A Single Intraperitoneal Dose of Carbon Monoxide-Saturated Ringer’s Lactate Solution Ameliorates Postoperative Ileus in Mice

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ABSTRACT

Treatment with inhaled carbon monoxide (CO) has been shown to ameliorate bowel dysmotility caused by surgical manipulation of the gut in experimental animals. We hypothesized that administration of CO dissolved in lactated Ringer’s solution (CO-LR) might provide similar protection to that observed with the inhaled gas while obviating some of its inherent problems. Postoperative gut dysmotility (ileus) was induced in mice by surgical manipulation of the small intestine. Some mice were treated with a single intraperitoneal dose of CO-LR immediately after the surgical procedure, whereas other mice received only the LR vehicle. Twenty-four hours later, intestinal transit of a nonabsorbable marker (70-kDa fluorescein isothiocyanate-labeled dextran) was delayed in mice subjected to intestinal manipulation but not the sham procedure. Gut manipulation also was associated with increased expression within the muscularis propria of transcripts for interleukin-1β, cyclooxygenase-2, inducible nitric-oxide synthase, intracellular adhesion molecule-1, and Toll-like receptor-4, as well as infiltration of the muscularis propria with polymorphonuclear leukocytes and activation of mitogen-activated protein kinases and nuclear factor-κB. All of these effects were attenuated by treatment with CO-LR. The salutary effect of CO-LR on gut motility, as well as many of the anti-inflammatory effects of CO-LR, was diminished by treatment with a soluble guanylyl cyclase (sGC) inhibitor, suggesting that the effects of CO are mediated via activation of sGC. These data support the view that a single intraperitoneal dose of CO-LR ameliorates postoperative ileus in mice by inhibiting the inflammatory response in the gut wall, possibly in a sGC-dependent fashion.

A transient episode of ileus, defined as impaired propulsive bowel motility, is common after abdominal surgery (Livingston and Passaro, 1990). However, the development of ileus can contribute to discomfort during the postoperative period as a result of abdominal distention, nausea, and emesis. In some instances, postoperative ileus can lead to more serious complications, including acute gastric dilatation, pulmonary aspiration, respiratory compromise, cardiac arrhythmias, anastomotic dehiscence, or intestinal perforation.

The mechanism(s) responsible for the development of postoperative ileus are not completely understood. However, simple manipulation of the stomach, intestine, or colon is sufficient to cause a period of gut dysmotility, and inflammation within the smooth muscle coats of the bowel (Kalff et al., 1998, 2003) and activation of the nonadrenergic noncholinergic neuronal pathway (De Winter et al., 1997) and activation of the nonadrenergic noncholinergic neuronal pathway (De Winter et al., 1997) have been identified as potential mediators of ileus.

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ABBREVIATIONS: CO, carbon monoxide; CO-LR, saturated solution of carbon monoxide in Ringer’s lactate solution; COHb, carboxyhemoglobin; SM, surgical manipulation; COX-2, cyclooxygenase-2; EMSA, electrophoretic mobility shift assay; FD70, fluorescein isothiocyanate-labeled dextran with an average molecular mass of 70 kDa; GC, geometric center; GI, gastrointestinal; HO-1, heme oxygenase-1; ICAM-1, intracellular adhesion molecule-1; iNOS, inducible nitric-oxide synthase; MCP-1, monocyte chemoattractant protein-1; LR, Ringer’s lactate solution; sGC, soluble guanylyl cyclase; MAPK, mitogen-activated protein kinase; MPO, myeloperoxidase; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; RT-PCR, reverse transcription-polymerase chain reaction; ERK, extracellular signal-regulated kinase; NF-κB, nuclear factor-κB; TLR-4, Toll-like receptor-4; JNK, c-Jun NH2-terminal kinase; LPS, lipopolysaccharide; t, total; IL, interleukin; UW, University of Wisconsin; tyrphostin AG 126, α-cyano-(3-hydroxy-4-nitro)cinnaminitrile.
implicated as being pathophysiologically important. Because of the clinical importance of ileus, numerous pharmacological strategies to ameliorate this problem have been investigated (Wolff et al., 2004; Delaney et al., 2005).

