Suppression of Inflammatory Cytokine Production by Carbon Monoxide Involves the JNK Pathway and AP-1*

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The stress-inducible protein heme oxygenase-1 provides protection against oxidative stress and modulates pro-inflammatory cytokines. As the sepsis syndrome results from the release of pro-inflammatory mediators, we postulated that heme oxygenase-1 and its enzymatic product CO would protect against lethality in a murine model of sepsis. Mice treated with a lethal dose of lipopolysaccharide (LPS) and subsequently exposed to inhaled CO had significantly better survival and lower serum interleukin (IL)-6 and IL-1β levels than their untreated counterparts. In vitro, mouse macrophages exposed to LPS and CO had significantly attenuated IL-6 production; this effect was concentration-dependent and occurred at a transcriptional level. The same effect was seen with increased endogenous CO production through overexpression of heme oxygenase-1. Mutation within the AP-1-binding site in the IL-6 promoter diminished the effect of CO on promoter activity, and treatment of macrophages with CO decreased AP-1 binding in an electrophoretic mobility shift assay. Electrophoretic mobility supershift assay indicated that the JunB, JunD, and c-Fos components of AP-1 were particularly affected. Upstream of AP-1, CO decreased JNK phosphorylation in murine macrophages and lung endothelial cells. Mice deficient in the JNK pathway had decreased serum levels of IL-6 and IL-1β in response to LPS compared with control mice, and no effect of CO on these cytokine levels was seen in Jnk1 or Jnk2 gene-deleted mice. In summary, these results suggest that CO provides protection in a murine model of sepsis through modulation of inflammatory cytokine production. For the first time, the effect of CO is shown to be mediated via the JNK signaling pathway and the transcription factor AP-1.

Heme oxygenase-1 (HO-1) is a microsomal enzyme responsible for the degradation of heme, generating biliverdin, iron, and carbon monoxide (CO). Induction of HO-1 has been reported in response to a wide variety of oxidant stimuli, and this enzyme is a vital component of cellular adaptation to stress. There is growing interest in the role of CO in the anti-inflammatory and cytotoxic function of HO-1. It has recently been demonstrated that the administration of exogenous CO inhibits lipopolysaccharide (LPS)-induced production of tumor necrosis factor-α while increasing interleukin (IL)-10 production both in vitro and in vivo (1). This effect is independent of the guanylyl cyclase/cGMP pathway, and at least in the case of tumor necrosis factor-α, the effect is post-transcriptional. More recently, Fujita et al. (2) demonstrated that inhaled CO can rescue HO-1-deficient mice from lethal ischemic lung injury, further strengthening the paradigm of CO as a direct mediator of cellular protection.

The sepsis syndrome is the leading cause of death in intensive care units in the United States, and its incidence continues to rise. Despite an increasing incidence, little impact on associated mortality rates has been made over the past several decades (3). It has long been recognized that invading microorganisms induce the release of a large number of humoral and cellular pro-inflammatory mediators, causing a systemic inflammatory response syndrome (4). Elevated plasma concentrations of a variety of cytokines and chemokines such as tumor necrosis factor-α, IL-1, and IL-6 have been described in septic patients (5, 6). IL-6 is one of the most reliably elevated markers in sepsis (7) and one of the few serum markers that has a demonstrable correlation with mortality rates in humans (8). Knowing the anti-inflammatory effects of CO, we hypothesized that CO would protect against lethality in an animal model of sepsis. We further postulated that modulation of IL-6 production may contribute to the salutary effects of CO. We tested these hypotheses using a model of LPS-induced sepsis syndrome.

MATERIALS AND METHODS

Animals—Male C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME); male Jnk1−/− and Jnk2−/− mice and the corresponding control mice were generated as previously described (9). Mice were allowed to acclimate for 1 week with rodent chow and water ad libitum.

Cell Culture Experiments—RAW 264.7 mouse peritoneal macrophages and MH-S mouse alveolar macrophages were purchased from American Type Culture Collection (Manassas, VA). Both cell types were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 100 μg/ml gentamycin in a humidified atmosphere of

enhancer-binding protein; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase.
CO Protects against Lethality in a Mouse Model of the Sepsis Syndrome—We used a murine LPS model to assess the effect of inhaled low dose CO on survival. C57BL/6 mice were treated with a lethal dose of intraperitoneal LPS and subsequently exposed to 250 ppm CO or ambient air. As shown in Fig. 1, the CO-treated animals had significantly better survival than their untreated counterparts. A low dose of CO is thus able to rescue animals from lethality due to LPS.

