did not alter the baseline bile secretion. When the CO concentration was increased to 4 μM, a time-dependent decline in the bile output was observed. Administration of this concentration of CO did not inhibit the oxygen consumption in the perfused liver (data not shown). We have also examined effects of supplement of unconjugated bilirubin on the ZnPP-induced choleretic response. Administration of unconjugated bilirubin at 500 nM in the perfusate did not attenuate the ZnPP-elicited choleresis. These results suggest that the decrease in production of CO, rather than of bile pigments such as biliverdin or bilirubin, accounts for the ZnPP-elicited choleretic changes. ZnPP administration did not significantly alter baseline bile output in the liver perfused without taurocholate during the experiments (data not shown), suggesting that suppression of endogenous CO specifically enhances the bile acid-dependent fraction of biliary transport.

ZnPP-enhanced transcellular fraction of HRP transport and its inhibition by CO. As shown in Fig. 1, the ZnPP-induced increase in bile output was apparently small and transient. Nevertheless, the response indicated a marked functional alteration in the bile excretion, when the biliary HRP excretion was examined. Figure 2 illustrates the effects of ZnPP administration on temporal alterations in biliary excretion of HRP. As previously described (25), the time history of HRP excretion showed two peaks. The first peak was at 4 min and the second was at 15–20 min after the HRP injection, reflecting paracellular and transcellular fractions of the HRP tranport, respectively. The ZnPP administration evoked a 40% elevation of the second peak but did not significantly elicit the increase in the first peak. Supplement of 2.5 μM CO significantly attenuated the ZnPP-induced elevation of the second peak. Table 1 shows the effects of 1 μM ZnPP on the first 6-min fraction (0–6 min, mainly paracellular fraction) and the later 25-min fraction after the second peak (15–40 min, mainly transcellular fraction) of the total HRP excretion. ZnPP induced an ~40% increase in the first fraction of the HRP excretion but with no statistical significance. On the other hand, it elicited a

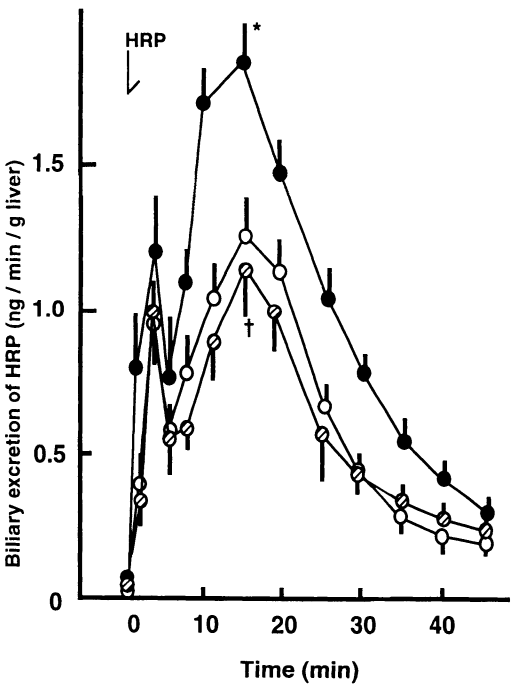

Fig. 2. Time history of the efflux of transportally administered horseradish peroxidase (HRP) into bile. Groups were treated with vehicle (control; open circles), 1 μM ZnPP (filled circles), and 1 μM ZnPP + 2.5 μM CO (hatched circles). Note marked elevation of the second peak, suggesting increasing transcellular fraction of bile transport. *$P < 0.001$ compared with control. †$P < 0.01$ compared with ZnPP-treated group.

significant increase in the later fraction, indicating >50% elevation as compared with the control value. Supplement of 2.5 μM CO significantly attenuated the ZnPP-elicited increase in the later fraction. In addition, supplement of colchicine, an inhibitor of microtubular assembly for vesicular transport, significantly attenuated the ZnPP-induced transcellular fraction of the HRP transport in agreement with the previous report (Table 1) (7). On the other hand, the increase in the transcellular HRP transport observed in the taurocholate-perfused liver treated with ZnPP was little, if any, when perfused with ZnPP in the absence of taurocholate (data not shown). These results collectively suggest that endogenous CO suppression elicits a marked increase in the transcellular transport of HRP as a

