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Original Article

Carbon monoxide may enhance bile secretion by increasing glutathione excretion and Mrp2 expression in rats

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Abstract

Background: Nitric oxide (NO) donors have been reported to induce choleresis via an increased excretion of glutathione. The effects of another gas molecule, carbon monoxide (CO), on bile formation are, however, inconsistent among previous reports. We investigated the sequential changes of bile output and the biliary contents in rats with or without CO supplementation to elucidate the mechanism of CO on bile excretion.
Methods: Dichloromethane (DCM) was gastrically fed to male Sprague–Dawley rats to yield CO by liver biotransformation. The rats were divided into DCM-treated ($n = 7$), DCM plus L-NAME-treated ($n = 6$), and corn oil-treated-($n = 8$) groups. Bile samples were collected hourly to examine the flow rate and bile content. Serum levels of nitrite and nitrate 4 hours after DCM supplementation with or without NO synthase (NOS) inhibition were measured by capillary electrophoresis. The expression of hepatic inducible NOS was evaluated by Western blotting 6 hours after DCM administration.
Results: Levels of carboxyhemoglobin rose to around 10% at 4 hours after DCM supplementation and were maintained until the end of the experiments. Bile flow increased after DCM supplementation and was associated with a concomitant increase of biliary glutathione and higher hepatic multidrug resistance-associated protein 2 (Mrp2) expression. Hepatic inducible NOS expression and serum nitrate/nitrite levels were also increased. Treatment with an NOS inhibitor (L-NAME) abolished the CO-induced glutathione excretion and choleresis, but not Mrp2 expression.
Conclusion: The present study demonstrated that CO enhanced biliary output in conjunction with NO by increasing the biliary excretion of glutathione. The increment in biliary glutathione was associated with an increased expression of hepatic Mrp2.

Keywords: biliary secretion; carbon monoxide; glutathione; Mrp2

1. Introduction

Carbon monoxide (CO) is well-known for its toxicity at high concentrations; however, blood levels of carboxyhemoglobin (COHb) below 20% are well tolerated in humans and are considered as the "biological threshold".[1] Extensive evidence examining large and small animals has shown the beneficial effects of CO in cardiovascular disease, sepsis and shock, cancer, transplantation, and acute lung, kidney, and liver injury.[2] The first randomized, placebo-controlled phase I trial to use inhaled CO in healthy subjects has recently been completed using the Covox DS delivery device (Ikaria, Hampton, NJ, USA).[3]

The effects of CO on bile excretion are, however, inconsistent among previous studies. Abolishing CO from the perfused liver led to an increase in bile acid-dependent bile flow, indicating a negative effect of CO on bile formation.[4] However, other studies have demonstrated that CO enhanced bile excretion by inducing bilirubin and glutathione (GSH)[5] or bicarbonate excretion.[6] Similar to CO, the gaseous signal molecule nitric oxide (NO) also modulates the formation of

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bile and has been reported to induce choleresis in rats by increasing biliary GSH excretion.[7] As CO has been reported to induce NO production,[8] CO may therefore enhance bile excretion through the action of NO.

The customary bile output in healthy subjects ranges from 500 mL to 700 mL a day. Such between-subject variations in bile excretion may be also present in animals and cause difficulties in comparing bile output after different treatments. Moreover, CO also acts on other organs including the cardiovascular system, lung, kidney, and intestine.[2] However, using a liver perfusion (i.e., *ex vivo*) model may not reflect the effect of CO on bile formation under physiologic conditions. As a consequence, an examination of sequential changes in bile output and biliary contents in the same animal pre- and post-CO administration would be more relevant to investigate the *in vivo* effects of CO on bile excretion.

Dichloromethane (DCM) is oxidized by liver cytochrome P450 to yield CO and carbon dioxide.[9] A mean maximum COHb level of 9.3% was detected in rats 6 hours after a single oral dose of DCM (6.2 mmol/kg) with a protective effect against immune-mediated liver damage.[10]

Bile formation is an osmotic process driven by the active transport of solutes into the bile. Water moves from blood into bile in response to osmotic gradients.[11,12] Membrane transporters such as multidrug resistance-associated protein 2 (Mrp2) are responsible for regulating the uptake and excretion of various biliary solutes.[13,14] By examining the sequential changes in biliary content, bile output, hepatic inducible NO synthase (iNOS), and Mrp2 expression in DCM-fed rats, we aimed to investigate the effect of CO on bile output and its underlying mechanism.

