Carbon monoxide liberated from carbon monoxide-releasing molecule CORM-2 attenuates inflammation in the liver of septic mice

Gediminas Cepinskas, Kazuhiro Katada, Aurelia Bihari, and Richard F. Potter
Centre for Critical Illness Research, Lawson Health Research Institute, London, Ontario, Canada
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Cepinskas G, Katada K, Bihari A, Potter RF. Carbon monoxide liberated from carbon monoxide-releasing molecule CORM-2 attenuates inflammation in the liver of septic mice. Am J Physiol Gastrointest Liver Physiol 294: G184–G191, 2008. First published November 8, 2007; doi:10.1152/ajpgi.00348.2007.—Recent studies suggest that exogenously administered CO is beneficial for the resolution of acute inflammation. In this study, we assessed the role of CO liberated from a systemically administered tricarbonyldichlororuthenium-(II)-dimer (CORM-2) on modulation of liver inflammation during sepsis. Polymicrobial sepsis in mice was induced by cecal ligation and perforation (CLP). CORM-2 (8 mg/kg iv) was administered immediately after CLP induction, and neutrophil [polymorphonuclear leukocyte (PMN)] tissue accumulation, activation of transcription factor, NF-κB, and changes in adhesion molecule ICAM-1 expression (inflammation-relevant markers) were assessed in murine liver 24 h later. In addition, the effects and potential mechanisms of CORM-2-released CO in modulation of vascular endothelial cell proinflammatory responses were assessed in vitro. To this end, human umbilical vein endothelial cells (HUVEC) were stimulated with LPS (1 μg/ml) in the presence or absence of CORM-2 (10–100 μM) and production of intracellular reactive oxygen species (ROS), (DHR123 oxidation) and NO (DAF-FM nitrosation) and subsequent activation of NF-κB were assessed 4 h later. In parallel, expression of ICAM-1 and inducible NO synthase (iNOS) proteins along with PMN adhesion to LPS-challenged HUVEC were also assessed. Induction of CLP resulted in increased PMN accumulation, ICAM-1 expression, and activation of NF-κB in the liver of septic mice. These effects were significantly attenuated by systemic administration of CORM-2. In vitro experiments, CORM-2-released CO attenuated LPS-induced production of ROS and NO, activation of NF-κB, increase in ICAM-1 and iNOS protein expression and PMN adhesion to LPS-stimulated HUVEC. Taken together, these findings indicate that CO released from systemically administered CORM-2 provides anti-inflammatory effects by interfering with NF-κB activation and subsequent downregulation of proinflammatory vascular endothelial cell phenotype in the liver of septic mice.

In general, one of the hallmarks of systemic inflammation as a consequence of bacterial infection (i.e., sepsis) is an increase in polymorphonuclear leukocyte (PMN) accumulation in the affected systemic organs, such as lung, heart and liver (2, 11, 34, 53, 57, 59, 62, 65). The mechanism(s) of PMN recruitment to the affected organs is a complex multistep process and involves series of adhesive interactions (rolling, firm adhesion, and migration) between PMN and vascular endothelial cells (15, 16, 19, 67, 68). Neutrophil sequestration has been associated with the increased chemokine production, augmented expression of the β2-integrin CD11b/CD18 and upregulation of vascular proadhesive phenotype [e.g., increased expression of E-selectin and vascular cell adhesion molecules, such as ICAM-1 and VCAM-1 (34, 17, 40)].

Although PMN recruitment to the affected organs is part of the natural defense mechanism, overwhelming PMN infiltration can potentially be detrimental to the host. Therefore, mechanisms exist to counteract increased PMN-endothelial cell adhesive interaction. One candidate capable of influencing PMN recruitment into affected organs, e.g., liver, is nitric oxide (NO) (9, 20). However, the role of NO with respect to the above remains controversial since the most recent findings suggest that NO exhibits both anti- and proinflammatory effects with respect to leukocyte-endothelial cell adhesive interactions, a phenomenon largely dependent on the time, quantity, and place of NO production (3, 4, 21, 32). Although there is a general agreement in regard to the anti-inflammatory effects of NO derived from constitutively expressed nitric oxide synthase (NOS) (e.g., endothelial NOS; eNOS), recent evidence strongly supports the notion that NO, particularly produced by inducible nitric oxide synthase (iNOS), is injurious and contributes to PMN recruitment and thereby further amplifies inflammation (57, 58). Conversely, NO cognate gaseous molecule, carbon monoxide (CO), a by-product of heme oxygenase (HO-1) activity, has been shown to offer both protection to microvascular perfusion and anti-inflammatory benefits during systemic inflammatory response syndrome (SIRS) (42, 43, 62, 70). In addition, recent studies have shown that exogenous administration of CO (250 ppm) inhibits lipopolysaccharide (LPS)-induced production of cytokines both in vivo and in vitro and consequently exhibits important cytoprotective function and anti-inflammatory properties that are beneficial for the resolution of acute inflammation (24, 33, 46, 54, 55). Although the exogenous administration of CO via inhalation has been shown beneficial during SIRS, such method of administration results in increased carboxyhemoglobin concentration (53), thus presenting a potential threat to the host.