Carbon monoxide (CO) is an invisible colorless and odorless gas. CO is commonly regarded as a poison, because inhalation of relatively high concentrations of the gas can interfere with the delivery of oxygen to cells. However, mammalian cells generate CO endogenously from the degradation of heme in a reaction catalyzed by various isoforms of the enzyme heme oxygenase (Wu and Wang, 2005). CO serves as a signaling molecule, mainly by activating the enzyme soluble guanylyl cyclase (sGC) (Pilz and Casteel, 2003; Wu and Wang, 2005). Stressful stimuli can lead to increased expression of heme oxygenase-1, an inducible enzyme, promoting the formation of several products with cytoprotective, anti-inflammatory, and/or antioxidant properties, including bilirubin, biliverdin, and CO.

During the past few years, inhaled CO has been shown to provide therapeutic benefit in numerous different animal models of disease. For example, exposure to an atmosphere containing a relatively low concentration of CO has been shown to improve survival in mice challenged with a lethal dose of lipopolysaccharide (Saeaday et al., 2004), prevent multiple system organ dysfunction after hemorrhagic shock in mice (Zuckerbraun et al., 2005), ameliorate pulmonary fibrosis in mice treated with the chemotherapeutic agent bleomycin (Zhou et al., 2005), and protect solid organ grafts against ischemia/reperfusion injury or graft dysfunction after transplantation in rodents (Nakao et al., 2003, 2005; Neto et al., 2006). Negative results with inhaled CO also have been reported previously (Clayton et al., 2001; Ghosh et al., 2005).

Recently, Moore et al. (2003, 2005) reported that inhalation of CO prevents the development of postoperative ileus in both rodents and swine. Although these results support the view that CO might have therapeutic potential for preventing or ameliorating ileus after abdominal operations, translating these results into the clinic might be difficult because of the practical problems associated with scavenging and monitoring levels of a potentially toxic gas. To develop a more practical means of delivering the gas, we hypothesized that transitory inhalation of relatively high concentrations of the gas might be therapeutic. Herein, we present data showing that manipulation of the small intestine in a standardized fashion promoted development of postoperative ileus in mice, but this effect was ameliorated when the open peritoneal cavity was filled briefly with Ringer's lactate solution (LR) saturated with CO.

**Materials and Methods**

**Animals and Materials.** The research protocol complied with the regulations regarding animal care as published by the National Institutes of Health and was approved by the Institutional Animal Use and Care Committee of the University of Pittsburgh. Male C57BL/6 mice weighing 20 to 25 g (Jackson Laboratories, Bar Harbor, ME) were used in this study. The animals were maintained at the University of Pittsburgh Animal Research Center with a 12-h light/dark cycle and had free access to standard laboratory feed and water. Animals were not fasted before the experiments. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

**Surgery.** Ileus was induced in mice by gentle surgical manipulation of the small intestine using a slightly modified version of the procedure as described previously (Kalfa et al., 1998). In brief, under general anesthesia induced with sodium pentobarbital (90 mg/kg i.m.), a midline laparotomy was carried out under sterile conditions. The small intestine was carefully compressed along its length from the duodenal-jejunal junction to the ileocecal junction using a rolling motion with two cotton-tipped applicators. To ensure even manipulation in all sections of the small intestine, this procedure was repeated three times. After completion of the standardized gut manipulation procedure, the intestine was returned to the peritoneal cavity, and the incision was closed in two layers using running 4-0 silk suture. All surgeries were performed in the morning.

**Preparation of CO Solutions.** CO gas (0.1–100%; Praxair, Inc., Danbury, CT) was bubbled into LR in a 15-ml plastic tube for 5 min at room temperature. LR equilibrated with CO (CO-LR) was kept in a tightly capped tube without a gas layer.

**Measurement of Dissolved CO Concentration.** The concentrations of dissolved CO in LR were determined using a CO analyzer (Taiyo Instruments, Inc., Osaka, Japan), as described previously (Nakao et al., 2006). Samples (0.5 ml) of the LR previously equilibrated with different concentrations of CO gas were transferred to a 10-ml vacuum vial, and then 1 ml of the headspace gas was transferred to the analytical instrument for determination of CO content.

