Carbon monoxide protects against the development of experimental necrotizing enterocolitis

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Zuckerbraun, Brian S., Leo E. Otterbein, Patricia Boyle, Ronald Jaffe, Jeffrey Upperman, Ruben Zamora, and Henri R. Ford. Carbon monoxide protects against the development of experimental necrotizing enterocolitis. Am J Physiol Gastrointest Liver Physiol 289: G607–G613, 2005. First published May 12, 2005; doi:10.1152/ajpgi.00055.2005.—Necrotizing enterocolitis (NEC) is a disease of neonates that is increasing in incidence and often results in significant morbidity and mortality. Carbon monoxide (CO), a by-product of the catabolism of heme, is known to have anti-inflammatory and antiapoptotic properties. In this study, we aimed to demonstrate that inhaled CO protects against the development of intestinal inflammation in a model of experimental NEC as well as decreases enterocyte cell death in vitro. Additionally, we also aimed to demonstrate that CO decreases enterocyte production of inducible nitric oxide synthase (iNOS) and nitric oxide (NO). Neonatal rats were exposed to intermittent hypoxia exposure and formula feeding to induce experimental NEC. Animals randomized to CO treatment were put in an environment containing 0.025% CO for 1 h/day on days 1–3 of life. All animals were killed on day 4 of life. In vitro experiments were performed with IEC-6 cells, a rat enterocyte cell line. Cells were examined for viability, iNOS production, and elaboration of NO. We found that CO diminished levels of serum inflammatory cytokines and nitrites, protected against intestinal inflammation, and decreased ileal iNOS production and protein nitration in a model of experimental NEC. In vitro, CO decreased cytokine- or hypoxia/endotoxin-induced iNOS and NO production. CO also abrogated TNF-α and actinomycin D-induced apoptosis or hypoxia/endotoxin-induced cell death. In conclusion, 1 h of daily low-dose inhaled CO protected against the development of intestinal inflammation in a model of experimental NEC. iNOS and NO production were decreased by CO both in vivo and in vitro. CO may prove to be a useful clinical adjunct in the treatment of NEC.

Heme oxygenase; nitric oxide

NECROTIZING ENTEROCOLITIS (NEC) is a disease that affects the gastrointestinal tract of premature infants and is characterized by varying degrees of tissue necrosis that results in intestinal barrier failure. NEC is often complicated by the development of systemic sepsis and multisystem organ dysfunction, which, in part, account for the significant fatality rate (10–50%) associated with the disease (17, 35). Although the etiology of NEC is multifactorial, central to its development is the complex and intricate interaction between the intestinal microbial flora, the immature intestinal epithelial barrier, and the host defense mechanisms (17, 35).

Mild mucosal damage resulting from perinatal hypoxia or infection may contribute to the initial insult to the intestinal epithelium that permits bacteria to breach the gut barrier and incite a local inflammatory response (18). The cellular and molecular pathways involved in the pathogenesis of NEC have been partially elucidated. Animal models and human specimens have shown that NEC is associated with upregulation of proinflammatory molecules, such as platelet activating factor (7), IL-8 (27, 39), and cyclooxygenase-2 (8), as well as concurrent downregulation of anti-inflammatory mediators such as IL-11 and IL-12 (26, 27). Therapeutic deliveries of trophic agents such as erythropoietin, epidermal growth factor, and hepatocyte growth factor to the intestine have been demonstrated to be protective (1, 10, 21). Preventive strategies, including pharmacological delivery of immunoglobulins (11, 20), antibiotic prophylaxis (37), and postnatal steroids (16), have shown some success in clinical trials, presumably by modulating the intestinal microbial flora, the epithelial barrier, or the host immune response.

