Low-dose inhaled carbon monoxide attenuates the remote intestinal inflammatory response elicited by hindlimb ischemia-reperfusion

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1Medical Biophysics, 2Surgery, and 3Critical Care, University of Western Ontario; 4The Centre for Critical Illness Research, and Lawson Health Research Institute, London, Ontario, Canada; 5Immunobiology Research Center, Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts

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Scott JR, Cukiernik MA, Ott MC, Bihari A, Badhwar A, Gray DK, Harris KA, Parry NG, Potter RF. Low-dose inhaled carbon monoxide attenuates the remote intestinal inflammatory response elicited by hindlimb ischemia-reperfusion. Am J Physiol Gastrointest Liver Physiol 296: G9–G14, 2009. First published October 16, 2008; doi:10.1152/ajpgi.90243.2008.—Heme oxygenase (HO) represents the rate-limiting enzyme in the degradation of heme into carbon monoxide (CO), iron, and biliverdin. Recent evidence suggests that several of the beneficial properties of HO, may be linked to CO. The objectives of this study were to determine if low-dose inhaled CO reduces remote intestinal leukocyte recruitment, proinflammatory cytokine expression, and oxidative stress elicited by hindlimb ischemia-reperfusion (I/R). Male mice underwent 1 h of hindlimb ischemia, followed by 3 h of reperfusion. Throughout reperfusion, mice were exposed to AIR or AIR + CO (250 ppm). Following reperfusion, the distal ileum was exteriorized to assess the intestinal inflammatory response by quantifying leukocyte rolling and adhesion in submucosal postcapillary venules with the use of intravital microscopy. Ileum samples were also analyzed for proinflammatory cytokine expression [tumor necrosis factor (TNF)-α and interleukin (IL)-1β] and malondialdehyde (MDA) with the use of enzyme-linked immunosorbent assay and thiobarbituric acid reactive substances assays, respectively. I/R + AIR led to a significant decrease in leukocyte rolling velocity and a sevenfold increase in leukocyte adhesion. This was also accompanied by a significant 1.3-fold increase in ileum MDA and 2.3-fold increase in TNF-α expression. Treatment with AIR + CO led to a significant reduction in leukocyte recruitment and TNF-α expression elicited by I/R; however, MDA levels remained unchanged. Our data suggest that low-dose inhaled CO selectively attenuates the remote intestinal inflammatory response elicited by hindlimb I/R, yet does not provide protection against intestinal lipid peroxidation. CO may represent a novel anti-inflammatory therapeutic treatment to target remote organs following acute trauma and/or I/R injury.

carbon monoxide; inflammation; ischemia-reperfusion; systemic inflammatory response syndrome

IT IS GENERALLY ACCEPTED THAT injury to organs remote from a focus of infection or trauma is a major cause of morbidity and mortality in Intensive Care Units across the world. Such injury, in the absence of infection, results as a consequence of a whole body inflammatory response defined as the systemic inflammatory response syndrome (SIRS) (7). Several studies have demonstrated that the small intestine represents a particularly vulnerable organ during SIRS and that damage to this crucial barrier may lead to the development of multiple organ dysfunction syndrome (MODS) (4). However, despite extensive investigation, few clinical therapeutic options currently exist to prevent and/or alleviate the detrimental consequences of this response. Experimental evidence suggests that blocking key components of the inflammatory cascade, such as proinflammatory cytokine expression, leukocyte recruitment, and/or lipid peroxidation, may represent effective strategies to preserve the integrity of such organs during SIRS (3, 5, 6, 10, 12, 13, 23, 24, 31). Therefore, the discovery of novel therapeutics that mimic this protective phenotype may represent essential tools in the prevention of MODS.