**Carboxyhemoglobin Measurements.** To determine the effect of peritoneal lavage with LR solution equilibrated with 100% CO on circulating concentrations of carboxyhemoglobin (COHb), mice were injected i.p. with 1.5 ml of CO-LR. Heparinized arterial blood samples (0.2 ml) were at indicated time points, and blood COHb levels were measured using an OSM3 hemoximeter (Radiometer, Copenhagen, Denmark).

**Experimental Design.** Just after induction of ileus (or the sham procedure) as described above, the peritoneal cavity was filled with 1.5 ml of either LR or CO-LR followed by closure of the abdomen. Six groups of mice were examined (sample sizes are shown in the legends for the figures). Mice in the Sham/LR group were subjected to general anesthesia and celiotomy but not gut manipulation. These mice were treated with LR before closure of the abdominal incision. Mice in the Sham/CO group were subjected to the sham procedure and treated with CO-LR. Mice in the SM/LR group were subjected to surgical manipulation and treated with LR before closure of the incision. Mice in the SM/CO group were subjected to surgical manipulation and treated with CO-LR. Animals in the Sham/LR group were treated the same as those in the Sham/LR group, except that these mice were pretreated with ODQ (20 mg/kg i.p.) 30 min before operation. Likewise, animals in the SM/LR/ODQ group were treated the same as those in the SM/CO group, except that these mice were pretreated with ODQ (20 mg/kg i.p.) 30 min before operation. Changes in intestinal motility (see below) were assessed 24 h after gut manipulation or the sham procedure.

**Determination of Intestinal Motility.** To determine the effect of surgical manipulation of the small intestine on gut motility, we measured the aboral transit of a nonabsorbable tracer fluorescein isothiocyanate-labeled dextran with an average molecular mass of 70 kDa (FD70), as described previously (Harada et al., 2005). In brief, mice were gavaged with 200 μl of FD70 dissolved in distilled water (2.5 mg/ml). Ninety minutes later, the animals were killed with an overdose of sodium pentobarbital. The entire gastrointestinal tract from stomach to distal colon was excised and divided into 14 segments: stomach, small intestine (divided into 10 segments of equal length), cecum, and colon (two segments of equal length). The luminal content of each segment was collected into a small tube and suspended in 1 ml of distilled water. The samples were mixed vigorously and then clarified by centrifugation. The supernatants were
collected and fluorometrically assayed for FD70 concentration. The transit of FD70 along the gastrointestinal tract was summarized by calculating the geometric center (GC) for the distribution of the fluorescent signal per segment × segment number/100 (Miller et al., 1981). Samples used for the measurement of gastrointestinal (GI) transit were not processed for any other evaluations.

Immunohistochemistry for Myeloperoxidase. Mid-segments of the small bowel were immersed in Krebs-Ringer buffer in a Sylgard glass dish with a silicone gel bottom. The gut segments were longitudinally opened along the mesenteric border and fastened down at the surrounding edges with micropins. The secured tissues were fixed in 100% ethanol for 10 min, and mucosa and submucosa were then removed carefully from the muscularis under a microscope. After washing with Krebs-Ringer buffer, the tissue was treated with Harker-Yates reagent for detection of polymorphonuclear neutrophils exhibiting myeloperoxidase (MPO) activity. Tissues were mounted on glass slides using GelMount (Biomedica Corp., Foster City, CA), overslipped, and inspected by light microscopy (Nikon FXA; Fryer, Huntley, IL) at a magnification of 200×. The number of MPO-polymorphonuclear leukocytes infiltrating the muscularis externa was counted in a blind manner by J. Schmidt and B. Stoffels from five randomly selected optical fields.