Table 1. *Effects of ZnPP on fractional amounts of biliary HRP excretion in perfused rat liver*

Group	First Peak (0–6 min)	Second Peak (15–40 min)	n
Control	3.81 ± 0.52	16.01 ± 1.95	8
ZnPP	5.57 ± 0.65	24.53 ± 1.79*	6
ZnPP + 2.5 μM CO	5.11 ± 0.20	16.70 ± 1.41†	6
CO (2.5 μM)	4.40 ± 1.70	17.68 ± 0.79†	6
ZnPP + colchicine	4.22 ± 0.83	10.86 ± 2.26‡	5

Values are means ± SE [in ng horseradish peroxidase (HRP)/g liver]; n = no. of experiments. All experiments were performed with 30 μM taurocholate in perfusate. CO, carbon monoxide. *$P < 0.01$ as compared with control. †$P < 0.02$ as compared with zinc protoporphyrin IX (ZnPP)-treated group. ‡$P < 0.001$ as compared with ZnPP-treated group.

Table 2. *Flux of CO and NO_2^- in venous perfusate collected from perfused rat liver*

Group	CO	NO_2^-
Control	620 ± 120	330 ± 190
ZnPP (1 μM)	50 ± 24*	300 ± 130

Values are means ± SE of measurements (in pmol·min^{-1}·g liver^{-1}) in bile samples collected at 5–10 min after the start of 1 μM ZnPP administration; n = 6 experiments for each group. Note that baseline NO_2^- level in control was not suppressed by ZnPP administration. *$P < 0.01$ as compared with control.

consequence of alterations in bile acid transport across hepatocytes under the current experimental conditions.

ZnPP abolishes CO and bilirubin excretion without altering NO generation. We examined whether ZnPP administration specifically suppressed levels of endogenously generated CO in the perfused rat liver (Table 2). The taurocholate-treated liver excreted ~0.6 nmol·min^{-1}·g liver^{-1} of CO into the venous perfusate. This level of CO flux was equivalent to that measured in the taurocholate-free perfused liver, as reported previously (28). As shown, the treatment with 1 μM ZnPP abolished detectable levels of CO in the hepatic venous effluent. On the other hand, the same dose of ZnPP did not suppress the basal flux of NO_2^- in the venous perfusate.

The ZnPP administration also abolished the bilirubin flux in bile samples. Figure 3 illustrates the time history of bilirubin excretion into bile in the control and ZnPP-treated groups. At 10 min after the start of perfusion in the control liver, the biliary efflux of bilirubin was ~0.35 nmol·min^{-1}·g liver^{-1}, which decreased in a time-dependent manner. In the liver treated with 1 μM ZnPP for 10 min, >90% of the baseline flux of bilirubin was eliminated. As described elsewhere (28), the flux of unconjugated bilirubin in the

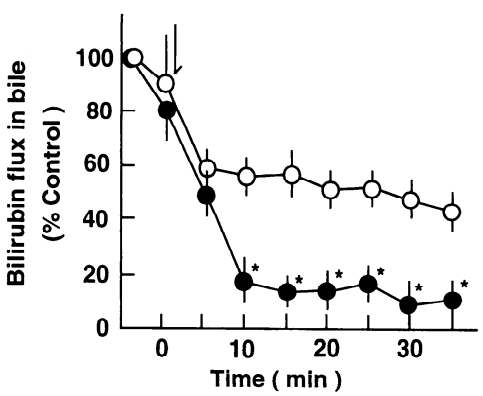

Fig. 3. Effects of ZnPP administration on biliary excretion of bilirubin. Concentrations of bilirubin were determined by enzyme-linked immunosorbent assay using 24G7, an anti-bilirubin monoclonal antibody. Data represent means ± SE of 6–8 experiments. Control, open circles; ZnPP-treated groups, filled circles. Because initial values of bilirubin flux during stabilizing period varied among experiments, data were expressed as the values normalized by the initial levels of bilirubin flux (0.45 nmol·min^{-1}·g liver^{-1} in average) measured 10 min before *time 0*. Note that bilirubin efflux was eliminated at 10 min after the start of ZnPP administration at 1 μM. *$P < 0.01$ compared with time-match values in control.