Recently, transitional metal carbonyls, CO-releasing molecules (CORMs), have been used to deliver CO in a more controlled manner without altering carboxyhemoglobin levels. As a consequence, these molecules have received increasing attention for the potential pharmaceutical application (48). In regard to the latter, CORMs have been shown to act pharmacologically in rat aortic and cardiac tissue where liberation of CO produced vasorelaxant effects (18, 30, 47, 49), decreased myocardial ischemia-reperfusion damage (13, 23), and reduced inflammatory response in LPS-stimulated macrophages (61).
In addition, recent findings indicate that CORM-liberated CO inhibits CD11b expression in platelet-activating factor (PAF)-
activated PMN (66).

However, the role of CORM-released CO in regulation of the
systemic inflammation during sepsis has not been investi-
gated yet. In this study we employed tricarboxyldi-
chlororuthenium (II) dimer (CORM-2), one of the novel CO
donors, to assess the effects and potential mechanisms of
CORM-released CO in modulation of vascular proadhesive
phenotype in the liver of septic [cecal ligation and perforation
(CLP)-challenged] mice.

MATERIALS AND METHODS

Medium 199 (M199), fetal calf serum (FCS), penicillin, and strep-
tomycin were purchased from Wisent (St-Bruno, Canada). CORM-2
(mol wt 512.18) was purchased from Sigma-Aldrich (St. Louis, MO)
and solubilized in dimethyl sulfoxide (DMSO). LPS (Escherichia coli
serotype 055:B5) was purchased from Sigma. Hamster anti-mouse
ICAM-1 antibody (clone 3E2) and mouse anti-human iNOS antibody
(clone 54) were purchased from BD Biosciences. Mouse anti-human
ICAM-1 (clone 15.2) was purchased from Santa Cruz, and rabbit
polyclonal anti-β-actin antibody were obtained from Sigma. Second-
ary biotin-conjugated goat anti-hamster antibody were purchased
from Rockland, and mouse/rabbit extravidin peroxidase kits were
obtained from Rockland.

Animals. C57BL/6 mice (6-wk-old; Jackson Laboratories) were
used in the experiments. All animal studies were performed in
accordance with the University of Western Ontario Animal Care and
Use Committee approved protocols.

CLP. Mice were anesthetized with 2% isoflurane in oxygen via a
facemask. A 1- to 2-cm midline incision was made through the
abdominal wall; the cecum was identified and ligated with a 3-0
tsilk tie 1 cm from the tip. Care was taken not to cause bowel obstruction.
A single puncture of the cecal wall was performed with a 20-gauge
needle. The cecum was lightly squeezed to express a small amount of
stool from the puncture site to assure a full-thickness perforation.
Great care was taken to preserve continuity of flow between the small
and large bowels. Inspection of mice at various intervals after CLP did
not reveal evidence of bowel obstruction. The cecum was returned to
the abdominal cavity, and the incision was closed with surgical clips.
Sham mice underwent anesthesia and midline laparotomy; the cecum
was exteriorized and returned to the abdomen, and the wound was
closed with surgical clips.

Experimental mice were injected with CORM-2 (8.0 mg/kg iv, tail
vein, to achieve 180 μM CORM-2 concentration in systemic circu-
lation) in 0.16 ml of normal saline immediately after CLP induction.
Sham mice were injected with the vehicle (0.25% DMSO in saline).
 Twenty-four hours following CLP induction mice were euthanized by
cervical dislocation. Subsequently, livers were collected and assessed
for the various experimental endpoints.