2. Methods

2.1. Experimental animals and procedures

Male Sprague–Dawley rats, each weighing 270–300 g, were used. After injection of 1 mL/kg of Zoletil 50 (Virbac, Carros, France) intraperitoneally for anesthesia, a midline laparotomy was performed. The common bile duct was explored and a tiny hole was created over its middle part. Through this hole, a PE 10 polyethelene tube (0.011 inch inner diameter, 0.024 inch outer diameter; Clay Adams, Parsippany, NJ, USA) was inserted. The PE 10 tube and the common bile duct were then tied together with 4-0 silk at both ends of the insertion site to prevent bile leakage. Finally, the PE 10 tube was connected to a light-shielded Eppendorf tube for bile collection. For gastric feeding, a plastic tube (the extension tube of an infusion set, 12 inch; Sigma Medical Supplies, Taipei, Taiwan) was inserted into the stomach via an incision made over the anterior wall of the stomach. The abdominal wall was closed in layers after completing the above surgical procedures.

DCM (6.2 mmol/kg; Sigma Chemical Co., St. Louis, MO, USA) in corn oil, or corn oil alone was infused into the rats' stomachs via a gastric tube. To investigate the interaction of NO and CO, a dose of 30 mg/kg of the NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME; Sigma Chemical Co.) was injected into the peritoneal cavity 1 hour after gastric feeding of DCM. Thereafter, the rats were divided into a DCM-fed group (DCM group, $n = 7$), a corn oil-fed group (control group, $n = 8$), and a DCM-fed plus L-NAME-treated group (L-NAME–DCM group, $n = 6$).

During the experiment, anesthesia was maintained with supplemental bolus injections of ketamine (30 mg/kg) when any spontaneous muscle activity or lessening of the anesthetic plane occurred. Bile was collected hourly for 7 hours. A volume of 1 mL of 5% glucose was administered every hour via the gastric tube to avoid dehydration and starvation in the rats. When the experiment concluded, the rats were sacrificed by anesthesia overdose. The liver was harvested after portal venous perfusion with phosphate buffer solution, and blood was drawn from the abdominal aorta. The protocol was approved by the Animal Care and Use Committee at our university, and all animals received care according to the animal center's regulations.

2.2. Measurement of hepatic iNOS expression and NO production

To study whether CO induces NO production, the rats were fed with DCM (6.2 mmol/kg) in corn oil ($n = 7$) or corn oil alone ($n = 6$) as described above and were sacrificed at the 4th hour of the experiment. Induction of NO was assessed by examining the expression of iNOS in liver extract and serum levels of nitrate and nitrite. The expression of iNOS was determined by Western blotting on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using antibody to inducible NOS (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Serum samples were pretreated by Microcon YM-3 ultrafiltration devices (Millipore Corporation, Bedford, MA, USA), and serum levels of nitrite and nitrate were measured by a high-performance capillary electrophoresis system (P/ACE MDQ; Beckman Coulter, Fullerton, CA, USA) with an ultraviolet detector set at 214 nm. The imprecision of the method was less than 8.5%, and the mean recovery was 115% (range 112.8–119.3%, $n = 3$).

2.3. Measurement of COHb

Blood levels of COHb were measured 1, 2, 4, and 6 hours after gastric feeding by sampling blood from the retro-orbital plexus of a rat with or without DCM treatment and from the abdominal aorta at sacrifice. A Co-Oximeter IL-1725 (Instrumentation Laboratories, Lexington, MA, USA) was used to measure COHb levels.

2.4. Measurement of bile output and biliary solutes

Bile output was determined gravimetrically, assuming a density of 1.0 g/mL. Biliary bicarbonate concentration and pH were measured immediately by a blood gas analyzer (IL Synthesis-25; Instrumentation Laboratories). Bile samples were centrifuged at 7000 rpm for 10 minutes, and the supernatants were stored at −8°C until analysis. For GSH measurement, bile samples were treated with 10% trichloroacetic

acid (TCA) to deproteinize them, and were centrifuged at 7000 rpm for 10 minutes. The supernatant was aspirated to analyze reduced and oxidized GSH.

