Carbon liberated from CO-releasing molecules attenuates leukocyte infiltration in the small intestine of thermally injured mice

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AIM: To determine whether Carbon (CO) liberated from CO-releasing molecules attenuates leukocyte infiltration in the small intestine of thermally injured mice.

METHODS: Thirty-six mice were assigned to four groups. Mice in the sham group (n = 9) were underwent to sham thermal injury; mice in the burn group (n = 9) received 15% total body surface area full-thickness thermal injury; mice in the burn + CORM-2 group (n = 9) were underwent to the same thermal injury with immediate administration of tricarbonyldichlororuthenium (I) dimer CORM-2 (8 mg/kg, i.v.); and mice in the burn+DMSO group (n = 9) were underwent to the same thermal injury with immediate administration of 160 μL bolus injection of 0.5% DMSO/saline. Histological alterations and granulocyte infiltration of the small intestine were assessed. Polymorphonuclear neutrophil (PMN) accumulation (myeloperoxidase assay) was assessed in mice mid-ileum. Activation of nuclear factor (NF)-κB, expression levels of intercellular adhesion molecule-1 (ICAM-1), and inducible heme oxygenase in mid-ileum were assessed.

RESULTS: Treatment of thermally injured mice with CORM-2 attenuated PMN accumulation and prevented activation of NF-κB in the small intestine. This was accompanied by a decrease in the expression of ICAM-1. In parallel, burn-induced granulocyte infiltration in mid-ileum was markedly decreased in the burn mice treated with CORM-2.

CONCLUSION: CORM-released CO attenuates leukocyte infiltration in the small intestine of thermally injured mice by interfering with NF-κB activation and protein expression of ICAM-1, and therefore suppressing the pro-adhesive phenotype of endothelial cells.

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Key words: Leukocyte infiltration; Carbon monoxide; Thermal injury; Small intestine

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INTRODUCTION

Systemic inflammatory response syndrome (SIRS) and multiple organ failure (MOF) still continue to be leading causes of morbidity and mortality in severe burn patients[1,2]. The intestine is considered to be the critical organ in the development of organ dysfunction in trauma, burns, and intensive care unit patients[3]. Thermal injury is accompanied by complex events that exert deleterious effects on various organs, such as the small intestine, distant from the original burn wound. Following thermal injury, the small intestine is subjected to ischemia, and consequently, especially during burn resuscitation, reperfusion injury occurs[4]. Intestinal ischemia-reperfusion results in organ injury through both tissue hypoxia and reperfusion phenomena mediated by neutrophils[5]. A variety of cytokines are released into the microcirculation by neutrophils, endothelial cells and monocytes during phases of hypoxia and reperfusion[6,7]. Although the pathophysiological basis of organ damage remains unclear, there is increasing evidence that leukocyte infiltration into intestinal tissue plays an important role in bacterial or endotoxin translocation and development of SIRS after thermal injury[8-12].

A lot of evidence indicates that endogenous Carbon (CO), a by-product of inducible heme oxygenase (HO-1), modulates inflammation. In addition, some experiments have determined that the administration of exogenous CO inhibits lipopolysaccharide (LPS)-induced production...
of cytokines both in vivo and in vitro, and consequently exhibits an important cytoprotective function and anti-inflammatory properties that are beneficial for the resolution of acute inflammation\(^\text{[13-15]}\).

Recently, transitional metal carbonyls have been identified as potential CO-releasing molecules (CORMs), with the potential to facilitate the pharmacological use of CO by delivering it to tissues and organs\(^\text{[16]}\). CORMs have been shown to act pharmacologically in rat aorta and cardiac tissue in which liberation of CO induced vasorelaxant effects\(^\text{[17-20]}\) and decreased myocardial ischemia-reperfusion injury\(^\text{[21,22]}\), respectively. Our previous studies\(^\text{[23,24]}\) have shown that burn-induced overexpression of adhesion molecules [such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1] on endothelial cells and leukocytes may contribute to liver and lung tissue injury, subsequently leading to multiple organ dysfunction syndrome (MODS). We also confirmed that CORM-released CO attenuated leukocyte sequestration in the liver and lungs of burned mice by interfering with NF-kappaB activation and protein expression of ICAM-1, therefore suppressing the pro-adhesive phenotype of endothelial cells. However, it is still unknown if CORM-released CO can exert its anti-inflammatory and protective effects in the small intestine. Based on these preliminary observations, in this study we employed tricarbonyldichlororuthenium (II) dimer (CORM-2), one of the novel group of CORMs, to determine whether it attenuated leukocyte infiltration to the intestinal tissue of thermally injured mice.