MPO activity assay. MPO activity as an index of neutrophil influx
was assessed as previously described by us (11). In brief, tissue was
homogenized in 0.5 ml of 50 mM potassium phosphate buffer (pH
7.4) and centrifuged at 10,000 g at 4°C for 30 min. The remaining
pellet was resuspended in 0.5 ml of 50 mM potassium buffer (pH 6.0)
containing 0.5% hexadecyltrimethylammonium bromide, sonicated,
and centrifuged at 12,000 g at 4°C for 10 min. MPO activity in the
supernatants was assessed spectrophotometrically at 460-nm wave-
length (Bio-Rad-680 microplate reader) with o-dianisidine as a sub-
strate. MPO activity was expressed as units per gram of tissue.

Cells. Human umbilical vein endothelial cells (HUVEC) were
harvested from the human umbilical cord veins by collagenase treat-
ment (Worthington Biochem, Freehold, NJ) as previously described
by us (10). The cells were grown in M199 (GIBCO, Burlington,
Canada) supplemented with 10% heat-inactivated FCS (Wisent), 2.4
mg/ml thymidine (Sigma Chemical, Oakville, Canada), 10 IU/ml hep-
arin sodium, antibiotics (100 U/ml penicillin and 100 μg/ml strepto-
mycin; Gibco), and 80 μg/ml endothelial mitogen (Biomedical
Technologies, Stoughton, MA). The cell cultures were incubated in
room air with 5% CO2 at 37°C and 95% humidity and were expanded by
brief trypsinization with 0.25% trypsin in PBS containing 0.025%
EDTA.

For the experiments, passage 1–3 HUVEC were stimulated with
LPS (1 μg/ml) for up to 4 h in the presence or absence of CORM-2 (10, 50,
and 100 μM). Control cells were treated with 0.25% DMSO
(CORM-2 vehicle), corresponding to the highest (100 μM) CORM-2
concentration used in the experiments. CORM-2 had no cytotoxic
effects on HUVEC up to the maximum concentration of 180 μM
(Trypan blue exclusion assay).

Cell ELISA. For assessment of ICAM-1 surface expression levels
cell ELISA was performed on HUVEC grown in 96-well cell culture
plates (Falcon). HUVEC were fixed in 3% paraformaldehyde for 30
min. The cells were then washed with PBS and incubated with the
primary mouse anti-human ICAM-1 monoclonal antibody at a con-
centration of 10 μg/ml for 1 h at room temperature. Anti-ICAM-1
antibody binding to HUVEC was quantitated spectrophotometrically
at 450-nm wavelength (Bio-Rad-680 microplate reader) by use of mouse
extravidin-peroxidase staining kit (Sigma) and 3,3′,5,5′-tetra-
methylbenzidine (TMB) as peroxidase substrate.

ROS and NO production. Oxidant production in HUVEC was as-
sembled by measuring the oxidation of intracellular dihydrodorhodamine 123
(DHR 123; Molecular Probes), an oxidant-sensitive fluorochrome, as
described previously (8). In parallel, intracellular production of NO was
assessed by nitration of NO-sensitive fluorochrome, 4-amino-5-methyl-
ylaminono-2′,7′-dichlorofluorescein diacetate (DAF-FM diacetate; Molec-
ular Probes) (60). To this end, HUVEC were loaded with DHR 123 (5
μM) or DAF-FM diacetate (10 μM) for 1 h before stimulation of
HUVEC with LPS. Following LPS stimulation the cells were washed
with PBS, lysed in 0.5% CHAPS buffer, and analyzed spectrophoto-
metrically (RF-1501 spectrophotometer, Shimadzu) at excitation and
emission wavelengths of 502/523 nm (for DHR123) and 495/515 nm (for
DAF-FM), respectively. Reactive oxygen species (ROS) and NO pro-
duction were expressed as fluorescence emission per microgram of
protein of flow cytometry.

SDS-PAGE and Western blotting. SDS-PAGE and Western blott-
ing were performed as described elsewhere. Samples (10 μg of
protein) were subjected to 7% SDS-PAGE, transferred onto PVDF
membranes (Amersham Pharma Biotech, Piscataway, NJ), and
assayed for ICAM-1, iNOS, and β-actin (loading control) protein
expression by chemiluminescence detection (Amersham ECL kit)
according to the manufacturer’s instructions. The specific protein
bands were quantified by densitometric analysis (GS-690 Image
Densitometer; Bio-Rad).