There is a growing body of evidence suggesting that heme oxygenase-1 (HO-1) may represent an endogenous host defense mechanism with potent anti-inflammatory and antioxidant properties (2, 11, 14, 35, 36). HO-1 represents an inducible rate-limiting enzyme in the degradation of heme into carbon monoxide (CO), iron, and bilirubin and has been shown to be endogenously upregulated and potentially cytoprotective in response to various proinflammatory stimuli (1, 21, 33). Interestingly, recent experimental findings suggest that providing exogenous byproducts of the heme degradation pathway (i.e., CO and/or biliverdin/bilirubin) may mimic this protective phenotype and suppress the inflammatory response associated with various disease states such as, hyperoxia, organ transplantation, endotoxemia, and ischemia-reperfusion (I/R) injury (1, 19, 20, 22, 25–28). Our hypothesis is that low-dose inhaled CO may represent a novel gaseous therapy to target, not only the lung, but also the microcirculation of remote organs, like the small intestine to attenuate the inflammatory response. Thus, using a murine model of trauma-induced SIRS, our objectives were to determine if low-dose [250 parts/million (ppm)] inhaled CO provided protection to the small intestine by altering (1) leukocyte recruitment, (2) proinflammatory cytokine expression, (3) lipid peroxidation, and (4) microvascular perfusion deficits elicited by hindlimb I/R.

MATERIALS AND METHODS

Experimental protocol. This study was approved by the Animal Research Ethics Board of the University of Western Ontario and met the guidelines of the Canadian Council on Animal Care. Male C57Bl/6 mice (20–25 g) were randomized into the following groups before experimentation. Sham (no treatment in room air) (n = 5), Sham + CO (no treatment + 3 h exposure to CO) (n = 5), I/R (1 h of hindlimb ischemia + 3 h of reperfusion in room air) (n = 5), and

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was visualized under trans-illumination using a Nikon Eclipse TE200 bathed in warm 37°C saline solution. The intestinal microcirculation overhead heating lamp, and the exposed segment of the ileum was While on the microscope stage, animal temperature was monitored with plastic film (Dow Chemical, Paris, ON, Canada) to avoid midline incision, and mice were transferred to the microscope stage obtained via cardiac puncture following intravital microscopy were Measurements of carboxyhemoglobin (COHb) in blood samples obtained via cardiac puncture following intravital microscopy were determined by a hemoximeter by the blood gas laboratory at the London Health Sciences Centre.

Intravital microscopy. The abdomen was opened via a longitudinal midline incision, and mice were transferred to the microscope stage for placement in a left lateral position. Saline (0.9% NaCl)-soaked applicators were used to gently exteriorize a segment of the distal ileum on a mounted glass slide. The ileum was immediately covered with plastic film (Dow Chemical, Paris, ON, Canada) to avoid dehydration and exposure to ambient air and to minimize peristalsis. While on the microscope stage, animal temperature was monitored with a rectal temperature probe maintained at 37°C with the use of an overhead heating lamp, and the exposed segment of the ileum was bathed warm saline solution. The intestinal microcirculation was visualized under trans-illumination using a Nikon Eclipse TE200 inverted microscope (×40 objective), captured with a b/w camera (MTI VE1000), projected on a monitor (Panasonic WV-BM1410), and recorded with a videocassette recorder (Panasonic AG-1980) for offline analysis (final magnification: ×900). After isolation of the ileum, mice were allowed to equilibrate on the microscope stage for a period of 15 min. Following this stabilization period, four submucosal postcapillary venules/mouse were randomly selected and recorded for offline analysis of leukocyte-endothelial interactions and venular volumetric blood flow. Preparation in this manner allowed for the visualization of intestinal leukocyte and red blood cell flow without the use of fluorescent dyes. Great care was taken to ensure that all venules in all mice were of very similar diameter (~40 µm diameter).

Adhesion molecule antibody injection. Four additional groups of mice were also treated with an intraperitoneal injection of anti-intercellular adhesion molecule (ICAM)-1 monoclonal antibody (40 µg/mouse; BD Pharmingen) or isotype control antibody (IgG) (Sham + ICAM-1, I/R + ICAM-1, Sham + IgG, I/R + IgG) immediately following reperfusion (n = 5). As previously shown, this dose was effective for ICAM-1 immunoblockade (9). Intravital microscopy of ileal submucosal venules was performed as described above.