Upregulation of inducible nitric oxide synthase (iNOS) and sustained overproduction of nitric oxide (NO) have been implicated in the intestinal mucosal injury associated with NEC (15, 26) and other intestinal diseases, including inflammatory bowel disease (23, 34, 36). Previous studies from our laboratory (15, 26) have demonstrated increased intestinal iNOS mRNA and protein expression in infants with NEC as well as in experimental animal models of NEC. Moreover, LPS-induced bacterial translocation has been shown to be ameliorated by scavenging NO (9) and is significantly reduced in iNOS knockout mice (22).

Heme oxygenase-1 (HO-1) is the rate-limiting enzyme in the breakdown of heme to free iron, biliverdin, and carbon monoxide (CO) (31). HO-1 is induced by cellular stresses, including hypoxia, LPS, reactive oxygen species, and NO. Previous studies have demonstrated that HO-1 is upregulated in the intestine following ischemia-reperfusion and that inhibition of HO-1 activity exacerbates intestinal injury (38). The by-products of heme degradation by HO-1 have been shown to mediate the protective properties of this enzyme. Potent antioxidant functions have been attributed to biliverdin, which is converted to bilirubin (2), and ferritin, which is upregulated after the release of free iron (14). Others, as well as our group, have shown that CO can inhibit apoptosis and inflammation (6, 30, 42). The purpose of our investigations was to test the hypoth-
esis that exogenous CO delivery reduces intestinal inflammation in an experimental model of NEC, decreases intestinal iNOS protein levels, and protects against subsequent enterocyte cell death.

MATERIALS AND METHODS

Collection of human intestinal specimens. The Human Rights Committee of Children’s Hospital of Pittsburgh approved collection of operative specimens for experimental purposes. Intestinal specimens were collected and snap frozen at the time of operation.

Animal model of necrotizing enterocolitis. The Institutional Animal Care and Use Committee of the University of Pittsburgh School of Medicine and Children’s Hospital of Pittsburgh approved the experimental protocol. Pregnant time-dated Sprague-Dawley rats were induced at term using subcutaneous injection of Pitocin (1 U). Immediately after birth (day 0), the neonates were weighed and randomized into two groups. Animals were either left with their mothers and thus breast fed or separated from their mothers, housed in an incubator (Ohio Medical Products, Madison, WI), subjected to 10 min of hypoxia three times daily (5% O₂–95% N₂; Prax Air, Pittsburgh, PA), and gavage fed with a special rodent formula two times daily (3, 26). Rats from each group were further randomized to receive no additional treatment or 1 l/day of CO treatment [250 parts per million (ppm) days 1–3]. The neonatal rats were killed on day 4. The intestines were inspected by a blinded reviewer for the presence of gross necrotic changes or Pneumatosis intestinalis. The last 2 cm of terminal ileum were harvested for morphological studies, and mucosal scrapings were collected for protein analysis.

CO exposure. CO at a concentration of 1% (10,000 ppm) in compressed air was mixed with balanced air (21% oxygen) as described previously (30). CO concentration for cell and animal exposure was maintained at 250 ppm at all times. For cell culture experiments, the system is similar except that the gas mixtures used also contained 5% CO₂ and the chambers were humidified and maintained at 37°C. Flows into the chambers were at a rate of 12 and 2 l/min for the animal and cell chambers, respectively. A CO analyzer (Interscan, Chatsworth, CA) was used to measure CO levels in the chambers, and there were no fluctuations in the CO concentrations after the chamber had equilibrated.

Morphological studies. Intestinal specimens were harvested as described previously (26). Hematoxylin and eosin slides were prepared as per standard protocol and examined by light microscopy. The presence of morphological changes in the intestinal epithelium, including separation of the villous core, submucosal edema, and epithelial sloughing, was determined by a pathologist in a blinded fashion. Additionally, mucosal injury was graded by assignment of a Chiu score ranging from 0 to 5.

Determination of serum nitrate levels. Total serum nitrate and nitrate levels were measured by the Griess reaction following cadmium-based conversion of nitrates to nitrites as described previously (40).

