Title: A single intraperitoneal dose of carbon monoxide-saturated Ringer’s lactate solution ameliorates post-operative ileus in mice

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Intraperitoneal CO Solution Prevents Ileus

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CO, carbon monoxide; CO-LR, saturated solution of carbon monoxide in Ringer’s lactate solution; COHb, carboxyhemoglobin; COX-2, cyclo-oxygenase-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; MPO, myeloperoxidase; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PMN, polymorphonuclear leukocyte; RT-PCR, reverse transcription polymerase chain reaction; TLR-4, Toll-like receptor-4;
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 its inherent problems. Post-operative 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 h later, intestinal transit of a nonabsorbable marker (70 kDa FITC-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 IL-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 post-operative ileus in mice, by inhibiting the inflammatory response in the gut wall induced by surgical manipulation, possibly in a sGC-dependent fashion.
Introduction

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 post-operative period as a result of abdominal distention, nausea, and emesis. In some instances, post-operative 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 post-operative ileus are not completely understood. Simple manipulation of the stomach, intestine or colon, however, is sufficient to cause a period of gut dysmotility, and inflammation within the smooth muscle coats of the bowel (Kalff et al., 1998; Kalff et al., 2003) as well as activation of the nonadrenergic noncholinergic neuronal pathway (De Winter et al., 1997) have been 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 anti-oxidant 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 (Sarady 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; Nakao et al., 2005; Neto et al., 2006). Negative results with inhaled CO also have been reported (Clayton et al., 2001; Ghosh et al., 2005).

Recently, Moore and colleagues reported that inhalation of CO prevents the development of post-operative ileus in both rodents and swine (Moore et al., 2003; Moore et al., 2005). 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. In order to develop a more practical means of delivering the gas, we hypothesized that peritoneal lavage with a solution containing dissolved CO might be therapeutic. Herein, we present data, showing that manipulation of the small intestine in a standardized fashion promoted development of post-operative 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 20–25 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 prior to 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 (Kalff et al., 1998). Briefly, 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 spread out onto a gauze sponge moistened with 0.9% saline. The small intestine was gently 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 is 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, 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 was determined using a TRIlyzer (Taiyo, Osaka, Japan), as previously described (Nakao et al., 2006). Samples (0.5 mL) of the LR previously equilibrated with different concentrations of CO of 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 (COHb) measurements. To determine the effect of peritoneal lavage with Ringer’s lactate solution equilibrated with 100% CO (CO-LR) on circulating concentrations of 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 prior to 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 prior to 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/ODQ group were treated the same as those in the Sham/LR group, except these mice were pretreated with 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one (ODQ; 20 mg/kg i.p.) 30 min before operation. Similarly, animals in the SM/CO/ODQ group were treated the same as those in the SM/CO group, except 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 non-absorbable tracer, fluorescein isothiocyanate-labeled dextran with an average molecular mass of 70 kDa (FD70), as previously described (Harada et al., 2005). Briefly, 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, 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 fluorescein-labeled dextran using the following formula; GC=\( \Sigma (\% \text{ of total fluorescent signal per} \)
segment x 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 (MPO).** 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 micro-pins. 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 KRB, the tissue was treated with Hanker-Yates reagent for detection of polymorphonuclear neutrophils exhibiting myeloperoxidase (MPO) activity. Tissues were mounted on glass slides using Gel/MountTM (Biomedia Corp., Foster City, CA), coverslipped and inspected by light microscopy (Nikon FXA, Fryer, Huntley, IL) at a magnification of 200X. The number of MPO-PMNs infiltrating the muscularis externa were counted in a blind manner by two of the authors (JS and BS) from five randomly selected optical fields.

**SYBR green real-time reverse transcriptase polymerase chain reaction (RT-PCR).**

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 slipping 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.
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 RT-PCR as previously described (Nakao et al., 2003).