CO Suppresses IL-6 Production via JNK—As inflammatory cytokines are known to play a role in the pathogenesis of the sepsis syndrome, we chose to study the effects of CO on cytokine production in our murine model. It has previously been demonstrated by our laboratory (1) that CO attenuates LPS-induced production of tumor necrosis factor-α and augments the production of IL-10 in vitro. To assess whether the effect of CO extends to IL-6 and IL-1β, we administered a sublethal dose of LPS (1 mg/kg) to mice in the presence or absence of inhaled CO (250 ppm). Mice treated with CO had lower serum levels of IL-6 and IL-1β 5 h after LPS injection than control mice as measured by ELISA (Fig. 2).

HO-1 and CO Decrease IL-6 Expression in Vitro—To confirm the effect of CO on IL-6 in vitro, we exposed two cell lines of mouse macrophages (RAW 264.7 and MH-S) to 1 μg/ml LPS in the presence or absence of CO and assessed IL-6 production by ELISA. No IL-6 was detectable in the cell medium from untransfected macrophages or from macrophages treated with CO alone. Cells exposed to LPS had an elevated level of IL-6 in the medium after 5 h, but this increase in IL-6 was significantly attenuated by exposure to CO (Fig. 3, A and B). The effect of CO was dose-dependent over a range of 50–500 ppm (Fig. 3C). To determine whether increased intracellular CO production would have the same effect as exogenously administered CO, we exposed a macrophage cell line (RAW 264.7) overexpressing HO-1 to 1 μg/ml LPS and assayed for IL-6 by ELISA. The control cells were transfected with the neomycin gene. The control cells exhibited a much greater increase in IL-6 levels than did the HO-1-overexpressing cells (Fig. 3D), suggesting that augmented intracellular CO production has an effect similar to that of exogenous administration of CO.

CO Suppresses IL-6 mRNA Expression in RAW 264.7 Macrophages—We assessed the expression of IL-6 mRNA in RAW 264.7 macrophages exposed to 1 μg/ml LPS in the presence or absence of CO. Cells were harvested 5 h after LPS treatment for RNA extraction and analysis by Northern blotting. As shown in Fig. 4, the untreated cells and those treated with CO alone did not have detectable IL-6 mRNA by Northern blotting. LPS caused a large increase in IL-6 mRNA production, and this was abrogated by treatment with CO. This demonstrates that CO affects IL-6 production in RAW 264.7 cells at a transcriptional level.

CO Exerts Its Greatest Effect via AP-1—Regulation of IL-6 gene expression is controlled by the binding of transcription factors to known consensus sequences within the promoter region, including binding sites for AP-1, NF-κB, or C/EBP-β, C/EBP-δ, or c-Fos-δ. In our experiments, we have shown that CO inhibits the binding of these factors to their consensus sequences in the IL-6 promoter. This suggests that CO may act to repress IL-6 gene expression by inhibiting the binding of AP-1, NF-κB, or C/EBP-β to their consensus sequences.
To identify which cis-regulatory sequences might be responsible for down-regulation of IL-6 by CO, RAW 264.7 macrophages were transiently transfected with wild-type or mutant constructs of the human IL-6 promoter bound to a luciferase reporter. Four promoter constructs were used, three with site-directed mutations in the AP-1-, NF-kB-, or C/EBP-β-binding sequence and a parental construct. Luciferase activity was induced by LPS treatment of cells transfected with all mutant constructs to at least the same level as the parental construct. CO treatment decreased luciferase activity to a significant degree in all constructs except the AP-1 mutant construct (Fig. 5).

**CO Attenuates AP-1 Binding in Vitro**

To confirm that CO can inhibit the activation of AP-1, EMSAs were performed.
Northern blot analysis. CO treatment resulted in decreased IL-6 mRNA
expression of 250 ppm CO and was analyzed for IL-6 mRNA expression by
in vitro
mutated NF-
phages were transiently transfected with wild-type or mutant con-
structs. CO treatment decreased luciferase activity to the least extent
activity was induced by LPS treatment of cells transfected with all con-
structs. CO treatment decreased luciferase activity to the least extent in
the AP-1 mutant construct, where the effect of CO was not statisti-
cally significant. *, p < 0.05 compared with LPS alone; #, p < 0.005
compared with LPS alone.

using nuclear extracts from RAW 264.7 macrophages treated
with LPS alone or with LPS and CO (250 ppm). Cells were
harvested 30 min after LPS treatment. As shown in Fig. 6, this
concentration of CO was capable of attenuating LPS-stimu-
lated AP-1 binding in macrophages.