Serum cytokine determination. Serum was collected for determination of rat TNF-α and IL-1β levels using Quantikine ELISA (R&D Systems, Minneapolis, MN) per manufacturer’s instructions.

Cell culture. The rat small-intestinal epithelial cell line, IEC-6, was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in DMEM with 4.5 g/l glucose (Bio-Whittaker, Walkersville, MD) supplemented with 5% fetal bovine serum, 0.02 mM glutamine (GIBCO, Grand Island, NY), 0.1 U/ml insulin, and 100 U/ml penicillin-100 μg/ml streptomycin at 37°C and 10% CO₂. Cells from passages 3 through 20 were used for experiments. CO was delivered as described previously (30).

Viability. Cell viability was determined by measuring ATP levels (CellTiter-Glo; Promega) as per the manufacturer’s protocol.

Western blot analysis. IEC-6 cells or ileal mucosal scrapings were collected in lysis buffer containing 20 mMol/l Tris with 100 μmol/l PMSF (Sigma), 1 μmol/l leupeptin (Sigma), and 1 μmol/l sodium orthovanadate (Sigma). Protein was quantified by bicinchoninic acid protein assay (Pierce, Rockford, IL). Lysates (30 μg) were subjected to SDS-PAGE (43). Immunoblotting was performed with mouse anti-HO-1 (Calbiochem; 1:1,000), rabbit anti-iNOS (BD Transduction; 1:1,000), or rabbit anti-nitrosotyrosine (Alexis; 1:500) followed by horseradish peroxidase-linked goat anti-rabbit or goat anti-mouse antibodies (1:10,000; Pierce).

Reporter assay. pGL3/2 rat iNOS promoter-luciferase reporter (gift from J. Pfeilshifter; Frankfurt, Germany) was constructed as described previously (5). IEC-6 cells grown in 35-mm wells were transfected with 4 μl Lipofectamine-2000 (Invitrogen), 0.15 μg pGL3/2 DNA, and 0.5 μg pEFP-Luc Z DNA, which was used as a control for transfection efficiency. Twenty-four hours after transfection, cells were treated for 6 h, lysates were collected, and luciferase assay (Promega) was performed with a luminometer (Berthold).

Cell culture nitrite determination. Nitrite was measured in the culture medium using the Griess method as described previously (9, 33).

Statistical analysis. Results are expressed as means ± SE. Differences among groups were analyzed with one-way ANOVA with Student-Newman-Keuls post hoc test for all pairwise comparisons (SigmaStat; SPSS, Chicago, IL). Statistical significance was accepted at P < 0.05.

RESULTS

Intestinal HO-1 protein is increased in NEC. Human intestinal specimens from patients with acute NEC and control intestinal specimens from patients with noninflammatory conditions (stoma closure) were analyzed for expression of HO-1. Whole cell lysates from NEC specimens demonstrated increased expression of HO-1 compared with control samples (Fig. 1A). We also sought to determine whether intestinal HO-1 protein levels would be increased in experimental NEC. Neonatal rats were randomized to breast feeding ad libitum or to intermittent hypoxia and formula feeding as described above. All animals were killed on day 4 of life, and terminal ileal mucosa was collected and analyzed by Western blotting. Previous studies from our laboratory have shown that neonatal rats subjected to hypoxia that were breast fed do not develop intestinal inflammation. HO-1 protein expression was increased in the hypoxia plus formula-fed group compared with breast-fed control animals (Fig. 1B). This is consistent with the presence of morphological changes in the intestinal epithelium, including separation of the villous core, submucosal edema, and epithelial sloughing, was determined by a pathologist in a blinded fashion. Additionally, mucosal injury was graded by assignment of a Chiu score ranging from 0 to 5.