SYBR Green Real-Time Reverse Transcriptase-Polymerase Chain Reaction. Segments of small intestine from the ligament of Treitz ligament to the terminal ileum were cut into 5-cm lengths and pinned in a dissecting dish. The muscularis propria was isolated from the mucosa and submucosa by slitting the intestine over a glass rod and stripping the muscularis from the intestinal mucosa circumferentially with moist cotton applicators. The isolated intestinal muscularis propria was snap-frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted from the intestinal muscularis 6 or 24 h after surgery using the TRIzol reagent (Life Technologies, Inc., Grand Island, NY) according to the manufacturer’s instruction. The mRNAs for IL-1β, IL-6, IL-10, inducible nitric-oxide synthase (iNOS), intracellular adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein-1 (MCP-1), heme oxygenase (HO)-1, Toll-like receptor (TLR)-4, TLR-9, and β-actin were quantified in duplicate by SYBR Green two-step real-time reverse transcriptase-polymerase chain reaction (RT-PCR) as described previously (Nakao et al., 2003).

Western Blotting. Protein taken from snap-frozen samples of the intestinal muscularis 6 h after manipulation was assessed by Western blotting, as described previously (Nakao et al., 2005). Phosphorylated (p-) p38 mitogen-activated protein kinase (MAPK), p-c-Jun N-terminal kinase (JNK), p-extracellular signal-regulated kinase (ERK)1/2, and total (t-) p38, t-JNK, and t-ERK1/2 were detected using commercially available antibodies. The band intensities were quantified by NIH image analysis software (National Institutes of Health, Bethesda, MD).

Electrophoretic Mobility Shift Assay. NF-κB DNA binding activity was measured by electrophoretic mobility shift assay (EMSA) using nuclear extracts from intestinal muscularis obtained 6 h after surgical manipulation. The NF-κB oligonucleotide (Promega, Madison, WI) was based on the NF-κB sequence in the immunoglobulin light-chain enhancer. DNA probes were prepared by end-labeling with [γ-32P]ATP (PerkinElmer, Wellesley, MA) and T4 polynucleotide kinase (Boehringer-Mannheim Biomedical Products, Mannheim, Germany) and purified in Tris-EDTA buffer containing NaCl (100 mM) using G-50 resin columns (Whatman, Newton, MA). Typically, 5 µl (5–10 µg) of nuclear extract was incubated with 100,000 counts/min of [32P]-labeled oligonucleotides (0.5 ng) for 1 to 2 h at room temperature in a buffer containing 10 mM Tris, pH 7.6, 10% glycerol, 1 mM EDTA, 1 mg/ml bovine serum albumin, and 0.2% Nonidet P-40. Protein-DNA complexes were resolved on 4% nondenaturing polyacrylamide gels in 0.4× running buffer containing 450 mM Tris borate and 1 mM EDTA, pH 8.0. Gels were dried after electrophoresis and subjected to autoradiography. The band intensities were quantified by NIH image analysis software.

Statistical Methods. Results are presented as means ± S.E. The data were analyzed using Student’s t test or analysis of variance followed by Fisher’s least significant difference test as appropriate. The p values < 0.05 were considered significant.

Results

CO Content of LR Solutions. We measured the content of CO in the solutions prepared by bubbling LR with CO gas mixtures containing from 0.1 to 100% CO at 20°C. When LR was equilibrated with 100% CO, the solution contained approximately 1200 µM CO (Fig. 1A).

Effect of CO-LR on Blood COHb Content. We sought to determine whether i.p. administration of CO-LR leads to systemic absorption of CO. Accordingly, mice (n = 4) were injected with 1.5 ml of CO-LR, and circulating COHb levels were measured at various time points thereafter. COHb lev-

**Fig. 1.** A and B, solubility of CO in LR (A) and sequential changes of COHb concentration in arterial blood after administration of CO-LR to mice (B). The content of CO in LR was measured after the solution was bubbled for 5 min with graded concentrations of CO. Arterial COHb concentrations were determined at various time points after three normal mice were injected i.p. with 1.5 ml of CO-LR.
els in blood rapidly increased to almost 8% at 5 min after i.p. injection of CO-LR but decreased to less than 4% within 30 min and continued to gradually decrease to the baseline level by 120 min (Fig. 1B).