Offline analysis. Leukocyte rolling velocity, leukocyte adhesion, venular volumetric blood flow, and wall shear rate were all determined by offline video playback analysis. Leukocytes were quantified by defining their behavior along a 200-µm length of submucosal postcapillary venule. Leukocytes were classified as rolling if they were observed moving with a torsional motion along the endothelium, whereas those that remained stationary for 30 s were defined as adherent. Leukocyte rolling velocity and centerline venular blood flow velocity (V) were measured using the use of a custom offline video velocimeter (37). The internal diameter (D) of captured venule images was also measured using ImageJ software (National Institutes of Health). Both venular volumetric blood flow and wall shear rate were calculated using the following formulas: volumetric blood flow (Q) = \( \pi r^2 \); wall shear rate (\( \gamma \)) = 8 × [V/D]. A correction factor of 1.6 was applied to the centerline velocities estimate mean venular blood velocities. Thus the formulas become Q = \( (V/1.6)\pi r^2 \) and \( \gamma = 8 \times [(V/1.6)/D] \) for volumetric blood flow and shear rate, respectively.

Measurement of ileum TNF-α and IL-1β expression. Frozen ileum samples were homogenized in homogenizing buffer, and the homogenate was centrifuged at 1,000 g for 15 min at 4°C. The resulting supernatant was then analyzed for total protein concentration using the Bradford assay, and as well as the expression of tumor necrosis factor (TNF)-α and interleukin (IL)-1β using commercially available enzyme-linked immunosorbent assay kits (Biosource International, Camarillo, CA).

Measurement of ileum ICAM-1 protein expression. Frozen tissue samples were first homogenized and then centrifuged at 10,000 g for 20 min at 4°C. The protein concentration was estimated using the Bradford assay. Protein (20 g) was electrophoretically resolved on 12.5% polyacrylamide gel and transferred to a nitrocellulose membrane. The membranes were blocked in 5% nonfat dry milk with 0.1% Tween 20 for 1 h at room temperature and incubated with an anti-mouse ICAM-1 polyclonal antibody (1:1,000 dilution in 5% nonfat dry milk; BD Biosciences, Missauga, ON, Canada) for 2 h at room temperature. Membranes were washed with Tween-PBS and incubated in horseradish peroxidase-conjugated hamster IgG (1:1,000 dilution in 5 nonfat dry milk; Amersham Biosciences, Missauga, ON, Canada) for 1 h at room temperature. Blots were developed by enhanced chemiluminescence and visualized on radiograph film. The bands were analyzed using image densitometry and expressed as relative optical density per pixel.

Estimate of intestinal lipid peroxidation-thiobarbituric acid reactive substances assay. Whole homogenate from harvested ileum samples was analyzed for lipid peroxidation as an indicator of oxidative stress. Malondialdehyde (MDA) content was determined by using the OXitec thiobarbituric acid reactive substances assay kit (Zeptometrix, Buffalo, NY). Briefly, 100 µl of homogenate were added to a reaction mixture containing 100 µl 8.1% SDS and 2.5 ml of acetic acid/thiobarbituric acid. Samples were then boiled at 95°C for 1 h and subsequently centrifuged at 3,000 g for 15 min. The absorbance of the resulting supernatant was measured at 532 nm, compared with a MDA standard curve, and expressed as nanomoles per milligram protein.

Measurement of hematological parameters. All hematological parameters, including hemoglobin, hematocrit, mean corpuscular volume, platelets, and neutrophils, were analyzed from blood samples obtained via cardiac puncture immediately following intravital microscopy using a Beckman Coulter STKS Hematology Flow Cytometer.

Statistical analysis. Data are presented as means ± SE. ANOVA with the Student-Newman-Keuls post hoc test was used for multiple comparisons. Statistical significance was set at P < 0.05.
RESULTS

To determine if CO exposure led to an increase in CO concentration within the bloodstream, the levels of COHb were analyzed in blood samples obtained via cardiac puncture immediately following the intravital microscopy protocol. We demonstrate a six- to eightfold significant increase in COHb levels following Sham/CO (5.48 ± 0.98%) and I/R/CO (8.35 ± 1.10%) compared with Sham (0.92 ± 0.11%) and I/R (1.0 ± 0.12%), respectively (Fig. 1).