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![Fig. 1. Intestinal heme oxygenase-1 (HO-1) is upregulated in necrotizing enterocolitis (NEC). A: mucosal protein was extracted from intestinal specimens from human neonates with acute NEC or controls without inflammatory disease (non-NEC) and subjected to Western blot analysis for HO-1 protein. β-Actin served as an internal control (n = 3/group). B: mucosal protein was extracted from neonatal rats subjected to breast feeding or to hypoxia and formula feeding at day 4 (n = 4/group).](Image)
previous findings in the intestine and other organs that illustrate upregulation of HO-1 following a variety of injuries.

**CO decreases mortality and protects against the development of intestinal inflammation induced by hypoxia and formula feeding.** Based on the demonstration that HO-1 protein is increased in experimental NEC and that HO-1 and CO have been shown to be cytoprotective, we hypothesized that delivery of a low dose of inhaled CO could protect against the development of intestinal inflammation in a model of experimental NEC. Neonatal rats from each group were randomized to receive either 1 h of CO (250 ppm) per day on days 1–3 of life or no additional therapy. There was a mortality rate of 40% (12/30 animals) before death on day 4 of life in neonatal rat pups exposed to hypoxia and formula feeding. The mortality rate in hypoxia plus formula-fed randomized rat pups treated with CO was 21.4% (6/28 pups; P < 0.05 compared with untreated hypoxia plus formula-fed rats). There was no mortality in breast-fed rats in either the nontreated or CO-treated groups by the time of death. All surviving animals were killed on day 4 of life. Gross pathological characteristics including *Pneumatosis intestinalis* and necrosis (Table 1) as well as morphological changes consistent with experimental NEC were identified in the hypoxia plus formula-fed group compared with breast-fed controls (Fig. 2). CO treatment had no effect on gross or microscopic evaluation of terminal ilea from breast-fed animals and significantly protected against the development of epithelial injury in the hypoxia plus formula-fed group (Table 1).

**CO decreases systemic markers of inflammation.** At the time of death, plasma was collected for determination of TNF-α and IL-1β levels as systemic markers of inflammation. Levels of both TNF-α and IL-1β were increased by 33.2- and 18.5-fold, respectively, in the hypoxia plus formula-fed group compared with breast-fed controls (Fig. 3; P < 0.05). CO significantly blunted these increases, resulting in only 3- and 2.5-fold increases in TNF-α and IL-1β, respectively (P < 0.05). These findings suggest that exogenous CO can attenuate systemic inflammation in this animal model.

**CO inhibits generation of intestinal iNOS.** Our previous investigations have demonstrated that iNOS is increased in intestinal specimens from neonates with acute NEC as well as in the intestines of rats in our experimental model of NEC. Induction of iNOS and NO generation are believed to directly contribute to tissue damage via formation of reactive nitrogen species. Western blot analysis of ileal mucosal samples from rats subjected to hypoxia plus formula-fed shows increased levels of iNOS and protein nitration (Fig. 4A). Nitration or nitrotyrosine formation is a marker for nitrosative and oxidative stress, as well as peroxynitrite formation, a potent oxidant formed by the reaction of NO with superoxide. Administration of CO abrogated iNOS expression and protein nitration in the hypoxia plus formula-fed groups, suggesting that CO may be protective in the intestines by decreasing or preventing the generation of NO. Serum nitrite plus nitrate levels correlated to the changes in iNOS protein levels seen in the ileal lysates (Fig. 4B).

**HO-1 and/or CO inhibits IEC-6 cell death.** Given the protective effects of CO in our animal model, we sought to determine whether induction of HO-1 or exogenous CO could inhibit enterocyte cell death in vitro. The rat intestinal epithelial cell line IEC-6 was utilized, and apoptosis was induced by treatment with TNF-α (10 ng/ml) plus actinomycin D (ActD; 200 ng/ml). Cell viability was analyzed by crystal violet staining of adherent cells and assessment of cellular ATP content. TNF-α plus ActD decreased IEC-6 cell viability to 24 ± 2.1% of that observed in untreated controls as determined by ATP content (Fig. 5; P < 0.05). CO significantly inhibited the TNF-α- and ActD-induced cell death, resulting in 54 ± 5.6% viability compared with untreated controls (P < 0.05).