High-performance liquid chromatography–tandem mass spectrometry was used to quantitate the GSH as previously described.[15] The chromatographic separation was performed at 45°C using a MS C18 column (2.1 mm × 150 mm, 3.5 μm particle size; Waters, Milford, MA, USA). The mobile phase consisted of 0.1% acetic acid in (1) H_2O and in (2) acetonitrile at a flow rate of 300 μL/min. The transitions *m/z* (Q1 to Q3) for GSH, GSH disulfide (GSSG), and internal standard, respectively, were 308.4/162.3, 613.7/354.8, and 311.4/181.8 on an API 5000 tandem mass spectrometer (Sciex, ON, Canada). The TCA-treated bile was mixed with 1:10 volume of the internal standard (glycine-$^{13}C_2$,^{15}N). The filtered supernatant was used for GSH quantification. The method was linear over a range of 5–5000 ng/mL with a correlation coefficient (*r*) greater than 0.99. The intrarun imprecision was less than 5% at the mean levels of 339 and 1758 ng/mL, and the interrun was less than 9%. The average recovery of GSH and GSSG was 104.8% and 123.5%, respectively.

Total bile acids were determined by an enzymatic method (Bio-Quant, San Diego, CA, USA) using 3-α-hydroxysteroid dehydrogenase. In the presence of thio-NAD, this enzyme converts bile acid to 3-keto steroids and thio-NADH. The amount of thio-NADH formed was measured at 405 nm. Bilirubin was measured by a diazo method on the Modular DP analyzer (Roche Diagnostics, Mannheim, Germany).

Sequential changes in bile output and biliary contents were expressed in relative fold changes, compared to those levels at the first hour. Differences in bile output and biliary contents between rats receiving corn oil, DCM, or DCM plus L-NAME treatment were compared at hourly intervals and in total by calculating the area under the curve (AUC).

2.5. Measurement of liver Mrp2 expression

Protein concentration was determined by the bicinchoninic acid protein method (Pierce Biotechnology, Rockford, IL, USA) using albumin as a standard. Western blot analysis was carried out with 50 μg protein of liver homogenates per well on 8% SDS-PAGE. After electrophoresis, the proteins were transferred onto a polyvinylidene difluoride membrane for 1.5 hours at 100 V. The membranes were then blocked with 5% milk/TBS–0.1% Tween solution and incubated with primary antibodies to Mrp2 (1:2,000; Alexis Biochemicals, San Diego, CA, USA) or β-actin (1:5000; Sigma Chemical Co.). After washing, the membrane was incubated with horseradish-peroxidase-coupled anti-mouse (1:50,000) or anti-rabbit IgG (1:5,000) antibodies at room temperature for 1 hour. Immunoreactive bands were visualized using enhanced chemiluminescence Western blotting detection reagents (Pierce Biotechnology) on a luminescent image analyzer LAS-3000 (Fujifilm Life Science, Stamford, CT, USA). Western blot analysis was repeated twice to confirm the initial results.

2.6. Statistical analysis

Data are expressed as mean ± standard error of the mean. The differences between groups were analyzed by Mann–Whitney *U* test using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). A *p* value less than 0.05 was considered as significant.

3. Results

3.1. Blood COHb after DCM treatment

Blood COHb was around 3% at the basal level in both the control and DCM groups and began to rise in DCM-fed rats at 2 hours after DCM feeding (Fig. 1A). As shown in Fig. 1A and 1B, COHb was significantly increased at 4 hours after DCM treatment, reached a peak level (12%) at 6 hours, and persisted at this level until the end of the experiment (11.2 ± 0.7% at 7 hours). Treatment with L-NAME did not affect the biotransformation of CO from DCM, and the mean level of COHb in this group rats was 12.1 ± 1.3% at 7 hours post DCM feeding (Fig. 1B). Blood COHb in the control group did not change,