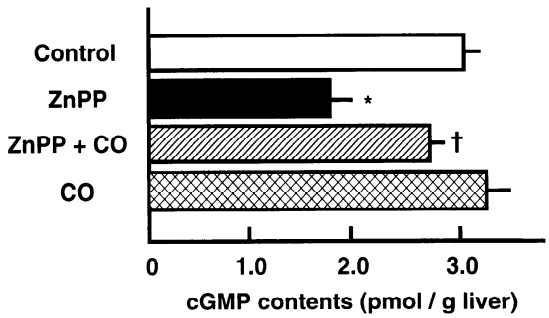

Fig. 4. Effects of ZnPP administration on tissue guanosine 3′,5′-cyclic monophosphate (cGMP) levels in isolated perfused liver. Data represent means ± SE of measurements at 10 min after start of 1 μM ZnPP administration. Control, perfusion without ZnPP (n = 5); ZnPP, application of 1 μM ZnPP (n = 6); ZnPP + CO, 1 μM ZnPP with 2.5 μM CO-containing perfusion (n = 7); CO, 2.5 μM CO alone. *$P < 0.05$ as compared with control. †$P < 0.05$ as compared with ZnPP-treated group.

venous effluent ranged at ~0.015 nmol·min^{-1}·g liver^{-1}. In addition, the baseline efflux of CO in the venous effluent of the taurocholate-treated perfused liver ranged at 0.5–0.6 nmol·min^{-1}·g liver^{-1} and no measurable levels of CO were detected in bile samples (28). Namely, the total CO flux roughly matched that of total amounts of bilirubin in agreement with the stoichiometric relationship between CO and bilirubin produced in the heme oxygenase reaction. These results confirm that, under the current experimental conditions, the heme oxygenase reaction was completely inhibited by the administration of 1 μM ZnPP.

ZnPP-induced cGMP reduction, and its recovery by CO. Effects of ZnPP administration on tissue cGMP levels were examined in the isolated perfused liver (Fig. 4). ZnPP administration elicited a 35% decrease in tissue cGMP contents. The ZnPP-induced reduction of cGMP was attenuated significantly by 2.5 μM CO, whereas the same dose of CO alone did not alter the baseline cGMP levels. These findings illustrate that endogenous CO suppression actually alters the tissue cGMP levels. Collectively, the decrease in endogenous generation of CO, but not of NO, accounts for the mechanism through which ZnPP reduces tissue cGMP levels.

NO donor and cGMP analog mimic modulatory action of CO on bile excretion. Figure 5 illustrates the effects of several reagents that mimic the repressive action of CO on the ZnPP-induced changes in bile output and amounts of bile acids and phospholipids. These measurements were carried out using bile samples collected at 10 min after the start of 1 μM ZnPP administration. As seen, the ZnPP-induced elevation of bile output coincided with an ~30% elevation in the flux of bile acids and phospholipids, and such increases were again abolished by supplement of 2.5 μM CO. The ZnPP-induced changes in the excretion of bile acids and phospholipids were significantly attenuated by 10 μM SNP, an NO donor, suggesting that NO, when administered exogenously, can share the inhibitory action on the choleresis with CO.

Pretreatment with 1 μM 8-BrcGMP exhibited ~65% repression of ZnPP-induced elevation in the bile out-

