Ketamine-induced hepatoprotection: the role of heme oxygenase-1

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Lipopolysaccharide (LPS) causes hepatic injury that is mediated, in part, by upregulation of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). Ketamine has been shown to prevent these effects. Because upregulation of heme oxygenase-1 (HO-1) has hepatoprotective effects, as does carbon monoxide (CO), an end product of the HO-1 catalytic reaction, we examined the effects of HO-1 inhibition on ketamine-induced hepatoprotection and assessed whether CO could attenuate LPS-induced hepatic injury. One group of rats received ketamine (70 mg/kg ip) or saline concurrently with either the HO-1 inhibitor tin protoporphyrin IX (50 μmol/kg ip) or saline. Another group of rats received inhalational CO (250 ppm over 1 h) or room air. All rats were given LPS (20 mg/kg ip) or saline 1 h later and euthanized 5 h after LPS or saline. Liver was collected for iNOS, COX-2, and HO-1 (Western blot), NF-κB and PPAR-γ analysis (EMSA), and iNOS and COX-2 mRNA analysis (RT-PCR). Serum was collected to measure alanine aminotransferase as an index of hepatic injury in combination with the selective heme oxygenase inhibitor tin protoporphyrin IX (SnPPN) to examine ketamine-induced hepatic injury from endotoxin shock.

Patients who have sustained severe injury, whether due to trauma or septic insult, frequently require multiple surgical procedures and have prolonged stays in critical care units. During this time they are exposed to a variety of anesthetics, sedatives, and analgesics. However, the effects that these drugs have on end-organ function is not completely understood.

Ketamine is an agent that possesses analgesic, sedative, and anesthetic properties. Additionally, it has been shown to exhibit anti-inflammatory effects in some models of shock. Our previous work has demonstrated that ketamine is able to attenuate LPS-induced hepatic injury from endotoxic shock. Although upregulation of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) play an important role in mediating hepatic injury from endotoxin, the precise mechanism of action to explain the hepatoprotective effects of ketamine remains to be fully elucidated.

Heme oxygenase is a protein known to have anti-inflammatory effects. Heme oxygenase is central to the heme catabolism pathway: it converts heme, in the presence of oxygen, to biliverdin, carbon monoxide (CO), and free iron (Fe2+). Subsequently, biliverdin is converted to bilirubin by bilirubin reductase (BVR). Heme oxygenase has two main isoforms, both of which are responsible for regulating the catabolism of heme and the production of CO. The first, heme oxygenase-1 (HO-1), is an inducible protein; its expression is upregulated in response to cellular stress. The second, heme oxygenase-2 (HO-2) is a constitutive protein; its levels of expression rarely change. Although biliverdin, BVR, and bilirubin all possess some degree of antioxidant activity, it is well demonstrated that the anti-inflammatory properties of HO-1 are manifest primarily through the actions of CO.

Previous studies in our laboratory have shown that ketamine administration upregulates the expression of HO-1. Given the potent anti-inflammatory effects associated with HO-1, we hypothesized that ketamine-induced hepatoprotection is due to upregulation of HO-1. To test this hypothesis we used our rat model of lipopolysaccharide (LPS)-induced hepatic injury in combination with the selective heme oxygenase inhibitor tin protoporphyrin IX (SnPPN) to examine ketamine-induced hepatoprotection and its potential mechanisms.

MATERIALS AND METHODS

Animal model. Male Sprague-Dawley rats weighing ~200 g were used for all studies. Rats were housed at constant room temperature with a 12:12-h light-dark cycle. All experiments were performed with rats fasted 18 h prior to experiment but allowed free access to water. Noninfectious inflammation was induced by intraperitoneal (ip) injection of LPS from Escherichia coli 0111:B4, given as a single dose...
of 20 mg/kg body wt, whereas control rats received a comparable volume of normal saline (0.9%). Since this dose of LPS has previously been shown to reproducibly cause significant liver injury 5 h after its administration (30), rats were euthanized 5 h later and liver tissue and serum obtained and stored at −80°C until analysis (see Measurement of hepatic injury, Cytosolic protein extraction, Unclear protein extraction, and RT-PCR analysis, below). The University of Texas at Houston Animal Welfare Committee approved all experiments before any studies were carried out. All experiments were performed in adherence to the National Institutes of Health Guidelines on the Use of Laboratory Animals.