**Western blotting.** Protein taken from snap-frozen samples of the intestinal muscularis 6 h after manipulation were analyzed by Western blotting, as previously described (Nakao et al., 2005a; Nakao et al., 2005b). Phosphorylated (p-) p38 mitogen-activated protein kinase (MAPK), p-c-Jun NH2-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 Institute of Health, Bethesda, MD).

**Electrophoretic mobility shift assay (EMSA).** NF-κB DNA binding activity was measured by 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]dATP (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 were incubated with 100,000 counts/min of 32P-
labeled oligonucleotides (0.5 ng) for 1-2 h at room temperature in a buffer containing 10 mM Tris (pH 7.6), 10% glycerol, 1 mM EDTA, 1 mg/ml BSA, 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 ± standard error (SE). The data were analyzed using student-t test or analysis of variance (ANOVA) followed by Fisher’s LSD test, as appropriate. *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 µmol/L of CO (Figure 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 levels 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 (Figure 1B).

**CO-LR prevents post-surgical 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 (Figures 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 post-surgical ileus. If the mice subjected to surgical manipulation were treated immediately after the procedure with a single i.p. dose of CO-LR, however, the distribution of the fluorescent tracer 24 h later was not different from the distribution observed in sham-operated mice. The
geometric center (GC) is the weighted distribution of the FD70 marker along the GI gastrointestinal 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 as compared to the sham group. In contrast, the mean GC for the SM/CO group as compared to 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 (Figure 3).

Many of the pharmacological effects of CO are mediated by activation of the enzyme, soluble guanylyl cyclase (sGC), that catalyzes the conversion of guanosine triphosphate to the second messenger, cyclic guanosine monophosphate (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 post-surgical ileus. In these groups, the animals were pre-treated prior to surgical manipulation with ODQ, a selective sGC inhibitor. In mice subjected to the sham procedure and treated with LR (Sham/LR/ODQ), pre-treatment with the sGC inhibitor did not affect gut motility (Figures 2 and 3). However, in mice subjected to surgical manipulation and treated with CO-LR and pre-treated with ODQ (SM/CO/ODQ), GI transit was impaired and not different from that observed in the SM/LR group.