Preparation of nuclear extracts and EMSA. Nuclear protein from
whole tissue (medial lobe of liver) was extracted as previously
described by us (39). Briefly, frozen tissue was homogenized in four
volumes (w/vol) of PBS containing the following proteinase inhibi-
tors: 2 mM 4-(2-aminoethy)benzenesulfonyl fluoride hydrochloride;
1 mM E-64; and 10 μg/ml each of pepstatin A, bestatin, leupeptin,
and aprotonin. The homogenate was centrifuged at 3,000 g for 10 min,
and the pellet was resuspended in 2 ml of buffer A (0.3 M mol/l sucrose,
5 mmol/l dithiothreitol, 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 obtained suspen-
sion was centrifuged at 1,000 g for 5 min at 4°C. The pellet (nuclei)
was washed in buffer A without 0.1% Triton X-405 and centrifuged
(1,000 g for 5 min at 4°C). Subsequently, the nuclei were extracted on
ice for 30 min in 60 μl of buffer containing 20 mmol/l HEPES, 0.75
mmol/l spermidine, 0.15 mmol/l spermine, 0.2 mmol/l ethylenedially-
iminotetraacetic acid, 2 mmol/l ethylene glycol-bis-(2-aminoethyl
ether)-N,N,N′,N′-tetraacetic acid, 2 mmol/l dithiothreitol, 20% glycer-
ol, and 1 mmol/l phenylmethylsulfonyl fluoride (4°C) in the pres-

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ence 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.

The nuclear proteins from cultured HUVEC were isolated in a similar manner as described in Ref. 11. For EMSA, 5 µg of total nuclear protein was incubated with 1.0 pmol of double-stranded γ-[^32P]ATP end-labeled oligonucleotides containing consensus binding sequences for NF-κB and electrophoresed on 4% polyacrylamide gel (PAG) under non-denaturing conditions. Subsequently, the gels were dried and exposed to X-ray film (Kodak) for 4–6 h at −80°C. The specific bands were quantified by densitometry (Bio-Rad GS-710 densitometer).

PMN adhesion assay. Human neutrophilic leukocytes (PMN) were isolated from the venous blood of healthy adults by 1% dextran sedimentation and gradient separation on Histopaque-1077. This procedure yields a PMN population that is 95–98% viable (Trypan blue exclusion) and 98% pure (acetic acid-crystal violet staining).

For the adhesion assay, 5 × 10⁶ PMN/ml were radiolabeled with 50 µCi Na⁵¹Cl in PBS for 60 min at 37°C. Radiolabeled PMN (5 × 10⁷/well) were added to HUVEC monolayers grown in 48-well plates (Falcon), and 30 min later the percentage of added PMN that remained adherent after a wash procedure was quantitated as follows: %PMN adherence = lysate (cpm)/[supernatant (cpm) + wash (cpm)] + lysate (cpm), where cpm is counts per minute (71).

Statistical analysis. All of the values are presented as means ± SE. Statistical analysis was performed by ANOVA and Student’s t-test for the comparisons. A value of \( P < 0.05 \) was considered to be statistically significant.

RESULTS

To determine whether PMN infiltration into liver during experimentally induced sepsis can be prevented by CORM-2-released CO the livers obtained from CLP-challenged (24 h) mice were assessed for MPO activity (as an index of PMN accumulation). As shown in Fig. 1, there was a significant accumulation of PMN in the livers of CLP-challenged mice compared with sham-operated animals, an effect, which was significantly reduced by the systemic (intravenous) administration of CORM-2.

The increase in MPO activity was associated with the upregulation of vascular endothelial cell proadhesive phenotype in the liver of septic mice, as assessed by the increase in ICAM-1 expression in the liver of CLP-challenged mice (Fig. 2).

The regulation of systemic inflammatory response with respect to the upregulation of the vascular proadhesive phenotype is intimately controlled by the nuclear transcription factor, NF-κB (11, 36). Previous studies have shown that activation of NF-κB is a key event in the pathogenesis of sepsis and is associated with the increased expression of adhesion molecules such as E-selectin and ICAM-1 (36, 38, 39). Therefore, in parallel experiments we assessed activation (nuclear localization) of the inflammation-relevant transcription factor NF-κB at the whole organ level. As shown in Fig. 3, the nuclear levels of NF-κB (EMSA assay) were elevated in the mouse liver as assessed 24 h following CLP induction. Interestingly, administration of CORM-2 was effective in attenuating NF-κB activation (Fig. 3).