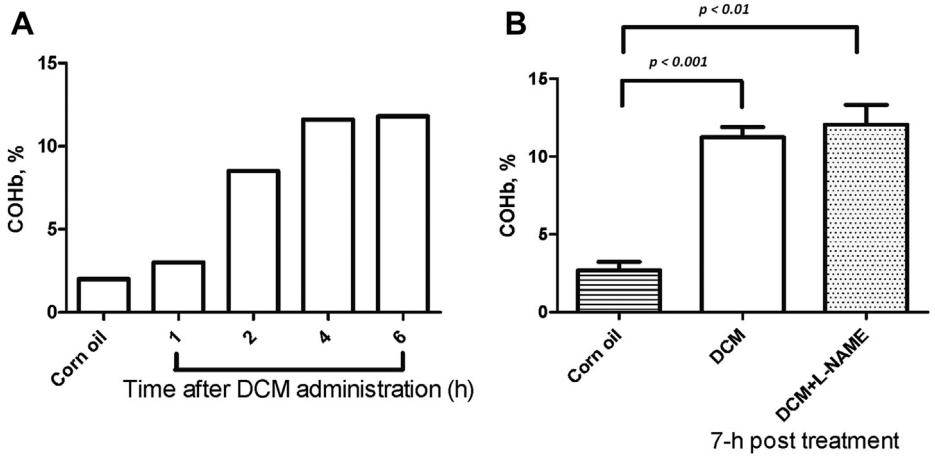

Fig. 1. Blood levels of carboxyhemoglobin (COHb) of experimental rats (A) from 1 hour to 6 hours after dichloromethane (DCM) administration, and (B) at the end of the experiment in DCM-treated, corn oil-treated, and L-NAME plus DCM-treated groups.

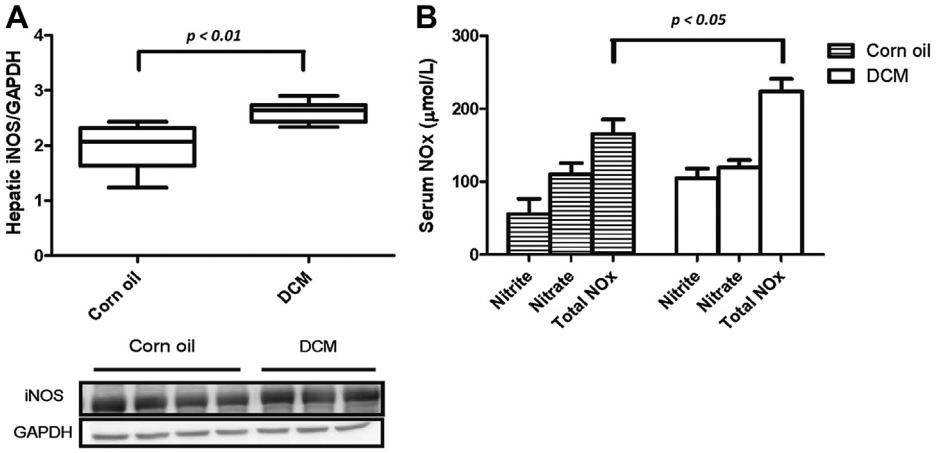

Fig. 2. Effects of dichloromethane (DCM) on (A) hepatic expression of inducible nitric oxide synthase (iNOS), and (B) serum levels of nitrite/nitrate (NOx) in rats 4 hours after treatment.

with a mean value of 2.7 ± 0.6% at the end of experiment (Fig. 1B).

3.2. CO-induced iNOS expression and NO production

Expression of iNOS was significantly higher in the liver homogenates of rats at 4 hours after DCM treatment compared to those of the control group (2.62 ± 0.07 vs. 1.98 ± 0.18; $p = 0.007$) (Fig. 2A). As shown in Fig. 2B, serum levels of nitrate and nitrite were significantly higher in the DCM group than the control group (224.0 ± 17.1 μmol/L vs. 165.6 ± 19.8 μmol/L; $p = 0.03$).

3.3. Changes in bile output by CO

As shown in Fig. 3A, the bile output of DCM-fed rats increased significantly compared to that of the control group (AUC 10.12 ± 0.69 vs. 6.77 ± 0.21; $p < 0.001$). L-NAME treatment abolished the choleretic effect of DCM (AUC for the L-NAME−DCM group vs. the DCM group 6.62 ± 0.20 vs. 10.12 ± 0.69; $p = 0.008$).