Intravital microscopy of submucosal postcapillary venules revealed that I/R led to a significant reduction in leukocyte rolling velocity (Fig. 2A) and 6.7-fold increase in venular leukocyte adhesion (Fig. 2B). I/R also led to a significant reduction in venular volumetric blood flow (Fig. 3A) and wall shear rate (Fig. 3B) compared with sham treatment. It should be noted that venules of similar diameter were selected in all animals, and, as such, there were no significant differences between groups (in μm: Sham: 36.9 ± 2.6, Sham + CO: 38.3 ± 1.6, I/R: 40.3 ± 1.1, I/R + CO: 36.6 ± 1.4). The increase in leukocyte adhesion following I/R was determined to be ICAM-1 dependent, since ICAM-1 immunoblockade led to a significant reduction in leukocyte adhesion, whereas IgG control antibody treatment did not (Fig. 4). Exposure to CO throughout reperfusion had a dramatic impact on ICAM-1-dependent leukocyte adhesion. This was evident as leukocyte adhesion and leukocyte rolling velocity were similar to sham levels with CO exposure throughout reperfusion. However, this change in leukocyte recruitment was determined to be independent of altered volumetric blood flow, since CO exposure in both sham and I/R mice did not significantly alter volumetric flow and wall shear rate in submucosal postcapillary venules (Fig. 3).

Hematological analysis of blood samples following intravital microscopy revealed a significant increase in hemoglobin, hematocrit, and neutrophils following I/R, with a significant decrease in circulating platelets. Interestingly, I/R + CO resulted in a trend toward an increase in circulating neutrophils compared with I/R mice; however, this was not significant.
Sham mice also showed a significant reduction in circulating platelets following CO exposure, yet there was not a further decrease in I/R + CO mice (Table 1).

Analysis of harvested ileum samples revealed that I/R led to a significant increase in intestinal proinflammatory cytokine expression (Fig. 5) and lipid peroxidation (Fig. 6), as indicated by elevated tissue TNF-α and MDA concentration (Fig. 6). IL-1β was not elevated at this early 3-h time point. CO exposure throughout reperfusion led to a significant reduction in I/R-induced proinflammatory cytokine expression (TNF-α), yet had no significant impact on lipid peroxidation (as indicated by no significant difference in ileum MDA concentration).

Expression of ICAM-1 protein as determined by Western blotting demonstrated a significant upregulation due to I/R that was abrogated by inhalation of 250 ppm CO (Fig. 7).

**DISCUSSION**

The overwhelming systemic inflammatory response that may develop following severe trauma and prolonged I/R continues to represent a major clinical challenge and remains one of the leading causes of death in Intensive Care Units. Trauma induced by lower limb I/R has been shown to initiate a systemic inflammatory response, resulting in remote organ inflammation and injury (29, 37). Significant multiple organ dysfunction can result following such an insult and can lead to the development of MODS. The small intestine has been implicated in the development of MODS, since this organ represents an essential barrier to intestinal bacteria and is a potential source of multiple proinflammatory stimuli (8, 30, 32). It is now generally accepted that intestinal leukocyte recruitment represents a critical factor leading to extensive intestinal injury and dysfunction. The discovery of a novel anti-inflammatory therapy to attenuate the overwhelming systemic inflammatory response and development of a remote intestinal inflammatory response during SIRS may hold vast therapeutic potential.

Using a direct in vivo approach, we hypothesized that low-dose inhaled CO would reduce the remote intestinal inflammatory response elicited by hindlimb I/R. We demonstrated a significant increase in COHb levels following CO exposure, confirming the delivery of CO in the vascular compartment for transport throughout the systemic circulation. An examination of multiple hematological parameters in Sham + CO mice suggested only a mild, but significant, reduction in the development of MODS. The small intestine has been implicated in the development of MODS, since this organ represents an essential barrier to intestinal bacteria and is a potential source of multiple proinflammatory stimuli (8, 30, 32). It is now generally accepted that intestinal leukocyte recruitment represents a critical factor leading to extensive intestinal injury and dysfunction. The discovery of a novel anti-inflammatory therapy to attenuate the overwhelming systemic inflammatory response and development of a remote intestinal inflammatory response during SIRS may hold vast therapeutic potential.