**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 following surgical manipulation of the gut have been shown to ameliorate post-surgical ileus in rodents (Turler et al., 2002). Accordingly, we sought to determine if 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 (Figure 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 prior to 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 pro-inflammatory 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 pro-inflammatory gene products within the *muscularis propria*, including IL-6, iNOS (Harada et al., 2005), cyclo-oxygenase (COX)-2 (Schwarz et al., 2001), and MCP-1 (Turler et al., 2002). Therefore, we performed studies to determine if 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 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 transcripts level based on the previous data (Moore et al., 2003). Relative to sham-operated controls, surgical manipulation followed by treatment with LR resulted in significant
upregulation of mRNA levels for several pro-inflammatory proteins, namely: IL-1β, IL-6, COX-2, iNOS, ICAM-1, MCP-1, TLR-4 and TLR-9 (Figures 5 and 6). Treatment with CO-LR effectively down-regulated 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 in the SM/LR group increased 179- and 24-fold, respectively, relative to the levels of these transcripts measured in normal intestine. Induction of IL-1β and ICAM-1 mRNA expression following surgical manipulation was significantly reduced to 63- and 13-fold, respectively, by CO-LR treatment. Twenty-four h after surgery in the SM/LR group, IL-1β and ICAM-1 mRNA levels were induced by less than 12-fold 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 post-surgical IL-1β, iNOS, COX-2, ICAM-1 and TLR-4 induction was not observed when mice were pre-treated 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 (Figure 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 following surgical manipulation. The effect of CO-LR on IL-10 mRNA up-regulation was partially reversed by pre-treatment with ODQ.
Treatment with CO-LR inhibits activation of NF-κB, ERK and JNK mitogen activated protein kinases (MAPK). Ileus induced by surgical manipulation of the gut has been associated with activation of the pro-inflammatory 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 pro-inflammatory signaling pathways (ERK, JNK and p38 MAPKs). Samples of mid-intestinal 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 (Figure 7A, B). Additionally, we observed increased phosphorylation of ERK, JNK and p38 MAPK after surgical manipulation of the gut in mice treated with LR (Figure 7C, 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. Pre-treatment with ODQ attenuated the effect of CO-LR on the activation of NF-κB and the phosphorylation of ERK.
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 post-operative ileus (Moore et al., 2003; Moore et al., 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. Additionally, careful monitoring of the administered CO concentration would be required, as would 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, the 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; Moore et al., 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 recently described (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 \textit{ex vivo} cold preservation of the donor small intestine prior to transplantation. Preservation of the intestine in UW solution containing dissolved CO compared to 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 a liter or two 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), tyrphostin 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 non-inflammed 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; Moore et al., 2005). Thus, we believe 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 pro-inflammatory 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 leukocytes (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. TLR4 is the receptor responsible for cellular responses to lipopolysaccharide (Medzhitov et al., 1997). More recently, TLR4-dependent signaling has been implicated in cellular responses to the pro-inflammatory 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 TLR4 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 TLR4 in postoperative ileus by visually showing the leakage of particles from the postoperative colon and by altering the pre-operative gut flora, using orally administered polymyxin B and neomycin and by using C3H/HeJ (TLR4-deficient and LPS-resistant) mice (Schwarz et al., 2002; Turler et al., 2006). As shown in these two studies, both gut decontamination and defective TLR4-dependent signaling resulted in significantly less postoperative smooth muscle motor functional impairment. Interestingly, intestinal expression
of TLR9, 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 pro-inflammatory 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 pro-inflammatory 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 appears 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, since pre-treatment 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 (NO), which like 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 NO 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 prior to the measurement of GI transit. In the model of post-surgical 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 pro-inflammatory transcription factors, cytokines, chemokines and adhesion molecules. We believe that the salutary effect of a single i.p. dose of CO-LR on post-surgical 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 post-surgical 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 (Ahluwalia 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, as well as 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 10-30% can cause headache, shortness of breath, and dizziness, and higher levels (30-50%) are
associated with severe headache, vomiting, syncope, and cardiac 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 to the level observed in cigarette smoking healthy volunteers (6±1%) (Zevin et al., 2001). Therefore, this is level of CO exposure is likely to be 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 post-operative ileus may be warranted.
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References


Footnotes

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*These authors are contributed equally to this work.
Legends for Figures

Figure 1. Solubility of CO in LR (A) and sequential changes of carboxyhemoglobin (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.

Figure 2. Distribution along the GI tract of FD70 90 min after enteral administration in normal animals and mice subjected to bowel manipulation. In the Sham/LR group (n=6) and the Sham/CO group (n=6), most of the fluorescent marker accumulated in the last two segments of the small bowel (sb) and cecum (c). Surgical manipulation (SM/LR, n=6) caused significant delay in the transit of the fluorescence. In contrast, SM/CO animals (n=6) treated with CO-LR, most of the fluorescent marker accumulated in distal segments of the small bowel. Although ODQ did not alter the transit in the Sham/LR animals (n=3), ODQ reversed the anti-ileus effects of CO in SM/CO/ODQ (n=3). The results are presented as histograms, showing the mean ± SE percentage of the total dose of administered FD70 in the stomach (st), 10 segments of small intestine from proximal to distal (sb1 through sb10), the cecum, the right half of the colon (col1) and the left half of the colon (col2).

Figure 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 ± SE 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. (* indicates p<0.05 for the contrast between SM/CO and SM/LR, # indicates p<0.05 between SM/CO and SM/CO/ODQ).