**CO-LR Prevents Postsurgical Ileus.** Motility was assessed 24 h after mice were subjected to surgical manipulation of the intestine or the sham procedure. In both the Sham/LR and Sham/CO groups, FD70 was rapidly transported aborally, such that the peak signal was in the distal ileum (Fig. 2). In contrast, in the SM/LR group (i.e., mice subjected to gut manipulation but treated only with LR), the distribution of FD70 was shifted toward the more proximal segments of the gastrointestinal tract, a finding that is consistent with the presence of postsurgical ileus. However, if the mice subjected to surgical manipulation were treated immediately after the procedure with a single i.p. dose of CO-LR, the distribution of the fluorescent tracer 24 h later was not different from the distribution observed in sham-operated mice. The GC is the weighted distribution of the FD70 marker along the GI tract. This parameter is a sensitive and reliable measurement of GI transit (Miller et al., 1981). Intestinal transit, as assessed by calculating the mean GC, was significantly delayed in the SM/LR group compared with the sham group. In contrast, the mean GC for the SM/CO group compared with the SM/LR group was located significantly more distally, a finding that is consistent with the view that treatment with CO-LR ameliorated gut dysmotility induced by surgical manipulation of the intestine (Fig. 3).

Many of the pharmacological effects of CO are mediated by activation of the enzyme sGC that catalyzes the conversion of guanosine triphosphate to the second messenger cGMP (Nakao et al., 2003). Accordingly, we studied two additional groups in an effort to determine whether the sGC/cGMP pathway was involved in the salutary effect of CO-LR on the development of postsurgical ileus. In these groups, the animals were pretreated before surgical manipulation with ODQ, a selective sGC inhibitor. In mice subjected to the sham procedure and treated with LR...
CO-LR decreases leukocytic infiltration of the muscularis propria. Kalff et al. previously reported that surgical manipulation of the gut in rodents (Kalff et al., 1999) or man (Kalff et al., 2003) is associated with subsequent leukocytic infiltration into the muscularis propria. Moreover, anti-inflammatory agents that decrease the extent of leukocytic infiltration into the muscularis propria after surgical manipulation of the gut have been shown to ameliorate post-surgical ileus in rodents (Turler et al., 2002). Accordingly, we sought to determine whether a single i.p. dose of CO-LR would affect leukocytic infiltration into the smooth muscle layers of the small intestine 24 h after surgical manipulation of the gut. The whole-mounts from sham-operated animals contained few MPO-positive cells and monocytes regardless of whether the mice were treated with LR, CO-LR, or LR and ODQ (Fig. 4). Surgical manipulation was associated with extensive leukocytic infiltration, but this effect was significantly decreased when the animals were treated with CO-LR. Pretreatment of mice with ODQ before treatment with CO-LR (SM/CO/ODQ group) abrogated the inhibition of leukocytic infiltration that was observed in the SM/CO group.

CO-LR ameliorates the expression of proinflammatory mediators in the muscularis propria. Previous studies have shown that surgical manipulation of the small intestine is associated with increased expression of a number of proinflammatory gene products within the muscularis propria, including IL-6, iNOS (Harada et al., 2005), cyclooxygenase (COX)-2 (Schwarz et al., 2001), and MCP-1 (Turler et al., 2002). Therefore, we performed studies to determine whether a single i.p. dose of CO-LR is sufficient to modulate the molecular inflammatory response to surgical manipulation of the gut. We used real-time quantitative RT-PCR to measure the expression of several transcripts in samples of isolated ileal muscularis propria obtained from mice that were subjected 6 h earlier to either surgical manipulation or the sham procedure. This time point was determined to be optimal for detecting changes in the expression of inflammation-related transcript levels based on previous data (Moore et al., 2003). Relative to sham-operated controls, surgical manipulation followed by treatment with LR resulted in significant up-regulation of mRNA levels for several proinflammatory proteins, namely IL-1β, IL-6, COX-2, iNOS, ICAM-1, MCP-1, TLR-4, and TLR-9 (Figs. 5 and 6). Treatment with CO-LR effectively down-regulated the expression of IL-1β, COX-2, iNOS, ICAM-1, and TLR-4 but failed to inhibit the increased expression of MCP-1, IL-6, and TLR-9. For example, 6 h after surgical manipulation, relative mRNA levels for IL-1β and ICAM-1 mRNA expression after surgical manipulation was significantly reduced to 63- and 13-fold, respectively, by CO-LR treatment. Twenty-four hours after surgery in the SM/LR group, IL-1β and ICAM-1 mRNA levels were induced by less than 12- and 4-fold, respectively, relative to the levels for these transcripts measured in normal intestine. Induction of IL-1β and ICAM-1 mRNA expression after surgical manipulation was significantly reduced to 63- and 13-fold, respectively, by CO-LR treatment. Twenty-four hours after surgery in the SM/LR group, IL-1β and ICAM-1 mRNA levels were induced by less than 12- and 4-fold, respectively, relative to the levels for these transcripts measured in normal intestine. At this time point (24 h after surgical manipulation), there were no differences in the levels of IL-1β and ICAM-1 mRNA between the SM/LR and SM/CO groups. The down-regulation of postsurgical IL-1α, iNOS, COX-2, ICAM-1, and TLR-4 induction was not observed when mice were pretreated with the sGC inhibitor ODQ.