In the next series of experiments we assessed the potential mechanisms associated to the anti-inflammatory effects of CORM-2-released CO by employing in vitro model of sepsis (LPS stimulation) and vascular endothelial cell (HUVEC) culture.

Our previous studies indicate that oxidants and nitric oxide are important mediators of tissue inflammation and injury during sepsis (39). Therefore, we assessed the potential role CORM-2-released CO on the production of intracellular ROS and NO. As shown in Figs. 4, stimulation of HUVEC with LPS resulted in an increased production of both, ROS (Fig. 4A) and NO (Fig. 4B).

A representative experiment is shown in A, and quantitative results (densitometric analysis) of 4 experiments are shown in B. \( *P < 0.01 \) compared with sham; \( #P < 0.05 \) compared with CLP; \( n = 4 \).
toxic effects on HUVEC as assessed by Trypan blue exclusion assay (data not shown).

Importantly, LPS-induced increase in NO production was associated with the increase in iNOS protein expression (Fig. 4, C and D) and elevation of NO2/NO3 levels (Griess reaction) in the cell culture medium (data not shown), the effects, which were significantly reduced in LPS-stimulated CORM-2-treated HUVEC.

It has been demonstrated that increased production of ROS leads to activation of inflammation-relevant transcription factor, NF-κB (8, 9, 11, 36). Therefore, in the next series of experiments we assessed the effects of CORM-2-liberated CO on LPS-induced activation of NF-κB in HUVEC. As shown in Fig. 5, stimulation of HUVEC with LPS resulted in an induction of NF-κB activation (EMSA assay), which was prevented by administration of CORM-2 in a dose-dependent manner.

In parallel, inhibition of NF-κB activation by CORM-2-released CO was associated with the reduced expression of total ICAM-1 protein (Western blot) in LPS-stimulated HUVEC (Fig. 6, A and B). In addition, CORM-2 was also effective in reducing the cell surface levels of ICAM-1 (cell ELISA) in HUVEC stimulated with LPS (Fig. 6C). The latter changes were accompanied by CORM-2-dependent attenuation of PMN adhesion to LPS-stimulated HUVEC in a dose-dependent manner (Fig. 7).

DISCUSSION

The development of sepsis in surgical, burn, and trauma patients is a substantial cause of morbidity and the leading cause of mortality in the intensive care units worldwide. Sepsis-related mortality frequently results from multiple organ failure, which is characterized by hepatic failure, impaired pulmonary function (acute respiratory distress syndrome), cardiac dysfunction, acute renal failure, and disseminated intravascular coagulation (35, 40). The liver is a unique organ than houses an elaborate vasculature to handle an enormous volume of blood (as much as 25% of the cardiac output) and thus is one of the first organs affected by systemic inflammation (6). Induction of the proinflammatory response in the liver varies depending on the nature (e.g., bacterial vs. nonbacterial) and magnitude of the initial inflammatory stimulus, and is characterized by a marked hepatic infiltration of PMN.

![Fig. 4. Effects of CORM-2 on intracellular production of reactive oxygen species (ROS) and NO in LPS-stimulated human umbilical vein endothelial cells (HUVEC). HUVEC were grown to confluence in 48-well cell culture plates and loaded with dihydrorhodamine 123 (DHR 123) or diaminofluorescein-FM (DAF-FM) for 1 h. Subsequently, HUVEC were stimulated with LPS (1 μg/ml) for 4 h in the presence or absence of CORM-2 (10, 50, 100 μM). Control cells were treated with 0.25% DMSO. Oxidative stress (DHR 123 oxidation) (A) and NO production (DAF-FM nitrosation) (B) along with the expression of inducible nitric oxide synthase (iNOS) protein (Western blot; C) were assessed 4 h after LPS stimulation. Densitometric analysis of iNOS protein expression. For A and B, n = 5 in triplicate; for C and D, n = 3. *P < 0.05 compared with control (unstimulated cells); #P < 0.05 compared with LPS-only stimulated cells.

Fig. 3. Effects of CORM-2 on NF-κB activation in the liver of CLP-challenged mice. Experimental conditions were the same as described in Fig. 1. A: NF-κB activation (nuclear accumulation of NF-κB proteins) was assessed in the liver of sham and CLP-challenged mice by electrophoretic mobility shift assay (EMSA) followed by densitometric analysis (B); n = 4. *P < 0.01 compared with sham; #P < 0.05 compared with CLP.