To assess the specificity of the AP-1 complex and to identify
the contributing family members, supershift experiments were
conducted by adding antibodies against c-Jun, JunB, JunD,
and c-Fos to the nuclear extracts before EMSA. Protein-anti-
body recognition can be visualized by a decrease in the mobility
and c-Fos to the nuclear extracts before EMSA. Protein-anti-
carried out by adding antibodies against c-Jun, JunB, JunD,
and C/EBPα to a lesser extent, JunB and JunD. Correspondingly, treatment of cells
with CO appears to affect c-Fos to the greatest extent and JunB and
JunD to a lesser extent.

FIG. 5. CO exerts its greatest effect via AP-1. RAW 264.7 macro-
phages were transiently transfected with wild-type or mutant con-
structs of the human IL-6 promoter bound to a luciferase reporter. The
mutated NF-κB, C/EBPα, and AP-1 sites are indicated. Luciferase ac-
This figure shows the effect of CO on IL-6 mRNA expression in
vitro. Total RNA was isolated from RAW 264.7 cells after treatment with LPS (1 μg/ml) in the presence or absence of CO (250 ppm), and nuclei were extracted for AP-1 EMSA at 30 min. Lane 1, control; lane 2, LPS stimulation; lane 3, CO alone; lane 4, LPS + CO; lane 5, specific competitor; lane 6, nonspecific competitor. Comparing lanes 2 and 4, treatment with CO decreased AP-1 after LPS stimulation.

FIG. 6. CO inhibits AP-1 activation in vitro. RAW 264.7 cells were
exposed to LPS (1 μg/ml) in the presence or absence of CO (250 ppm),
and nuclei were extracted for AP-1 EMSA at 30 min. Lane 1, control; lane 2, LPS stimulation; lane 3, CO alone; lane 4, LPS + CO; lane 5, specific competitor; lane 6, nonspecific competitor. Comparing lanes 2 and 4, treatment with CO decreased AP-1 after LPS stimulation.

FIG. 7. Supershift EMSA indicating that the AP-1 complex is
composed of JunB, JunD, and c-Fos. Antibodies against c-Jun
(sc-44, first lanes) and sc-1894 (second lanes), JunB, JunD, and c-Fos
were added to nuclear extracts (prepared as described in the legend
to Fig. 6) prior to EMSA. The AP-1 complex consists mainly of c-Fos and,
to a lesser extent, JunB and JunD. Correspondingly, treatment of cells
with CO appears to affect c-Fos to the greatest extent and JunB and
JunD to a lesser extent. RA, room air.

Deletion of the Jnk1 or Jnk2 Gene Inhibits IL-1 and IL-6
Production in Vivo—To investigate whether the JNK pathway
may mediate the effect of CO on LPS-induced IL-6 and IL-1
production, we used mice deficient in either the Jnk1 or Jnk2
gene. Each gene is capable of making various isoforms of JNK,
which may mediate the effect of CO on LPS-induced IL-6 and IL-1
production. CO treatment of cells transfected with all constructs. CO treatment decreased luciferase activity to the least extent in the AP-1 mutant construct, where the effect of CO was not statistically significant. *, p < 0.05 compared with LPS alone; #, p < 0.005 compared with LPS alone.

later time points were also tested, but CO had no effect up to 3 h (data
not shown). p-JNK, phosphorylated JNK.

FIG. 8. CO inhibits JNK phosphorylation after stimulation with
LPS. RAW 264.7 macrophages were stimulated with LPS (1
μg/ml) and exposed to either CO (250 ppm) or ambient air (room air
(RA)). Cells were collected at times from 0 to 30 min for Western blot
analysis of JNK phosphorylation. JNK phosphorylation was inhibited
by CO treatment at 5 and 15 min, but this effect was lost by 30 min.
Later time points were also tested, but CO had no effect up to 3 h (data
not shown). p-JNK, phosphorylated JNK.

Deletion of the Jnk1 or Jnk2 Gene Inhibits IL-1 and IL-6
Production in Vivo—To investigate whether the JNK pathway
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gene. Each gene is capable of making various isoforms of JNK,
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production. CO treatment of cells transfected with all constructs. CO treatment decreased luciferase activity to the least extent in the AP-1 mutant construct, where the effect of CO was not statistically significant. *, p < 0.05 compared with LPS alone; #, p < 0.005 compared with LPS alone.