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**Table 1. Pathological changes in terminal ileum of surviving neonatal rats on day 4 of life**

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**Fig. 2. Carbon monoxide (CO) improves intestinal injury in a model of experimental NEC.** Representative hematoxylin and eosin staining images of ileum from breast-fed (A and B) and hypoxia plus formula-fed (C and D) neonatal rats on day 4 of life are shown. Animals were randomized to receive no additional treatment (A and C) or received inhaled CO (250 parts per million [ppm]) for 1 h/day on days 1–3 of life (B and D). Ileum from hypoxia and formula-fed rats (C) exhibits architectural changes, including villous atrophy and cellular vacuolization; however, CO treatment (D) improved these changes. All micrographs are ×40 magnification.

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CO alone had no measurable effect on viability of IEC-6 cells in vitro. Induction of HO-1 by cobalt protoporphyrin had similar protective effects. Additionally, CO diminished IEC-6 cell death induced by LPS or hypoxia (1% oxygen). This is primarily thought to result in cell death via necrosis as opposed to apoptosis. Of note, CO did not prevent IEC-6 cell death caused by direct exposure to peroxynitrite (data not shown).

**CO prevents iNOS upregulation and/or NO generation in vitro.** Given that iNOS and NO have been shown to be injurious to the intestines in multiple in vivo models and CO was able to decrease both iNOS and protein nitration in experimental NEC, as well as serum nitrite levels, we hypothesized that CO could inhibit the upregulation of iNOS in intestinal epithelial cells in vitro. The effects of LPS and/or 1% oxygen (hypoxia) were investigated on iNOS protein by Western blotting. LPS and hypoxia were chosen to be an in vitro mixture (TNF-α, IL-1β, interferon-γ) that is known to upregulate iNOS. Cytokine stimulation increased iNOS protein (Fig. 6C) as well as nitrite levels to 17.2 ± 0.9 μM compared with 1.4 ± 0.3 μM in unstimulated controls (P < 0.01; Fig. 6D). CO and cobalt protoporphyrin significantly suppressed this cytokine effect, resulting in nitrite levels of 9.8 ± 0.7 and 10.4 ± 1.0 μM, respectively (P < 0.05).
Griess assay. Cytokine stimulation increased nitrite to 17.2 μM compared with 10.4 ± 1.0 μM in unstimulated controls (P < 0.05). CO and CoPP significantly inhibited this cytokine effect, resulting in nitrite levels of 9.8 ± 0.7 and 10.4 ± 1.0 μM, respectively (P < 0.05 compared with CM-stimulated cells).

**DISCUSSION**

In this study, we report that HO-1 is upregulated in intestinal specimens from human neonates with acute NEC as well as in the ileal mucosa of neonatal rats in an experimental model of NEC. One hour of exogenous CO delivery per day protected animals against the development of intestinal inflammation in experimental NEC and decreased mortality rates in this model. These findings were associated with a decrease in systemic inflammation as evidenced by decreased serum TNF-α and IL-1β. The development of experimental NEC was also associated with increased ileal mucosal iNOS expression, protein nitration, and serum nitrites. CO therapy attenuated the increase in iNOS expression and in nitrosative stress. Furthermore, we report that induction of HO-1 or addition of exogenous CO protects IEC-6 cells from the cytopathic effects of TNF-α plus ActD or LPS and/or hypoxia in vitro. Additionally, CO prevented cytokine-induced iNOS upregulation and NO generation in IEC-6 cells in vitro.