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leukocyte integrin immunoglobulin superfamily and functions as a ligand for theleum is ICAM-1 (CD54). ICAM-1 is a member of the molecules (e.g., vascular P- and E-selectins and L-selectins) localized on circulating leukocytes). One of the key molecules responsible for the firm PMN adhesion to the vascular endothelium and PMN (19, 28, 40). Neutrophil tethering and the expression of adhesion molecules on the surface of vascular endothelium serves as a prime marker of vascular activation and correlates with the infiltration of PMN into affected organs (11, 16, 45, 50, 52). The mechanisms involved in organ-specific upregulation of the proinflammatory phenotype during systemic inflammation are unclear, but transcriptional events appear to be important (17, 37). One transcriptional factor that is believed to play a key role in the expression of proinflammatory response is the inducible nuclear transcription factor, NF-κB (1, 7, 29). NF-κB is a ubiquitous, rapidly acting transcription factor involved in immune and inflammatory reactions and has been implicated in the regulation of a number of genes products which contribute to the further amplification of inflammation. That would include induction of endothelial cell adhesion molecule (e.g., ICAM-1 and E-selectin) and proinflammatory cytokine expression (5, 11, 12, 36). In addition, a key role of NF-κB in modulation of inflammatory reactions and regulation of liver regeneration in experimental models of systemic inflammation has also been shown (27, 26, 57).

One of the potent regulators of the inflammatory response is inducible HO-1, an enzyme that catalyzes the formation of CO, biliverdin/bilirubin, and ferrous iron and exhibits anti-inflammatory properties that are beneficial for the resolution of inflammation (24, 33, 55, 62, 69). Several studies have demonstrated beneficial anti-inflammatory effects of CO in preventing microvascular perfusion deficits and cellular injury in different organs during SIRS (42– 44, 53, 54, 62, 70). In addition, it has been demonstrated that CO suppresses LPS-induced proinflammatory cytokine production by macrophages and interferes with the upregulation of proadhesive phenotype in vascular endothelial cells (54, 63). However, most of the studies addressing the role of CO in modulation of inflamma-

Fig. 5. Effects of CORM-2 on NF-κB activation in HUVEC stimulated with LPS. HUVEC were grown to confluence in a 35-mm Petri dish and stimulated with LPS (1 μg/ml) in the presence or absence of CORM-2 (10, 50, 100 μM). Control cells were treated with 0.25% DMSO. NF-κB activation was assessed by EMSA 4 h following LPS stimulation. A: representative EMSA from 3 experiments. B: densitometric analysis of NF-κB EMSAs, n = 3. *P < 0.05 compared with control (unstimulated cells); #P < 0.05 compared with LPS-only stimulated cells.

In general terms, the systemic inflammatory response, as a consequence of polymicrobial infection, is an entirely normal host response to remove pathogens (primarily by the increased PMN recruitment to the afflicted sites). However, overwhelming accumulation of PMN usually leads to the further damage of the affected organs, thus contributing significantly to the development of multiple organ dysfunction syndrome (25, 41). PMN recruitment within the microvasculature of the liver is a multistep process and can be achieved by both adhesion molecule-dependent and independent pathways. PMN recruitment to the portal venules and arterioles are directly controlled by the expression of adhesion molecules on the surface of vascular endothelium and PMN (19, 28, 40). Neutrophil tethering and rolling is mainly mediated by the selectin family of adhesion molecules (e.g., vascular P- and E-selectins and L-selectins localized on circulating leukocytes). One of the key molecules responsible for the firm PMN adhesion to the vascular endothelium is ICAM-1 (CD54). ICAM-1 is a member of the immunoglobulin superfamily and functions as a ligand for leukocyte integrin αLβ2 (lymphocyte function-associated antigen-1), which mediates leukocyte adhesion in response to proinflammatory stimulus(i) imposed by cytokines, e.g., TNF-α and IL-1β, or bacterial products such as LPS (14, 15, 35, 56, 68). It is also agreed that upregulation of ICAM-1 on microvascular endothelium serves as a prime marker of vascular activation and correlates with the infiltration of PMN into affected organs (11, 16, 45, 50, 52). The mechanisms involved in organ-specific upregulation of the proinflammatory phenotype during systemic inflammation are unclear, but transcriptional events appear to be important (17, 37). One transcriptional factor that is believed to play a key role in the expression of proinflammatory response is the inducible nuclear transcription factor, NF-κB (1, 7, 29). NF-κB is a ubiquitous, rapidly acting transcription factor involved in immune and inflammatory reactions and has been implicated in the regulation of a number of genes products which contribute to the further amplification of inflammation. That would include induction of endothelial cell adhesion molecule (e.g., ICAM-1 and E-selectin) and proinflammatory cytokine expression (5, 11, 12, 36). In addition, a key role of NF-κB in modulation of inflammatory reactions and regulation of liver regeneration in experimental models of systemic inflammation has also been shown (27, 26, 57).