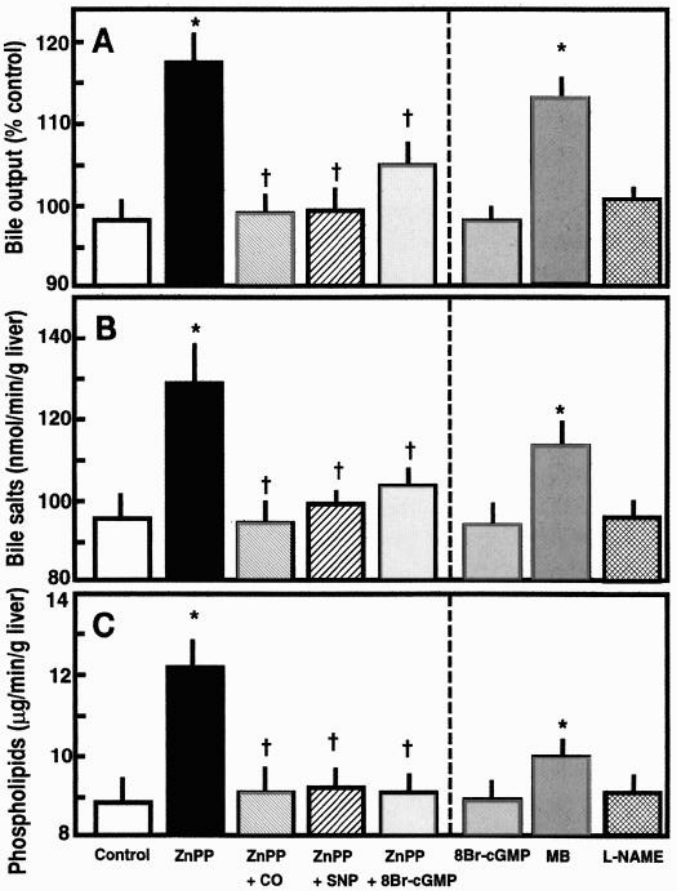

Fig. 5. Effects of supplement of CO, sodium nitroprusside (SNP), 8-bromoguanosine 3′,5′-cyclic monophosphate (8-BrcGMP), and N^G-nitro-L-arginine methyl ester (L-NAME) on ZnPP-induced elevation in bile output (*A*), bile salts (*B*), and phospholipids (*C*). ZnPP, application of 1 μM ZnPP (n = 12); ZnPP + CO, 1 μM ZnPP with 2.5 μM CO-containing perfusion (n = 8); ZnPP + SNP, 1 μM ZnPP with 10 μM SNP (n = 6); ZnPP + 8-BrcGMP, 1 μM ZnPP + 1 μM 8-BrcGMP (n = 8); MB, 250 nM methylene blue, a guanylate cyclase inhibitor (n = 6); L-NAME, 1 mM L-NAME, a NO synthase inhibitor (n = 8). Data represent means + SE measured in bile samples collected at 10 min after the start of reagent administration. Supplement with 2.5 μM CO, 10 μM SNP, 1 μM 8-BrcGMP, or 1 mM L-NAME alone did not significantly alter baseline bile output, bile acids, or phospholipids (data not shown). *$P < 0.01$ compared with control (n = 10). †$P < 0.05$ compared with ZnPP-treated group.

put. We were not able to observe any choleretic or cholestatic responses under administration of 1 μM 8-BrcGMP alone. Likewise, administration of 1 μM 8-BrcGMP alone did not alter the baseline vascular conductance, as shown previously (19). The absence of choleretic action of this concentration of 8-BrcGMP was reproducible in the taurocholate-free perfused liver. When 5 μM 8-BrcGMP was administered, the baseline vascular conductance exhibited a 10% increase without showing significant elevation in bile output (data not shown). On the other hand, administration of 250 nM methylene blue, a guanylate cyclase inhibitor, elicited a significant increase in bile output concurrent with the increasing excretion of bile salts and phospholipids. In addition, the methylene blue-induced choleresis coincided with the increase in the vascular resistance that was observed similarly in the ZnPP-treated liver. Under the same experimental conditions, the 10-min administration of methylene blue significantly lowered tissue cGMP levels to 1.1 ± 0.3 pmol/g liver (means ± SE of 6 experiments). It should be noted that administration of 1 mM L-NAME alone in the absence of ZnPP neither increased the baseline bile output nor enhanced transport of bile salts and phospholipids. These results along with those described earlier in RESULTS indicate that endogenous levels of NO generation play little role, if any, in regulation of bile transport under the current experimental conditions.