### MATERIALS AND METHODS

#### Materials

CORM-2 was obtained from Sigma-Aldrich and was solubilized in DMSO to obtain a 10 mmol/L stock solution. Polyclonal or monoclonal antibodies against ICAM-1 and HO-1 were purchased from Santa Cruz Biotechnology. All other chemicals were reagent grade and obtained from Sigma unless otherwise stated.

#### Animals and burn protocol

C57BL/6 mice (36 male; body weight 20 ± 2 g) were fed a standard laboratory diet and water ad libitum. Mice were assigned to four groups. Mice in the sham group \((n = 9)\) were underwent to sham thermal injury; mice in the burn group \((n = 9)\) received 15% total body surface area (TBSA) full-thickness thermal injury; mice in the burn + DMSO group \((n = 9)\) were underwent to the same thermal injury with immediate administration of 160 μL bolus injection of 0.5% DMSO/saline; and mice in the burn + CORM-2 group \((n = 9)\) were underwent to the same thermal injury with immediate administration of CORM-2 (8 mg/kg, i.v.). The experimental protocol was approved by The Council on Animal Care at Jiangsu University for the protection and welfare of animals. Under anesthesia with spontaneous inhalation of isoflurane/N₂O (Abbott Laboratories, Mississauga, ON, Canada) in a 60% oxygen/40% nitrogen mixture, the dorsum of each mouse was shaved and the animal was subjected to 15% TBSA full-thickness thermal injury as previously described\(^\text{[25,26]}\). Sham animals were immersed in a water bath at room temperature. All animals were resuscitated with 1.5 mL saline immediately after thermal (or sham) injury. No wound care was required for the burn wounds. This burn method achieves a histologically proven, full-thickness scald injury\(^\text{[27,28]}\). The animals were sacrificed at 24 h after experimental manipulation.

#### Ileum histologic studies

The mid-ileum specimens harvested from different groups of animals were immersed in 4% formaldehyde solution at 24 h after thermal injury. The tissue was embedded in paraffin wax, serially sectioned, and stained with hematoxylin-eosin. Ileal morphologic characteristics were evaluated by light microscopy. Ileum tissue was evaluated for density of granulocytes and degree of hydropic degeneration. Tissues were evaluated in a semi-quantitative manner by two experienced independent examiners that were blinded to the experimental groups (Table 1). A scoring system was used for each item using 0 up to 2 points for the different states of organ damage (with 2 being most granulocytes, edema and degeneration; Table 1). Afterwards, the mean ± SEM of each item was calculated.

### Preparation of intestinal homogenates

Immediately after withdrawing blood, the intestine was exposed. Leaving approximately the first 5-cm-long proximal segment of the intestine, 3-cm-long segments of jejunum and ileum were removed, cleaned, and snap-frozen in liquid nitrogen. The samples were stored at -70°C. Equal weights (100 mg wet weight) of intestine from various groups were suspended in 1 mL PBS and sonicated (30 cycles, twice, for 30 s) on ice\(^\text{[29]}\). Homogenates were cleared by centrifuging at 12 000 r/min at 4°C, and the supernatants were stored at -70°C. Protein levels in the homogenates were determined using the Bio-Rad (Hercules, CA, USA) assay kit.

#### Myeloperoxidase (MPO) activity

MPO activity was measured in ileum tissue using a procedure similar to that documented by Hillegas et al\(^\text{[30,31]}\). Tissue samples were homogenized in 50 mmol/L potassium phosphate buffer (PB) (pH 6.0), and centrifuged at 10 000 × g (10 min); pellets were suspended in 50 mmol/L PB containing 0.5% hexadecyltrimethylammonium bromide. After sonication, the samples were centrifuged at 10 000 × g for 10 min. Aliquots (0.3 mL) were added to 2.3 mL of reaction mixture containing 50 mmol/L PB, o-dianisidine, and 20 mmol/L H₂O₂ solution. One unit of enzyme activity was defined as the amount of MPO.

### Table 1 Histological scoring system for ileum and jejunum sections stained with hematoxylin/eosin

| Granulocyte infiltration | Hydroptic degeneration |
|---------------------------|------------------------|
| **Ileum**                 | **Jejunum**            |
| No                        | No                     |
| Moderate                  | Moderate               |
| Intense                   | Intense                |
|                           |                        |
| **0**                     | **1**                  |
| **2**                     |                        |

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present that caused a change in absorbance measured at 460 nm for 3 min. MPO activity was expressed as U/g tissue.