3.4. Changes of biliary contents by CO

As demonstrated in Fig. 3B, there was a significant increase in total glutathione (GSH + GSSG) excretion at 4 hours after DCM treatment (AUC for the DCM group vs. the control group 7.56 ± 1.34 vs. 4.39 ± 0.35; $p = 0.035$). The increase in GSH excretion in DCM-fed rats was diminished with L-NAME treatment (AUC for the L-NAME−DCM group 4.17 ± 0.30; $p = 0.026$).

As shown in Fig. 4A, CO did not alter the excretion of biliary bicarbonate (AUC for the DCM group vs. the control group 5.07 ± 0.12 vs. 5.41 ± 0.26; $p = 0.174$). However, the biliary excretion of bicarbonate showed a decline from the basal level until 7 hours in both the DCM and control groups. The decrement was significantly lessened in DCM-fed rats with L-NAME treatment (AUC for the L-NAME−DCM group 5.78 ± 0.22; $p = 0.008$).

Bile acid excretion decreased in all experimental rats, with biliary bile acids at 7 hours less than half of the initial levels (Fig. 4B). Similar to the changes in biliary excretion of bicarbonate, the decrement was lessened significantly in DCM-fed rats with L-NAME treatment (AUC for the

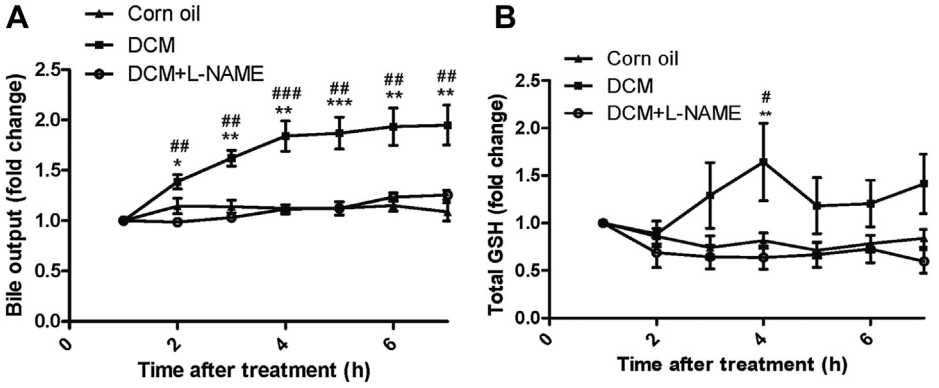

Fig. 3. Sequential changes in (A) bile output and (B) total glutathione excretion in dichloromethane (DCM)-treated, corn oil-treated, and L-NAME plus DCM-treated groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ between DCM- and corn oil-fed rats; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ between DCM- and L-NAME plus DCM-treated rats.