ZnPP-induced elevation of oxygen consumption and vascular resistance and their attenuation by CO. In another set of experiments, we examined the parameters indicating the viability of the perfused liver preparation, such as oxygen consumption and tissue ATP levels during the ZnPP administration (Table 3). At 10 min after the start of ZnPP administration, when bile output reached maximum level, the liver exhibited a 10% increase in oxygen consumption as compared with the baseline values measured before ZnPP administration. In concert with the increase in oxygen consumption, tissue ATP levels exhibited a slight elevation

Table 3. *Effects of ZnPP on oxygen consumption and tissue ATP contents in perfused rat liver*

Group	Oxygen Consumption, μmol·min^{-1}·g liver^{-1}	Tissue ATP Levels, μmol/g liver	Vascular Conductance, ml·min^{-1}·g^{-1}·cmH_2O^{-1}
Control	2.0 ± 0.1	1.9 ± 0.1	0.48 ± 0.07
ZnPP	2.3 ± 0.1*	2.1 ± 0.1	0.34 ± 0.03*
ZnPP + CO	1.9 ± 0.1†	1.8 ± 0.1	0.46 ± 0.03†
CO alone	2.0 ± 0.1	2.0 ± 0.1	0.49 ± 0.05

Values are means ± SE of measurements at 10 min after the start of 1 μM ZnPP administration; n = 6–8 experiments for each group. Note that ZnPP-elicited increase in oxygen consumption is not a consequence of uncoupled respiration, inasmuch as ZnPP treatment did not influence tissue ATP levels. *$P < 0.01$ compared with control group. †$P < 0.01$ compared with ZnPP-treated group.

but without statistical significance. The ZnPP-elicited elevation of oxygen consumption was attenuated by 2.5 μM CO, whereas the same dose of CO neither altered baseline oxygen consumption nor reduced ATP contents. This phenomenon is unlikely to result from uncoupling of mitochondrial respiration, because tissue ATP levels were well maintained during oxygen consumption. These results, taken together, indicate that ZnPP administration did not significantly affect the viability of the liver in the current experiments.

DISCUSSION

The current study provides evidence that CO modulates bile acid-dependent biliary transport in the liver. Because we previously demonstrated that endogenous CO production is necessary to maintain hepatic sinusoidal vessels in a relaxed state (19), the present findings suggest another biological action of endogenous CO as a novel regulator of bile transport in the liver. In other words, suppression of endogenous CO by ZnPP evokes a choleretic response coupled with the decrease in vascular conductance resulting from sinusoidal constriction. This is a unique phenomenon in terms of the balance between sinusoidal perfusion and biliary transport. The sinusoidal perfusion rate is one of the factors that determines the amount of bile flow. Most vasoconstrictors, including norepinephrine (11), leukotriene C_4 (10), and high doses of endothelin-1 (25), are known to diminish the bile output as a consequence of a decrease in the portal conductance.

Upregulation of cGMP via activation of soluble guanylate cyclase is considered to serve as a mechanism through which low molecular monoxides such as NO and CO exert their signal transducing activities to modulate cell functions (3, 14, 26). In fact, cGMP has been postulated to be a secondary signal molecule responsible for NO- or CO-dependent modulatory mechanisms for sinusoidal tone via relaxation of fat-storing Ito cells, liver-specific microvascular pericytes (9, 19). The present findings showing inhibitory effects of 8-BrcGMP on the ZnPP-induced choleretic response also suggest that cGMP plays a role in the suppressive mechanism for the ZnPP-induced choleresis. Endogenous generation of CO, but not of NO, is likely to account for the changes in the guanylate cyclase activity; the ZnPP administration suppressed hepatic cGMP levels without lowering the basal NO release. These results taken together suggest that under the current experimental conditions CO, rather than NO, serves as an endogenous effector for the cGMP-mediated regulation of the biliary transport.