later time points were also tested, but CO had no effect up to 3 h (data
not shown). p-JNK, phosphorylated JNK.
IL-1 and IL-6 in response to LPS injection and abrogates the effect of CO. Wild-type (WT), Jnk1−/−, or Jnk2−/− mice were injected with a non-lethal dose of LPS, and cytokine levels were measured at time 0, 1 h, and 5 h in the presence or absence of CO. A, IL-1β levels were significantly higher in control mice than in either the Jnk1−/− or Jnk2−/− mice at 1 h, but by 5 h, this difference was no longer present. At this same time point, the wild-type mice had significantly lower levels of IL-1β as described under Results, but CO had no effect on IL-β levels in the Jnk1−/− and Jnk2−/− mice. ⋆, p < 0.005 compared with the wild-type mice; #, p < 0.05 compared with the 5-h control; ¶, p < 0.05 compared with the wild-type mice in the presence of CO. B, 5 h after injection, IL-6 levels were significantly lower in the Jnk1−/− and Jnk2−/− mice than in the control mice, and there was no effect of CO on the levels of IL-6 in the Jnk1−/− and Jnk2−/− mice. ⋆, p < 0.065 compared with the wild-type mice; ¶, p < 0.07 compared with the wild-type mice; #, p < 0.05 compared with the wild-type mice without CO (n = four to five mice in each group).

IL-β levels in the Jnk1−/− and Jnk2−/− mice (Fig. 9A). Similarly, IL-6 (which is slower to increase after LPS injection compared with IL-1β) was significantly less in the Jnk1−/− and Jnk2−/− mice than in the control mice at 5 h, and there was no effect of CO on the levels of IL-6 in the Jnk1−/− and Jnk2−/− mice (Fig. 9B). This indicates not only that the JNK pathway is involved in the production of IL-1β and IL-6 in response to LPS, but that the attenuation of this pathway diminishes or abrogates the effect of CO.

Deletion of the Jnk1 Gene Abrogates the Survival Effect of CO in Response to a Lethal Dose of LPS—To determine whether deletion of the Jnk1 gene affects survival in response to LPS, these mice were injected with a lethal dose of LPS and subsequently exposed to room air or CO (250 ppm). At 24 h, the average length of survival was 28–29 h in both groups (Fig. 10), indicating that CO had no effect on survival. Interestingly, the Jnk1−/− mice had a longer survival than the wild-type mice shown in Fig. 1.

**Discussion**

The sepsis syndrome remains the leading cause of death in intensive care units in the United States, and efforts to impact mortality have been largely unsuccessful (3). Evidence from human and animal studies demonstrates that HO-1 is induced by sepsis, and this induction confers a survival advantage. Both increased carboxyhemoglobin concentration and increased breath CO excretion have been observed in patients with sepsis (15, 16). Bacterial LPS administration to rats induces HO-1 expression (17), and in a rodent model of hemorrhagic shock, induction of HO-1 has been shown to be protective and inhibition of HO-1 deleterious (18). Similarly, in a murine cecal ligation model, inhibition of HO-1 leads to increased mortality (19). The mechanism by which HO-1 provides cellular protection remains a subject of avid investigation; all of the products of heme catabolism have been implicated, including bilirubin, ferritin (stimulated by the release of iron), and CO (20–22). Here, we have examined the effect of exogenously administered CO in a murine LPS model of septic shock and have shown that CO reduces mortality.

There is a well described correlation between serum IL-6 levels and poor outcome in sepsis (8, 23–26), and increased IL-6 is an independent predictor of survival in human sepsis (27). In our mouse model, serum IL-6 and IL-1β were markedly attenuated by exogenous CO administration. The low cytokine levels could result from any indirect action of CO that improves the pathophysiology of sepsis, but our in vitro data suggest that CO has a direct effect on cytokine production. The attenuation of IL-6 production by exogenous CO in vitro occurred even at very low concentrations of CO (50 ppm). For comparison, this concentration of CO is close to the levels that can routinely be measured in the exhaled breath of healthy individuals who smoke (30 ppm) (28).

Using Northern blotting, we have shown that the in vitro attenuation of LPS-induced IL-6 production occurs at a transcriptional level. Our study using IL-6 promoter constructs with mutations at each of three transcription factor-binding sites (NF-κB, AP-1, and C/EBP) suggests that the primary action of CO may be through modulation of AP-1. This interpretation must be made cautiously, however, as mutation of any single binding site had no effect on the overall level of promoter stimulation by LPS, implying that no single transcription factor is absolutely required for IL-6 induction in response to LPS. Also, there was a trend toward decreased IL-6
CO Suppresses IL-6 Production via JNK

CO-pretreatment of human monocytes leads to a profound decrease in IL-6 production. The effect of CO is mediated by inhibition of AP-1 activity, as demonstrated by the following studies:

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