**Measurement of ICAM-1**

ICAM-1 levels in ileum tissue homogenates were measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer’s instructions.

**Western blot analysis**

Tissues were homogenized for extraction in ice-cold mild lysis buffer, containing 1% Nonidet P-40, 0.15 mol/L NaCl, 0.01 mol/L sodium phosphate (pH 7.2), 2 mmol/L EDTA, 50 mmol/L sodium fluoride, 0.2 mmol/L sodium vanadate, and 1 μg/mL aprotinin. The tissue homogenates were centrifuged at 20,000 × g for 15 min and supernatants were collected. SDS-PAGE was performed on equivalent amounts of protein samples using precast 7% resolving/4% stacking Tris/HCl gels (Bio-Rad, Hercules, CA, USA). Separated proteins were then transferred to PVDF membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Membranes were blocked in 5% non-fat milk in TBS buffer containing 0.1% Tween 20 (TBST) for 1 h at room temperature. Blocked membranes were incubated with primary antibodies specific for mouse ICAM-1 and HO-1 at a concentration of 1:1000 and 1:5000, respectively, in TBST overnight at 4°C. Then, the membranes were washed and probed with horseradish-peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 1 h at room temperature. Chemiluminescence detection was performed with the Amersham enhanced chemiluminescence detection kit according to the manufacturer’s instructions. To ensure a similar amount of protein in each sample, the membranes were “stripped off”, reprobed with actin, developed with horseradish-peroxidase-conjugated secondary antibody, and visualized by enhanced chemiluminescence.

**Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)**

Nuclear protein from ileum tissue was extracted using our previously described method[32,33]. Briefly, frozen tissues were weighed, transferred to Corex tubes and homogenized in four volumes (w/v) of PBS containing 2 mmol/L phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 3000 × g for 10 min, and the pellet was then resuspended in 2 mL buffer A [0.3 mol/L sucrose, 5 mmol/L dithiothreitol (DTT), 5 mmol/L MgCl2, 10 mmol/L Tris/HCl, 0.1% Triton X-405], and further homogenized using a Dounce homogenizer. After filtration through a 100-μm nylon mesh, the suspension was centrifuged at 1000 × g for 5 min at 4°C. The pellet (nuclei) was washed in buffer A (without 0.1% Triton X-405) and centrifuged (1000 × g for 5 min at 4°C), and then the nuclei were extracted on ice for 30 min in 60 μL buffer B containing 20 mmol/L HEPES, 0.75 mmol/L spermidine, 0.15 mmol/L spermine, 0.2 mmol/L EDTA, 2 mmol/L ethylene glycol-bis (b-aminoethyl ether)-N, N', N'-tetraacetic acid, 2 mmol/L DTT, 20% glycerol, and 1 mmol/L PMSF (4°C) in the presence of 0.4 mol/L NaCl. Finally, the samples were centrifuged for 10 min at 21,000 × g (4°C), and the supernatants were collected and stored at -80°C as the nuclear protein fraction.

For EMSA, 5 μg total nuclear proteins was incubated with 1.0 pmol double-stranded [32P] ATP end-labeled oligonucleotides containing consensus binding sequences for NF-κB (sense strand 5'-AGGGACTTTCCGGCTG GGGACTTTCC-3') in a binding buffer (10 mmol/L HEPES, pH 7.9, 80 mmol/L NaCl, 3 mmol/L MgCl2, 0.1 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L PMSF, and 10% glycerol), as described previously[34]. Samples were incubated for 30 min at room temperature and then run through a 4% non-denaturing polyacrylamide gel (0.5 × TBE buffer) at 280 V for 1 h. The gel was dried and then exposed to X-ray film (Kodak) for 4-6 h in cassettes at -80°C. Signal detection and quantification were performed by computer-assisted densitometry.

**Statistical analysis**

All the values are presented as mean ± SE. Statistical analysis was performed by ANOVA and Student’s t test for the comparisons. P < 0.05 was considered to be statistically significant.

**RESULTS**

**Histology**

Histological analysis showed that the ileum from sham mice had the normal architecture of the intestinal epithelium and wall, while thermal injury induced severe edema and sloughing of the villous tips, as well as infiltration of inflammatory cells into the mucosa (Figure 1). Semi-quantitative analysis of histological samples of ileum and jejunum showed that granulocyte infiltration in the burned mice was significantly increased compared to that in the sham group. Administration of CORM-2 (8 mg/kg, i.v.), significantly decreased granulocyte infiltration. However, CORM-2 did not improve the hydropic degeneration induced by thermal injury in either the ileum or jejunum (Table 2).