We also examined changes in the expression of two key anti-inflammatory gene products, namely IL-10 and HO-1. Transcripts for both of these genes were significantly up-regulated 6 h after surgical manipulation and treatment with LR (Fig. 6). Although treatment with CO-LR did not alter HO-1 mRNA expression, administration of CO-LR significantly increased IL-10 mRNA expression in the muscularis propria after surgical manipulation. The effect of CO-LR on IL-10 mRNA up-regulation was partially reversed by pretreatment with ODQ.

Treatment with CO-LR inhibits activation of NF-κB, ERK, and JNK MAPK. Ileus induced by surgical manipulation of the gut has been associated with activation of the proinflammatory transcription factor NF-κB within intestinal muscle tissue (Schwarz et al., 2002). Therefore, we sought to determine whether treatment with CO-LR would be able to block activation of this signaling pathway, as well as activation of other key proinflammatory signaling pathways (ERK, JNK, and p38 MAPKs). Samples of midintestinal smooth muscle tissue were obtained 6 h after surgical manipulation or the sham procedure. As expected, increased NF-κB DNA binding activity as detected by EMSA was observed in the SM/LR group (Fig. 7, A and B). Furthermore, we observed increased phosphorylation of ERK, JNK, and p38 MAPK after surgical manipulation of the gut in mice treated with LR (Fig. 7, C and D). Treatment with a single i.p. dose of CO-LR significantly down-regulated the activation of the NF-κB, ERK, and JNK signaling pathways but did not affect phosphorylation of p38 MAPK. Pretreatment with ODQ attenuated the effect of CO-LR on the activation of NF-κB and the phosphorylation of ERK.

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Fig. 3. Geometric center for the distribution along the GI tract of FD70 90 min after enteral administration. GI transit data are presented as the mean ± S.E. geometric center for the distribution of FD70 90 min after enteral administration of the tracer. Higher numbers indicate better progression of the FD70 tracer into the distal alimentary tract.
Discussion

Previously, a series of reports of the cytoprotective effects of CO generated considerable interest in the notion of using CO as a therapeutic agent to prevent the development of postoperative ileus (Moore et al., 2003, 2005). However, CO is a toxic gas that interferes with the oxygen-carrying capacity of the blood. Hence, using inhaled CO as a therapeutic agent before, during, and/or after surgical procedures would be a formidable undertaking, requiring the use of a closed circuit ventilation system that would minimize or remove the risk of contaminating the operating theater with the gas. In addition, careful monitoring of the administered CO concentration would be required, as would the monitoring of circulating COHb levels in patients. Finally, in the animal studies of CO for the prevention of ileus that have been reported so far, gas was administered for a prolonged period (3 h before anesthesia and surgery in one study and 1 h before and continuously for 24 h after surgery in the other studies) (Moore et al., 2003, 2005). Treating patients with CO for such prolonged periods would impose major logistical problems for most hospitals, further decreasing the attractiveness of this therapeutic strategy.