HO-1 and its catalytic by-products, including CO, likely play a defensive role after intestinal injury. In this study, we demonstrate increased levels of HO-1 protein in the ileal mucosa of animals with NEC, which is consistent with previous studies in which intestinal HO-1 in models of ischemia-reperfusion (38) and hemorrhagic shock (4, 32) were increased. Presumably, the delay in increased HO-1 expression is insufficient to provide protection as the inflammatory sequelae have already been initiated. Induction of HO-1 either pharmacologically or by preconditioning is cytoprotective after intestinal transplantation and ischemia-reperfusion. Our data suggest that HO-1 upregulation in acute NEC may be a compensatory mechanism to counteract the degree of intestinal inflammation by producing local endogenous CO within the intestinal epithelium. Of note, HO-1 may also be protective by other mechanisms, including the generation of biliverdin or via its role as a transcription factor.

The most striking finding in these studies is that 1 h/day of inhalational CO therapy (250 ppm) was sufficient to prevent the development of NEC in our animal model. Moreover, the exposure to CO was initiated 1-day postpartum. CO is a known anti-inflammatory and antiapoptotic agent. Otterbein et al. (30) illustrated in RAW cells that CO acts as a potent anti-inflammatory agent by decreasing TNF-α and increasing IL-10. Other studies have highlighted the anti-apoptotic role of CO both in vitro and in vivo (6, 42). The protective effects of CO in this animal model of NEC are likely to be multifactorial. In this study, we demonstrate an amelioration of TNF-α- plus ActD-induced apoptosis as well as LPS- and/or hypoxia-induced IEC-6 cell death in vitro. As reported, CO therapy was also associated with decreased elaboration of both systemic and local inflammatory mediators. Additional studies are required to determine the mechanism by which CO exerts these effects in the NEC model.

iNOS and NO are associated with mucosal damage and gut barrier failure in inflammatory bowel disease, experimental ileitis, endotoxic shock, and NEC (9, 15, 22). Our data show that CO decreases the upregulation of iNOS both in vitro and
in vivo and suppresses nitrosotyrosine formation in vivo. Peroxynitrite, which is formed by the interaction of NO and superoxide, is believed to be the reactive nitrogen species responsible for the nitration of tyrosine residues in this model (9). One mechanism by which CO-mediated enterocyte protection may occur is via inhibition of iNOS protein upregulation, thereby leading to decreased NO and peroxynitrite generation. The reduction in protein nitration seen in the experimental NEC group treated with NEC was dramatic. The effects of CO may be more pronounced in the setting of concomitant inflammation and injury, to reduce both the activation of inflammatory cells and the subsequent stimulation of intestinal parenchymal cells. Furthermore, the effects of CO on total serum nitrite levels (nitrite plus reduced nitrate) likely represent a systemic effect on inflammatory cells. Even with the upregulation of iNOS under these inflammatory conditions in the intestines, the amount of NO produced would comprise only a minor portion of total serum nitrogen oxides measured.

Interestingly, within the gastrointestinal tract, HO-2 has been shown to be constitutively expressed in enteric neurons and in the interstitial cells of Cajal (12, 13). Moore et al. (25) illustrated that exogenous CO can prevent ileus associated with intestinal manipulation, and Nakao et al. (28) showed similar effects in a model of small intestinal transplantation. These observations suggest that, in addition to attenuating the local inflammatory milieu, CO may also prevent the development of intestinal injury by maintaining gastrointestinal motility, which in turn may prevent local bacterial overgrowth in the intestine. Altered intestinal microflora is known to contribute to the development of NEC. The influence of CO on intestinal motility and microbiological flora requires further investigation.

In conclusion, we have shown that 1 h/day of CO therapy protected formula-fed plus hypoxic neonatal rats from the development of experimental NEC, even when the exposure was initiated 1-day postpartum. The mechanism appears to involve the inhibition of iNOS expression and protein nitration. CO may be a clinically useful therapeutic adjunct for the prevention of NEC. Additional studies are essential to elucidate the mechanisms involved and to determine optimal timing and dosing of CO delivery.

GRANTS

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