**Effect of CORM-2 on MPO activity in small intestine of thermally injured mice**

To determine whether the burn-induced increase in polymorphonuclear neutrophil (PMN) accumulation in the small intestine was effectively prevented by CORM-2,
the activity of MPO, an enzyme in azurophilic granules of neutrophils, was assessed. Extracts of the ileum samples were examined for content of MPO 24 h after thermal injury. The mean MPO levels are shown in Figure 2. MPO activity in organs obtained from burned mice was markedly increased compared to that in the sham group ($P < 0.01$), while it was significantly decreased by treatment with CORM-2 ($P < 0.05$).

**Effect of CORM-2 on expression of ICAM-1 in the small intestine of thermally injured mice**

At 24 h after a 15% TBSA full-thickness thermal injury, the expression of ICAM-1 in the ileum was significantly increased compared to that in the sham-treated mice. Administration of CORM-2 (8 mg/kg, i.v.) significantly decreased expression of ICAM-1 (Figure 3).

**Effect of CORM-2 on expression of HO-1 in the small intestine of thermally injured mice**

At 24 h after 15% TBSA full-thickness thermal injury, the expression of HO-1 in the small intestine significantly increased compared to that in the sham-treated animals. *In vitro* administration of CORM-2 (8 mg/kg, i.v.), expression of HO-1 in the ileum tissue of burn mice was more significantly increased compared to burn group (Figure 4).
CORMs have been demonstrated to be due to the CO vasoactive, antihypertensive and anti-rejection effects of systems have been identified and synthesized. The (CORMs) that have the ability to release CO in biological CO inhalation) in the modulation of inflammation. As a result, it is hypothesized that CORM-2 contributes to the attenuation of leukocyte injury.

**Effect of CORM-2 on activity of NF-κB in the small intestine of thermally injured mice**

Binding of nuclear protein to the radiolabeled consensus binding sequences of NF-κB was assessed by EMSA. At 24 h after 15% TBSA full-thickness thermal injury, NF-κB activation in the ileum was markedly increased, and this was markedly inhibited by administration of CORM-2 (8 mg/kg i.v.) (Figure 5).

### DISCUSSION

Major burns alter immune function, which produces an imbalance between pro- and anti-inflammatory cytokine synthesis, and increases susceptibility to post-burn infection and sepsis[^35-37^]. Also, severe burns cause damage to multiple organs distant from the original burn wound, leading to MOF, a serious clinical problem. The intestine is one of the most sensitive tissues to ischemia and reperfusion induced by thermal injury. PMNs may play an important role in ischemic injury, and reperfusion of intestine is associated with accumulation of PMNs in the intestinal tissue. It has been suggested that tissue accumulation of PMNs is a key event that determines the severity of ischemia-reperfusion injury[^36^].

We report here that CORM-released CO exerts a protective effect against the pathological changes caused by thermal injury of the small intestine. Importantly, this exogenous CO showed effective inhibition of activation of NF-κB and expression of ICAM-1. Thus, we propose that CORM-2 contributes to the attenuation of leukocyte infiltration into the intestinal tissue after burn challenge.

What is, then, the mechanism by which attenuation of PMN infiltration to the intestine is caused by thermal injury?

Many experimental studies have highlighted the specific and independent role of exogenous CO (i.e. CO inhalation) in the modulation of inflammation[^38,39^]. Recently some new metal carbonyl-based compounds (CORMs) that have the ability to release CO in biological systems have been identified and synthesized. The vasoactive, antihypertensive and anti-rejection effects of CORMs have been demonstrated to be due to the CO liberated by the compounds. CORM-2, a DMSO-soluble CORM, also has exhibited anti-inflammatory actions in an in vitro model of LPS-stimulated murine macrophages[^40^].