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Fig. 6. Effects of CORM-2 on ICAM-1 protein expression in LPS-stimulated HUVEC. Experimental conditions were the same as described in Fig. 4. Total ICAM-1 protein expression and β-actin (loading control) (A) and cell surface levels of ICAM-1 (C) were assessed by Western blotting and cell ELISA, respectively. 4 h following LPS stimulation. A representative Western blot is shown in A, and quantitative results (densitometric analysis) are shown in B. *P < 0.01 compared with unstimulated cells; #P < 0.05 compared with LPS-only stimulated cells; n = 3.
Oxidative stress is believed to be the major causative mediator to directly (e.g., through cell membrane lipid peroxidation) or indirectly [through the cell signaling pathway(s), e.g., NF-κB-dependent signaling] affect organs during inflammation. Tissue or organ injury/dysfunction during SIRS appears to be mediated by both ROS, such as superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (OH$^-$), and also reactive nitrogen species, such as peroxynitrite (ONOO$^-$) (21, 22, 32).

As it has been shown previously, iNOS-derived NO is a major source for ONOO$^-$ formation and thus can be involved in modulation of inflammatory response (9, 32). However, the role of NO with respect to modulation of leukocyte recruitment to the affected organs remains controversial. In regard to the latter, numerous studies have demonstrated both pro- and anti-inflammatory effects of iNOS-derived NO, a phenomenon that appears to be largely dependent on the model of inflammation used (i.e., acute vs. chronic inflammation), organ and cell-type investigated, and even the route of drug (e.g., NOS inhibitors) administration. It also has been shown that dual action of NO (i.e., pro- vs. anti-inflammatory effects) depend on the quantity and intracellular location where NO is produced (3, 4, 21, 31, 32).

In the present study, we found that stimulation of HUVEC with LPS results in an increased production of intracellular ROS and NO, the effects of which were significantly reduced by CORM-2-liberated CO. In addition, treatment of HUVEC with CORM-2 during LPS stimulation resulted in attenuation of LPS-induced iNOS expression at the protein level and reduced NO production as assessed by NO$_2$ and NO$_3$ levels (Griess reaction) in the cell medium (data not shown).

The manner in which CO interferes with iNOS expression and/or activity remains unclear. However, several studies have demonstrated that CO directly [by interfering with the heme group activity of NOS enzyme (61)] or indirectly [through inhibition of NF-κB activation (63)] can modulate iNOS protein activity and expression, respectively. The results of the present study indicate that protective effects of CORM-2-derived CO are associated with the decreased iNOS expression most likely as a result of suppressed NF-κB activation in CORM-2-treated endothelial cells. Such reduced iNOS expression, therefore, may be associated with the decreased NO availability and potential decrease in peroxynitrite production during sepsis.

Although it is important to mention that the antioxidant potential of CO is a subject of some controversy, our data strongly suggest that CORM-2-released CO can directly interfere with the production of intracellular ROS. Recent findings indicate that CO derived from enhanced HO-1 activity or from CORM-2 inhibits activity of NADPH oxidase and therefore suppresses O$_2^-$ overproduction and the accumulation of ROS in LPS-stimulated macrophages (64).

In conclusion, we demonstrated that application of CO-releasing compounds, such as CORM-2, was a successful treatment attenuating the inflammatory effects normally associated with sepsis. The mechanism by which CORM-2-derived CO offers anti-inflammatory effects appears to be through the reduction of cellular oxidative stress, suppression of NF-κB activation, and subsequent downregulation of NF-κB-dependent expression of vascular endothelial cell adhesion molecule(s). These results suggest that use of such CO-releasing
molecules may find a place in the clinical management of sepsis or SIRS.

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