Rather than providing the gas by inhalation, another approach for using CO as a therapeutic agent was described recently (Nakao et al., 2006). Rather than administering CO by inhalation, these investigators dissolved the gas in an organ preservation solution [University of Wisconsin (UW) solution], which is commonly used for ex vivo cold preservation of the donor small intestine before transplantation. Preservation of the intestine in UW solution...
tion containing dissolved CO compared with control UW solution was associated with reduced small intestinal vascular resistance, improved graft blood flow, and improved graft mucosal barrier function. Prompted by these intriguing results and recognizing that most surgeons irrigate the peritoneal cavity with 1 or 2 liters of crystalloid solution at the conclusion of an open abdominal operation, we hypothesized that CO could be dissolved in LR and administered by lavaging the open peritoneal cavity with this solution after a standardized gut manipulation procedure. As our results indicate, this approach was quite effective in preventing the development of ileus 24 h after standardized surgical manipulation of the intestine. Indeed, a single dose of CO-LR at the conclusion of the surgical procedure can be added to the compendium of pharmacological agents that have been shown to ameliorate experimental postoperative ileus, such as ethyl pyruvate (Harada et al., 2005), α-cyano-(3-hydroxy-4-nitro)cinnamonic acid (tryphostin AG 126) (Moore et al., 2004), and the highly selective COX-2 inhibitor 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone (Schwarz et al., 2001).

CO is capable of hyperpolarizing enteric smooth muscle cells (Farrugia et al., 1998). In the noninflamed intestine, CO is produced primarily by HO-2, which is constitutively expressed in enteric neurons and in interstitial cells of Cajal. HO-2-dependent CO production seems to be crucial for the maintenance of normal gastrointestinal motility (Chen et al., 2002; Piotrowska et al., 2003). However, treatment with CO-LR did not accelerate the aboral transit of the FD70 marker in mice not subjected to surgical manipulation (i.e., the Sham/CO group). These findings are consistent with previously reported results regarding the effects of inhaled CO on intestinal motility in animals not subjected to surgical manipulation of the gut (Moore et al., 2003, 2005). Thus, we believe that it is unlikely that the therapeutic benefit of CO-LR observed in the present study reflects a direct effect of...
the dissolved gas on intestinal smooth muscle function and motility.

Inflammation involving the smooth muscle coats of the bowel has been implicated in the pathogenesis of ileus after surgical manipulation of the gastrointestinal tract (Schwarz et al., 2002). In this context, it is noteworthy that treatment with a single intraperitoneal dose of CO-LR at the conclusion of a standardized surgical procedure ameliorated the development of inflammation in the muscularis propria of the small intestine, as evidenced by significant decreases in the expression of transcripts for several proinflammatory proteins (COX-2, iNOS, and ICAM-1) and a significant increase in the expression of the transcript for a key anti-inflammatory cytokine IL-10. Treatment with this solution also significantly decreased the accumulation of leukocyte (MPO-positive) cells within the muscularis propria 24 h after the standardized surgical procedure.

TLRs are responsible for the ability of cells to respond to variety of microbial products. TLR-4 is the receptor responsible for cellular responses to lipopolysaccharide (Medzhitov et al., 1997). More recently, TLR-4-dependent signaling has been implicated in cellular responses to the proinflammatory cytokine-like molecule, high-mobility group box 1 (Park et al., 2004), as well as hepatic injury caused by ischemia and reperfusion (Tsung et al., 2005). Herein, we showed that surgical manipulation of the gut is sufficient to promote marked up-regulation of TLR-4 expression in the gut. Moreover, we showed that treatment with CO-LR significantly blunted this effect. This anti-inflammatory effect of CO-LR was reversed by pharmacological inhibition of sGC by pre-treatment with ODQ. We previously demonstrated the potential mechanistic involvement of TLR-4 in postoperative ileus by visually showing the leakage of particles from the postoperative colon and by altering the preoperative gut flora using orally administered polymyxin B and neomycin and by using C3H/HeJ (TLR-4-deficient and LPS-resistant) mice (Schwarz et al., 2002; Turler et al., 2006). As shown in these two studies, both gut decontamination and defective TLR-4-dependent signaling resulted in significantly less postoperative smooth muscle motor functional impairment. Interestingly, intestinal expression of TLR-9, which is responsible for cellular activation in response to bacterial DNA (Hemmi et al., 2000), was also up-regulated by surgical manipulation of the gut, but this effect was not modulated by treatment with CO-LR. Our data are insufficient to shed any light on why the regulation of these two genes was differentially affected by CO-LR treatment.