Inhibition of heme oxygenase activity. To examine the role of heme oxygenase in LPS-induced hepatic injury we utilized SnPPN (Frontier Scientific, Logan, UT), a potent competitive inhibitor of heme oxygenase activity that, when administered parenterally, produces a substantial time-dependent inhibition of heme oxygenase activity (3, 5, 20, 21). SnPPN was prepared within 1 h of use and protected from light by first dissolving it in 1 ml of 0.5 N NaOH, increasing the pH to 7.4 via the addition of 2 N HCl. Rats were given SnPPN (50 µmol/kg ip) or vehicle concurrently with ketamine (70 mg/kg ip) or saline 1 h prior to LPS or saline injection. Rats were euthanized 5 h after receiving LPS. Serum and tissue samples were collected.

Heme oxygenase activity. To confirm the inhibition of HO-1 activity by SnPPN in our animal model, we evaluated microsomal bilirubin production. Immediately after dissecting, segments of liver were separately snap frozen for analysis of bilirubin production as an index of heme oxygenase activity by measuring the rate of generation of bilirubin in microsomes isolated from the liver. Liver was homogenized in 5 vol of 0.1 M potassium phosphate buffer (pH 7.4) by using a Polytron followed by sonication on ice for 30 s. The homogenate was centrifuged at 3,000 × g at 4°C for 10 min, and the supernatant was removed. Protein concentrations of each sample were determined by the Lowry method.

Inhaled CO. To further examine the hepatoprotective role of HO-1, we administered inhaled CO, the end product of the HO-1 catalyzed reaction, and examined its effects in our LPS model of hepatic injury. Experimental rats were administered CO at a dose of 250 ppm over 1 h. Briefly, CO (1%) in air was premixed with filtered house air to deliver a final concentration of 250 ppm into a living environment. Flow into the environment was maintained at 12 l/min. A CO analyzer (Interscan, Chatsworth, CA) was used to confirm CO levels within the environment, and there were no fluctuations in the CO concentrations after the chambers had equilibrated. Control rats were administered room air only, also for 1 h. After the administration of CO or room air, rats received ip PBS or saline as described above. Rats were euthanized, and serum and tissue were collected as described above.

Measurement of hepatic injury. The release of the hepatocellular enzyme alanine aminotransferase (ALT) into the bloodstream was used as an index of hepatic injury. ALT was determined quantitatively by kinetic assays (Pointe Scientific, Canton, MI).

Cytosolic protein extraction. Extraction of cytosolic proteins for Western immunoblot was performed by grinding frozen tissue under mortar and pestle in liquid nitrogen slurry. Ground samples were added to 1 ml of lysis buffer (10 mM Tris, 100 mM polymethylsulfonyl fluoride, 10 µg/ml soybean trypsin inhibitor, 10 µg/ml protease inhibitor cocktail-P8340 (Sigma Chemical, St. Louis, MO), and 0.5% NP-40) and subjected to 20-s bursts of a Polytron. Homogenates were centrifuged at 11,000 × g for 12 min at 4°C and the supernatant was removed. Protein concentrations of each sample were determined by the bicinchoninic acid protein assay (Bio-Rad, Hercules, CA). Homogenate samples were then added to sample buffer (125 mM Tris, pH 6.8, 2% sodium dodecyl sulfate, 5% glycerol, 1% β-mercaptoethanol, and 0.003% bromphenol blue).

iNOS, COX-2, and HO-1 analysis: Western immunoblot. Proteins were separated via 10% polyacrylamide gel electrophoresis (PAGE) using 80 µg/well of sample for iNOS and COX-2 as well as 7.5% PAGE for HO-1. Resultant proteins were electroblotted onto nitrocellulose membranes and incubated for 1 h at room temperature in blocking solution (5% nonfat dried milk, 0.1% Tween 20, and PBS). The resultant blot was washed three times for 10 min each in 0.1% Tween 20 PBS, followed by an overnight incubation at 4°C with