The regulatory role of cGMP in hepatobiliary function has attracted great interest lately. Recent observation (15) has shown that exogenous administration of submillimolar orders of 8-BrcGMP (10–100 μM) stimulates a bile acid-independent fraction of the bile formation but does not affect the bile acid-dependent fraction. This observation suggesting choleretic action of cGMP is inconsistent with our present findings. However, such discrepancy in the cGMP action on bile transport might be ascribed to different sensitivities to exogenously applied cGMP between vascular and parenchymal compartments. It should be noted that the concentration of the cGMP analog used in the current study was as low as 1 μM. We previously showed that this concentration of 8-BrcGMP sufficiently attenuated the ZnPP-induced constriction of local sinusoidal segments colocalized with hepatic stellate cells, a cellular compartment producing cGMP in response to CO, whereas the same treatment per se did not alter the baseline microvascular tone in a steady state (19). Along with the lack of choleretic action of 1 μM 8-BrcGMP by itself, these facts tempted us to consider the possibility that micromolar levels of exogenous 8-BrcGMP attenuate the ZnPP-induced choleresis as a consequence of or in parallel with the inhibition of the stellate cell-mediated sinusoidal responses. Because we observed a significant elevation of the vascular conductance by 8-BrcGMP at concentrations >5 μM (see RESULTS), it is possible that in the study of Myers et al. (15), an excessive cGMP administration might have evoked maximum vasodilation and altered the hepatocellular function independently of the microvascular events. The role of the microvascular responses in the ZnPP-induced choleresis may also explain the mechanism for elevation of bile acid-dependent bile output by this reagent in that the decrease in the microvascular conductance would allow greater time for bile acid uptake and consequently stimulate the bile output. Further investigation will be needed to disclose such a cGMP-sensing mechanism localized in nonparenchymal cellular compartments for regulation of the bile-transporting ability of hepatocytes.

Although the current study provides evidence for cGMP involvement in the CO-mediated regulation of bile transport, there is still the possibility that CO modulates bile excretion through a cGMP-independent mechanism. Actually, the effect of 8-BrcGMP on the ZnPP-induced choleresis was limited only to the 65% inhibition, suggesting the presence of alternative mechanisms for the CO-mediated modulation of bile transport. Such an unknown action of CO that involves a cGMP-independent signal transduction mechanism may be in accordance with the recent observation by Rich et al. (17) that CO elicits an increase in potassium current and thereby hyperpolarizes the resting membrane potential in rabbit corneal epithelial cells. We have also examined effects of administration of Ba^{2+}, a broad-spectrum potassium-channel inhibitor, on bile transport in the perfused liver. This reagent, however, did not evoke any choleretic responses but elicited a marked chorestasis, presumably because of its vasoconstrictive action on the portal vein (T. Sano, S. Kashiwagi, Y. Ishimura, and M. Suematsu, unpublished observations). On the other hand, we have recently shown that glibenclamide significantly potentiates bile transport without affecting the vascular conductance, suggesting involvement of hepatocellular ATP-sensitive potassium channels in the control mechanisms of bile formation (18). Although the whole picture of CO-mediated modulatory mechanisms for bile transport has not been fully understood yet, our observation

provides new insight into the physiological significance of the heme oxygenase reaction in controlling cell functions in various organs.

Biological actions of CO as an endogenous regulator of bile transport led us to consider its possible pathophysiological relevances to homeostasis of hepatobiliary function. The enzyme activity of heme oxygenase in the liver is highly limited by the flux of NADPH from pentose-monophosphate shunt, particularly when the liver undergoes a fasted state (8). CO generation may be inhibited by acute NADPH consumption in other microsomal enzyme systems such as cytochrome *P*-450, which contributes to synthesis of bile acids (1) or xenobiotic metabolism (21). Under these circumstances, suppression of heme oxygenase activity might help facilitate biliary transport of these metabolites in accordance with cytochrome *P*-450 reactions. On the other hand, changes in the baseline activity of heme oxygenase have recently been reported to occur when the liver is exposed to ischemia-reperfusion (24) or to endotoxin (27). In this context, we speculate that CO not only serves as an endogenous regulator of biliary transport and sinusoidal perfusion in physiological conditions but also exerts its cholestatic action to jeopardize the integrity of hepatobiliary function when heme oxygenase is expressed excessively under disease conditions.

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