**Figure 4. Effect of surgical manipulation and CO-LR on infiltration of the smooth muscle coats of the small intestine by myeloperoxidase (MPO)-containing inflammatory cells.** The muscularis propria was extensively infiltrated by MPO-positive cells 24 h post-operatively in the SM group, although very few positive cells were found in whole mounts from sham-operated mice or sham-operated mice treated with CO-LR. Treatment with CO-LR reduced the number of infiltrating cells after surgical manipulation. Pre-treatment with ODQ prior to administration of CO-LR blocked the anti-inflammatory effects of CO. Represented images are depicted in Panel A, whereas Panel B depicts the mean ± SE number of MPO-positive cells per high powered field for each of the treatment groups (n=4-6 per condition; * indicates p<0.05 for the contrast between SM/CO and SM/LR, # indicates p<0.05 for the contrast between SM/CO and SM/CO/ODQ).

**Figure 5. Effect of surgical manipulation and CO-LR on the expression of transcripts for inflammatory mediators in the muscularis propria of the small intestine.** The groups (n=6 each) are the same as in Figure 2 although different animals were used for these studies. Samples of intestine were obtained 6 h after surgical manipulation or the sham procedure. Quantitative real-time RT-PCR was used to determine mRNA levels. (* indicates p<0.05 for the contrast between SM/CO and SM/LR, # indicates p<0.05 for the contrast between SM/CO and SM/CO/ODQ).
Figure 6. Effect of surgical manipulation and CO-LR on the expression of transcripts for IL-10, HO-1, TLR4 and TLR9 in the muscularis propria of the small intestine. The groups (n=6 each) are the same as in Figure 2 although different animals were used for these studies. Samples of intestine were obtained 6 h after surgical manipulation or the sham procedure. Quantitative real-time RT-PCR was used to determine mRNA levels. (* indicates p<0.05 for the contrast between SM/CO and SM/LR, # indicates p<0.05 for the contrast between SM/CO and SM/CO/ODQ).

Figure 7. Effect of surgical manipulation and CO-LR on NF-κB DNA binding as assessed by EMSA (A, B) and MAPKs phosphorylation as assessed by Western blotting (C, D) in the muscularis propria of the small intestine. The groups (n=6 each) are the same as in Figure 2 although different animals were used for these studies. Samples of intestine were obtained 6 h after surgical manipulation or the sham procedure. The panels A and C demonstrated representative pictures from 3 independent experiments. Arrow shows NF-κB binding. The panels B and D depicts mean ± SE relative band intensities; (* indicates p<0.05 for the contrast between SM/CO and SM/LR, # indicates p<0.05 for the contrast between SM/CO and SM/CO/ODQ).
Figure 1

A. CO solubility (µmol/L)

B. COHb (%) vs. minutes after ip injection
Figure 2

% Distribution of marker

Sham/LR

SM/LR

Sham/CO

SM/CO

Sham/LR/ODQ

SM/CO/ODQ
Figure 3
Figure 4

A.

Sham LR  Sham CO  Sham LR ODQ

Sham CO  Sham LR ODQ

SM LR  SM CO  SM LR ODQ

B.

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* #
Figure 5

IL-1β

iNOS

IL-6

ICAM-1

COX-2

MCP-1

Fold increase vs normal intestine

Sham

LR

CO

ODQ

SM

LR

CO

ODQ

LR

CO

ODQ

LR

CO

ODQ

Sham

SM
Figure 6

IL-10

Fold increase vs normal intestine

HO-1

TLR4

TLR9

Sham

LR CO LR CO LR CO

SM

LR CO LR CO LR CO

ODQ

Sham

LR CO LR CO LR CO

SM

LR CO LR CO LR CO
Figure 7AB

A.

![Image of Western blot showing NFκB expression across different conditions: Sham/LR, Sham/CO, Sham/LR/ODQ, SM/LR, SM/CO, cold comp., and Free probe mutant.]

B.

Bar chart showing band intensity (vs sham/LR) for different conditions: Sham/LR, Sham/CO, Sham/LR/ODQ, SM/LR, SM/CO, and SM/CO/ODQ. The chart includes error bars and indicates statistical significance with symbols * and #.
Figure 7C

C.

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Figure 7D

D.

ERK

Ratio of band intensity (p-/t-)

JNK

p38

Sham/LR
Sham/CO
Sham/LR/ODQ
SM/LR
SM/CO
SM/CO/ODQ