The biochemical basis for the anti-inflammatory effects of CO remains very poorly understood. In mice challenged with LPS, the effect of inhaled CO on the activation of the proinflammatory transcription factor NF-κB reportedly are tissue-specific; in the lung, CO inhibits LPS-induced NF-κB DNA binding, whereas in the liver, CO augments LPS-induced NF-κB DNA binding (Sarady et al., 2004). In our study, treatment with CO-LR inhibited intestinal NF-κB activation as assessed by EMSA, at least at the time point that was examined (6 h after surgical manipulation). At this same time point, treatment with CO-LR also inhibited activation of the proinflammatory MAPKs, ERK, and JNK. This finding is consistent with some prior
studies of the effects of CO on JNK-dependent signaling (Morse et al., 2003) but inconsistent with others (Ning et al., 2005). As it seems to be the case for the effect of CO on NF-κB-dependent signaling, the effect of CO on JNK activation may be cell- and/or tissue-specific. The mechanisms whereby CO modulates activation of NF-κB, ERK, or JNK remain to be elucidated. Nevertheless, because pretreatment with ODQ prevented the beneficial effects of CO-LR on gut motility and CO-induced alterations in molecular inflammatory events after surgical manipulation, our results are consistent with the idea that the salutary effects of intraperitoneal CO-LR are mediated via activation of sGC/cGMP pathway.

Exogenously supplied nitric oxide, which similar to CO binds to heme and activates sGC, has been shown to slow GI transit and decrease smooth muscle contractile activity via an sGC-dependent mechanism (Zyromski et al., 2001). Endogenously generated iNOS-derived nitric oxide has been implicated as being important in the pathogenesis of ileus after surgical manipulation (Turler et al., 2006) or hemorrhagic shock (Hierholzer et al., 2004) in rodents. In the present study, i.p. treatment with CO-LR did not affect...
GI transit in sham-operated animals, presumably because any sGC/cGMP-mediated inhibitory effects of CO on gut motility are relatively transient, and mice were treated with a single dose of CO-LR 24 h before the measurement of GI transit. In the model of postoperative ileus used for the studies reported here, impaired GI transit is a consequence of various inflammatory processes, including the recruitment of leukocytes into the muscularis propria and increased expression or activation of various proinflammatory mediator factors, such as cytokines, chemokines, and adhesion molecules. We believe that the salutary effect of a single i.p. dose of CO-LR on postsurgical GI motility was related to inhibition of inflammation involving the smooth muscle layers of the intestine. Since pretreatment with ODQ reversed the beneficial action of CO-LR on postsurgical ileus, as well as many of the anti-inflammatory effects of CO-LR, it is plausible that the sGC/cGMP pathway is important for these pharmacological effects. This notion is supported by recent data showing that cGMP-dependent signaling down-regulates leukocyte recruitment in vivo (Ahlawalia et al., 2004).

Despite its tantalizing potential as a therapeutic agent, translating the use of CO from the laboratory to the clinical arena has been a formidable challenge. Being a well known toxic agent, administration of CO to patients by inhalation will require careful measures to minimize the risk of environmental contamination and careful monitoring of the inhaled CO concentration and circulating levels of COHb. Blood COHb levels correlate with acute clinical symptoms. In normal healthy adults, COHb levels range between 0.4 and 3%. COHb levels of 10 to 30% can cause headache, shortness of breadth, and dizziness, and higher levels (30–50%) are associated with severe headache, vomiting, syncope, and cardiopulmonary dysrhythmias (Von Burg, 1999). Using CO dissolved in LR obviates the problems associated with scavenging a toxic gas. Furthermore, in our studies, a single i.p. dose of CO-LR was sufficient to provide a therapeutic effect without any observed adverse effects. The administration a single dose of CO-LR increased circulating COHb levels. However, the peak level (7.35%) was comparable with the level observed in cigarette smoking healthy volunteers (6 ± 1%) (Zevin et al., 2001). Therefore, this is level of CO exposure is probably safe. However, early careful clinical trials documenting the safety of intraperitoneal CO-LR administration will be necessary.

Based on the results presented here, we believe that a careful clinical evaluation of CO-LR for the prevention of postoperative ileus may be warranted.

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