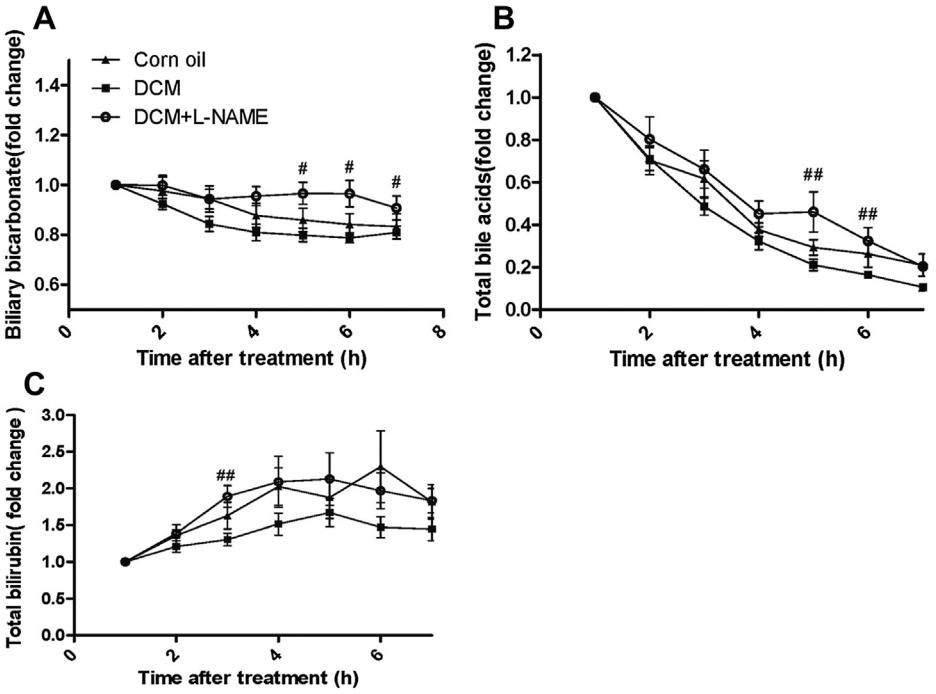

Fig. 4. Sequential changes in (A) bicarbonate, (B) total bile acids, and (C) total bilirubin excretion in dichloromethane (DCM)-treated, corn oil-treated, and L-NAME plus DCM-treated groups. #$p < 0.05$, ##$p < 0.01$ between DCM- and L-NAME plus DCM-treated rats.

L-NAME−DCM group vs. DCM group 3.30 ± 0.09 vs. 2.45 ± 0.12; $p = 0.026$).

Excretion of biliary bilirubin increased in all experimental rats with no significant difference among the different experimental groups (Fig. 4C; AUC for the control, DCM, and L-NAME−DCM groups 10.45 ± 0.99, 8.40 ± 0.49, and 10.89 ± 1.15, respectively).

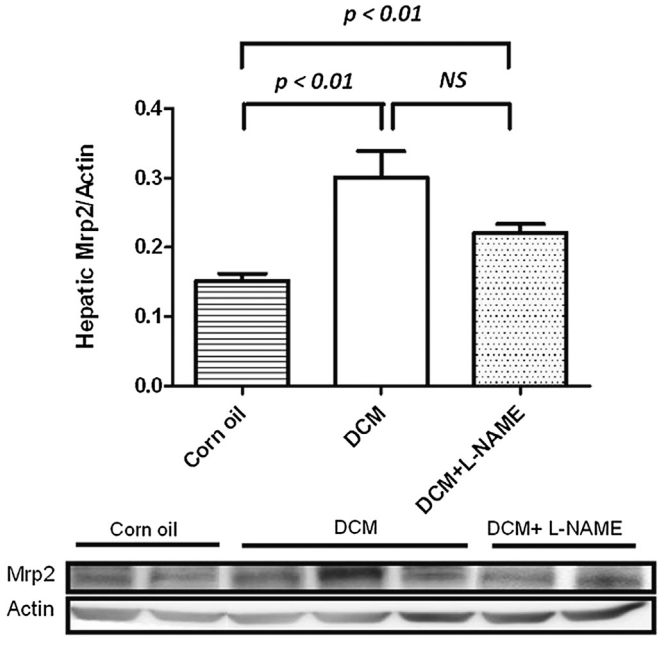

Fig. 5. Effects of dichloromethane (DCM) with or without nitric oxide synthase inhibitor (L-NAME) on Mrp2 expression.

3.5. Hepatic Mrp2 expression

As shown in Fig. 5, DCM treatment significantly increased hepatic Mrp2 expression (DCM group vs. control group 0.30 ± 0.04 vs. 0.15 ± 0.01; $p = 0.002$). Although the expression of hepatic Mrp2 tended to decrease after L-NAME treatment, this was not statistically significant (L-NAME−DCM group vs. DCM group 0.22 ± 0.01 vs. 0.30 ± 0.04).

4. Discussion

Our study demonstrates that biliary excretion was increased by CO at physiologically relevant concentrations. The choleretic effect of CO was caused by an increased excretion of total glutathione, which was associated with an enhanced expression of Mrp2. In addition, CO induced NO production; the inhibition of NO reduced the total glutathione excretion and diminished the choleretic effect of CO. NO, therefore, plays a role in the mechanism of CO-induced choleresis.