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**Table 1. Hematological analysis of blood samples**

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Sham + CO</th>
<th>I/R</th>
<th>I/R + CO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin, g/l</td>
<td>126.6±2.25</td>
<td>118.2±2.40</td>
<td>149.8±2.84</td>
<td>152.8±3.22</td>
</tr>
<tr>
<td>Hematocrit, l/l</td>
<td>0.34±0.010</td>
<td>0.32±0.006</td>
<td>0.42±0.014</td>
<td>0.41±0.010</td>
</tr>
<tr>
<td>MCV, fl</td>
<td>46.2±0.56</td>
<td>47.9±0.17</td>
<td>47.9±0.60</td>
<td>47.0±0.34</td>
</tr>
<tr>
<td>Neutrophils, ×10^9/l</td>
<td>0.3±0.08</td>
<td>0.2±0.09</td>
<td>1.6±0.13</td>
<td>2.0±0.32</td>
</tr>
<tr>
<td>Platelets, ×10^9/l</td>
<td>885.2±43.8</td>
<td>736.2±19.7</td>
<td>697.4±25.8</td>
<td>715.0±73.0</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5 mice. CO, carbon monoxide; I/R, ischemia-reperfusion; MCV, mean corpuscular volume. Shown is hematological analysis of blood samples obtained via cardiac puncture following Sham, Sham + CO, I/R, or I/R + CO treatment. P < 0.05 vs. Sham (*) and vs. +CO (†).
the number of circulating platelets. All other measured hematological parameters remained unchanged compared with sham mice. Zevin et al. (38) recently reported that CO inhalation (that mimicked CO exposure from cigarette smoking) at even higher concentrations (1,200–1,500 ppm) in human volunteers led to a similar increase in COHb and had no significant impact on blood pressure and heart rate. Interestingly, elevated COHb levels have also been reported in trauma victims and septic patients as an indirect measure of increased endogenous HO activity (15, 37). Taken together, these data suggest that low-dose inhaled CO may represent a therapeutic option with clinical utility to mimic the endogenous HO pathway.

Systemic inflammation, resulting in remote organ leukocyte recruitment, has been strongly linked to the development of MODS. The present study demonstrates the development of a remote intestinal inflammatory response as indicated by a reduction in venular volumetric blood flow, leukocyte rolling velocity, and wall shear rate in ileal submucosal postcapillary venules following hindlimb I/R. This was also accompanied by an increase in venular leukocyte adhesion, which was significantly attenuated following ICAM-1 immunoblockade. Interestingly, CO exposure following reperfusion completely blocked ICAM-1-dependent leukocyte adhesion elicited by hindlimb I/R and led to an increase in leukocyte rolling velocity in submucosal postcapillary venules. CO exposure also led to a normalization of ICAM-1 protein expression following hindlimb I/R. Morisaki et al. (17) demonstrated that superfusion with a buffered solution containing CO at micromolar concentrations was able to attenuate endotoxin-induced leukocyte recruitment in mesenteric venules. Nakao et al. (18) demonstrated that inhaled CO improved intestinal graft viability following transplantation, in part, by increasing laser Doppler blood flow in the wall of transplanted intestinal grafts. However, in the present study, we showed that the anti-inflammatory phenotype observed was independent of altered venular volumetric blood flow, since wall shear rate and volumetric blood flow were not significantly altered by CO inhalation. This discrepancy may reflect the severity of I/R insult between these two experimental models (direct I/R vs. remote I/R), sensitivity of observational technique (whole organ vs. direct observation of the microcirculation), or differential vasoactive properties of CO throughout the microvascular unit (arteriole-capillaries-venule). Interestingly, Ott et al. (20) reported a significant improvement in volumetric blood flow in the liver with low-dose CO exposure following hindlimb I/R characterized by increase sinusoidal diameter and red blood cell velocity. These results allude to particular organ-specific differences and/or a disparity of CO functionality within different microvascular beds. Furthermore, these observations suggest that CO may alter upstream signaling events involved in modulating leukocyte recruitment, vasomotor tone (in particular microvascular beds), and/or the expression of key leukocyte/endothelial cellular adhesion molecules.