MPO is an enzyme that is found predominantly in the azurophilic granules of PMNs. Tissue MPO activity is frequently utilized to estimate tissue PMN accumulation in inflamed tissues, and correlates significantly with the number of PMNs determined histochemically in tissues[^41^]. In the present study, we found that intestinal MPO activity was markedly elevated after thermal injury, and administration of CORM-2 led to significant down-regulation of MPO activity. This indicates that CORM-2 effectively prevents PMN chemotaxis and infiltration in the small intestine after thermal injury, which consequently decreases the production of oxidants and reduces tissue oxidative injury, which contributes to MODS. In parallel, histological analysis in this study indicated that mid-ileum sections from thermally injured mice showed inflammatory cell infiltration through the wall, concentrated below the epithelial layer, edema of the distal portion of the villi, and necrosis of the epithelium at the villous tips. On the contrary, ileum sections from mice treated with CORM-2 showed a significant decrease in leukocyte infiltration.

PMN-endothelial cell interactions are supposed to play a central role in the pathogenesis of intestinal barrier failure following thermal injury and ischemia-reperfusion[^42^]. The presence of ICAM-1, which mediates leukocyte adhesion, correlates with infiltration of leukocytes into inflammatory lesions[^43,44^]. It seems to be the initial marker of inflammatory reactions and is involved in the acute inflammatory reaction following burns[^45^]. ICAM-1 activates leukocytes and endothelial cells, which in turn, prompt the release of various inflammatory mediators. This may result in SIRS, acute respiratory distress syndrome, and MODS, which may develop further into progressive MOF and death[^46-48^]. The present
results showed that at 24 h post-burn, the expression of ICAM-1 in intestinal tissue was markedly up-regulated. CORM-2 was able to inhibit the up-regulation of ICAM-1 induced by thermal injury. Our findings strongly indicate that CORM-2 appears to inhibit leukocyte activation and adhesion, and consequently, might effectively decrease the inflammatory response in the small intestine induced by burns.

HO is a rate-limiting enzyme that is responsible for the catabolism of heme into bilirubin, free iron, and CO. Three HO isoforms have been identified: HO-2 and HO-3 isoforms are believed to be constitutive and physiologically expressed, whereas HO-1 isoform is a stress-responsive protein that is induced by various stimuli. The adaptive response of HO-1 to various stimuli suggests that it may play an important role in protection against the inflammatory response and oxidative injury. Other studies have shown that up-regulation of endogenous HO-1 ameliorates inflammatory responses and/or tissue damage. In this study, we found that HO-1 was significantly up-regulated by thermal injury. Interestingly, the expression of HO-1 in the small intestine of thermally injured mice treated with CORM-2 was more significantly increased compared to burned mice without CORM-2 (Figure 4). This result indicates that not only major burn injury might significantly induce the expression of HO-1, but also the increase in HO-1 expression can be further enhanced by the administration of CORM-2. Through the by-product (CO and/or biliverdin), the potent cytotoxic and anti-inflammatory functions were ultimately led to exert.

NF-κB family members control transcriptional activity of various promoters of proinflammatory cytokines, cell-surface receptors, transcription factors, and adhesion molecules that are involved in intestinal inflammation. Stimuli like oxidative stress, cytokines (interleukin-1, interleukin-6, tumor necrosis factor-2), bacteria and viruses can release NF-κB from its inactive cytoplasmic form to the nucleus. Thermal injury has been known to induce hepatic NF-κB expression associated with hepatic cell apoptosis and proliferation, but its effect on NF-κB activation in the intestine has never been clarified. Previously, using a thermal injury model in mice, we have shown that CORM-2 plays a pivotal role in inhibition of NF-κB activity in the liver, which subsequently decreases hepatocellular secretion of inflammatory cytokines and burn-related hepatic dysfunction. In this study, NF-κB activity in mid-ileum was elevated by thermal injury, while it was markedly inhibited by administration of CORM-2. These results show that CORM-2 plays, at least partly, an important role in inhibition of NF-κB activity in the small intestine. Therefore, the role of NF-κB activation and the regulation of CORM-2 in thermal-injury-induced intestinal damage requires further study.

In conclusion, the present study serves to clarify the role of CORM-2, one of the novel CORMs, on the mechanisms of anti-inflammation and cytoprotection. Application of CORM-2 to thermally injured mice attenuated PMN accumulation, and prevented activation of NF-κB in the small intestine. This was accompanied by a decrease in expression of ICAM-1, and an increase in expression of HO-1. Taken together, these findings indicate that CORM-released CO modulates gut inflammation in burned mice by interfering with NF-κB activation, and protein expression of ICAM-1 and HO-1, and therefore suppresses the pro-adhesive phenotype of endothelial cells. Further studies are now required to understand the detailed mechanisms of the anti-inflammatory effects mediated by CORMs, and to contribute to the development of a therapeutic approach to protect against gut damage during severe burn injury.

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