Sano et al, using zinc protoporphyrin IX to inhibit heme oxygenase (HO) and thereby abolishing CO in the venous perfusate, reported that bile acid-dependent bile output was increased by CO suppression.[4] Their results indicated that CO exerted a negative effect on bile formation.[4] However, two studies demonstrated that CO enhanced biliary output.[5,6] The controversies relating to the effect of CO on bile excretion may be attributed to the fact that CO regulates bile excretion biphasically in a dose-dependent manner. CO causes choleresis at a concentration of 4 μM, while higher concentrations of CO increase paracellular junctional permeability, decrease the

biliary excretion of bile acids, and abolish the choleretic effect of CO.[5] Our findings were consistent with those reports that CO induces choleresis at physiologically relevant concentrations.[5,6]

Bile is formed primarily by osmotic filtration resulting from active transport of biliary solutes.[16] Among the biliary contents, bile acids,[13] GSH (oxidized or reduced),[17] and perhaps bicarbonate[18] meet the criteria. HO catalyzes the degradation of heme to yield biliverdin, iron, and CO.[19] By intraperitoneal injection of hemin to induce HO-1, Shintani et al demonstrated that CO suppressed hydrogen sulfide generation and stimulated biliary output and bicarbonate excretion in mice.[6] However, by transportal administration of 4 μM of CO to perfused rat liver, Norimizu et al showed that CO increased biliary excretion of GSH and resulted in choleresis.[5]

Several factors may underlie the controversial results from the CO studies. First, the amount of CO induced by HO-1 is unknown, and CO has been shown to act differently on biliary excretion at different concentrations.[5] Second, the induction of HO-1 increases the concentrations of biliverdin, iron, and CO; the effect of CO alone on bile excretion is difficult to assess. Third, different species of animal (rats vs. mice) or experimental models (*in vivo* vs. *ex vivo*) were used and may cause variations among studies. Instead of HO-1, the present study used DCM to generate CO specifically, and the results indicate that the choleretic effect of CO on rats was caused by an increase in biliary total glutathione excretion.

GSH is mainly synthesized in the liver, and is excreted into bile in both its reduced and its oxidized forms.[17] A high-affinity electrogenic canalicular carrier and MRP2 that actively export both oxidized and reduced GSH into bile have been functionally characterized.[20,21] Our study showed that CO enhanced biliary output by increasing Mrp2 expression in rat liver, and hence biliary excretion of GSH. This finding was in accordance with that of Norimizu et al demonstrating that the choleretic effect of CO was abolished in mice genetically deficient for Mrp2.[5]

The gas molecules CO and NO are linked in that NO upregulates HO-1 expression and leads to the formation of endogenous CO,[22] whereas CO induces NO production through upregulation of nuclear factor-κB and iNOS.[23] Exogenous NO has been reported to induce choleresis in perfused rat liver by stimulating GSSG excretion, which is independent of guanosine 3′,5′-cyclic monophosphate.[7] Our study showed that CO induced hepatic iNOS expression and elevated biliary nitrate/nitrite levels. Although the expression of Mrp2 was mildly attenuated, the GSH excretion as well as the choleretic effect of CO was completely abolished by NOS inhibition. Infusion of nonspecific NO inhibitor reduces NO production and decreases hepatic perfusion, indicating that NO has a role in the liver microcirculation.[24] Other than the effects on Mrp2 expression and GSH excretion in our study, the reduced hepatic perfusion after the injection of nonspecific NOS inhibitor demonstrated that NO may have additional effects on the reduction of bile output. Despite the complex effects of CO and NO, our findings suggest that NO contributes at least in part to the CO-induced choleresis.

In addition to the liver, the exogenous application of low doses of CO exerts protective effects on the lung, intestine, blood vessels, heart, and kidney.[2] *In vivo* studies, using animal models similar to ours, are able to investigate the systemic effects of CO and evaluate the effect of CO on bile excretion under specific physiologic conditions. We examined sequential changes in bile output and biliary contents in the same rat before and after CO administration instead of observing changes in different rats at fixed intervals. Our results, therefore, may be more relevant to investigations of the actions of CO on biliary excretion than previous studies have been.

As the armamentarium of CO-releasing molecules now continues to grow and expand, CO has become easier and safer to administer. Our results indicate that CO would be a therapeutic strategy for cholestatic diseases caused by down-regulation of Mrp2, such as inflammation-induced cholestasis.[25] However, further studies are needed to reveal how CO acts with NO on bile excretion and how CO upregulates Mrp2. In conclusion, our study demonstrates that CO upregulates hepatic Mrp2 expression and induces NO formation. In conjunction with the action of NO, CO increases biliary excretion of GSH to produce the choleretic effect.

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