I/R is generally accepted to result in a profound increase in proinflammatory cytokine expression, leukocyte recruitment, and oxidative stress. We demonstrated a significant increase in remote intestinal (ileum) TNF-α expression following hindlimb I/R that was greatly attenuated with inhaled CO throughout reperfusion. Nakao et al. (18) also demonstrate a significant reduction in intestinal proinflammatory cytokine expression (TNF-α, IL-6) with inhaled CO treatment following transplant-induced I/R. These findings are further corroborated by Moore et al. (16), who report a significant reduction in proinflammatory cytokine expression (IL-1β, IL-6) in a murine model of postoperative ileus following administration of inhaled CO. Taken together, these data further suggest that low-dose inhaled CO may exhibit potent anti-inflammatory properties, in part, by inhibiting the production of proinflammatory cytokines. In the present study, we also demonstrated a significant increase in ileum lipid peroxidation/oxidative stress following hindlimb I/R, as indicated by elevated MDA concentration in ileum samples. However, CO exposure throughout reperfusion did not significantly alter ileum MDA. Several studies suggest that the antioxidant properties associated with elevated HO activity may be attributed to increased bilirubin production. Both in vitro and in vivo evidence suggest that bilirubin exhibits potent antioxidant properties (34). Thus our data supports the concept that low-dose inhaled CO may affect distinct components of the inflammatory cascade, independent of altering intestinal lipid peroxidation/oxidative stress.

In conclusion, hindlimb I/R was associated with an increase in remote intestinal proinflammatory cytokine expression, lipid peroxidation, and submucosal postcapillary venule ICAM-1-dependent leukocyte recruitment. Low-dose inhaled CO attenuated both proinflammatory cytokine expression and leukocyte recruitment between these two experimental models (direct I/R vs. remote I/R), sensitivity of observational technique (whole organ vs. direct observation of the microcirculation), or differential vasoactive properties of CO throughout the microvascular unit (arteriole-capillaries-venule). Interestingly, Ott et al. (20) reported a significant improvement in volumetric blood flow in the liver with low-dose CO exposure following hindlimb I/R that was greatly attenuated with inhaled CO throughout reperfusion. Nakao et al. (18) also demonstrated a significant reduction in intestinal proinflammatory cytokine expression (TNF-α, IL-6) with inhaled CO treatment following transplant-induced I/R. These findings are further corroborated by Moore et al. (16), who report a significant reduction in proinflammatory cytokine expression (IL-1β, IL-6) in a murine model of postoperative ileus following administration of inhaled CO. Taken together, these data further suggest that low-dose inhaled CO may exhibit potent anti-inflammatory properties, in part, by inhibiting the production of proinflammatory cytokines. In the present study, we also demonstrated a significant increase in ileum lipid peroxidation/oxidative stress following hindlimb I/R, as indicated by elevated MDA concentration in ileum samples. However, CO exposure throughout reperfusion did not significantly alter ileum MDA. Several studies suggest that the antioxidant properties associated with elevated HO activity may be attributed to increased bilirubin production. Both in vitro and in vivo evidence suggest that bilirubin exhibits potent antioxidant properties (34). Thus our data supports the concept that low-dose inhaled CO may affect distinct components of the inflammatory cascade, independent of altering intestinal lipid peroxidation/oxidative stress.

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recruitment elicited by hindlimb I/R. Our data suggest that the anti-inflammatory properties associated with low-dose inhaled CO were independent of altered volumetric blood flow, wall shear rate, and lipid peroxidation. Low-dose inhaled CO may represent a novel gaseous therapeutic strategy to alter key components of the inflammatory cascade during SIRS, particularly those that affect leukocyte recruitment at the microvascular level.

GRANTS

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