Table 1. Primers and probes used for quantitative real time RT-PCR

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Primer and Probe Sequences</th>
<th>Accession Number</th>
<th>Amplicon Length</th>
<th>Lowest Limit of Detection</th>
<th>PCR Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat iNOS</td>
<td>1694(+): GAGCCCGAGGAGGAGGAGG 1768(-): TtokcGATACGCGAGAGGAGG</td>
<td>NM_012611</td>
<td>86 bases</td>
<td>170 copies or molecules</td>
<td>100%</td>
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<tr>
<td>Rat COX-2</td>
<td>1708(+): GACCAGCAGCATACCAGG 1784(-): ACTGTAGTTAAATGTCTACAGC</td>
<td>NM_017232</td>
<td>80 bases</td>
<td>180 copies or molecules</td>
<td>100%</td>
</tr>
<tr>
<td>Rat 36B4</td>
<td>632(+): AGAGGTGTGACGACATGACAG 693(-): CAGGGCAGGACACTCTG</td>
<td>X15096</td>
<td>63 bases</td>
<td>230 copies or molecules</td>
<td>99%</td>
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<tr>
<td>Rat β-Actin</td>
<td>1264(+): TACCGGCTGCTGCTAGC</td>
<td>NM_031144</td>
<td>84 bases</td>
<td>170 copies or molecules</td>
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Table 2. Heme oxygenase activity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bilirubin Production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>Saline</td>
</tr>
<tr>
<td>SnPPN</td>
<td>Saline</td>
</tr>
</tbody>
</table>

Values are mean bilirubin production (pmol·h⁻¹·mg protein⁻¹) ± SE; n = 5 per group. LPS, lipopolysaccharide, 20 mg/kg intraperitoneal; SnPPN, tr protoporphyrin IX, 50 µmol/kg intraperitoneal. P < 0.05. *P < 0.05 vs. Saline-Saline-Saline. **P < 0.05 vs. Saline-Saline-LPS.
polyclonal antibody for iNOS and COX-2 (1:1,000 dilution, Cayman Chemical, Ann Arbor, MI) and monoclonal antibody for HO-1 (1:3,000 dilution, Stressgen Biotech, Sydney, BC, Canada). Blots were washed three times in 0.1% Tween 20 PBS and incubated for 2 h at room temperature with a horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin (Amersham, Arlington Heights, IL) as a secondary antibody (1:5,000 dilution) for iNOS and COX-2 and goat anti-mouse immunoglobulin (Santa Cruz Biotechnology, Santa Cruz, CA) as secondary antibody for HO-1 (1:5,000 dilution). After three final washes the immune complexes were visualized with the use of enhanced chemiluminescence detection (Amersham). Autoradiographs were assessed semiquantitatively by computer-assisted densitometry (Optimas 6.1) and reported as mean relative arbitrary units.

Nuclear protein extraction. Extraction of nuclear proteins was performed by use of a commercially available nuclear extraction kit (BioVision, Mountain View, CA). Cytosolic extraction buffer-A mix (CEB-A mix) was prepared using the provided cytosolic extraction buffer-A, protease inhibitor cocktail, and dithiothreitol (DTT). Cytosolic extraction buffer-B (CEB-B) was provided in the kit. Nuclear extraction buffer mix (NEB mix) was prepared using the provided nuclear extraction buffer, protease inhibitor cocktail, and DTT. Frozen tissue was ground under mortar and pestle in liquid nitrogen slurry. Ground tissue was suspended in 1 ml of cold PBS and homogenized with two 20-s bursts of the Polytron. Homogenates were centrifuged at 500 g for 3 min and the supernatant discarded. Pellets were suspended in 0.2 ml CEB-A mix, vortexed for 15 s, and then incubated on ice for 10 min. CEB-B (11 μlH9262) was added to the sample. After 1 min incubation on ice, the samples were again vortexed on high for 5 s and then centrifuged for 10 min at 16,000 g. The supernatant (cytosol) was collected and stored at −80°C. When the samples were repeatedly vortexed on high for 15 s and then incubated on ice...
for 10 min. Samples were then centrifuged at 16,000 g for 10 min, and the supernatant (nuclear extract) was collected and stored at −80°C.

**NF-κB and PPAR-γ analysis: EMSA.** To determine nuclear factor-κB (NF-κB) and peroxisome proliferator-activated receptor-γ (PPAR-γ) DNA binding activity within the nuclear fractions of gastric mucosa, electrophoretic mobility shift assays (EMSA) were performed. Ten micrograms of nuclear protein extract from each sample were incubated at room temperature for 10 min in binding buffer [20% glycerol, 0.25 mg/ml poly(dI-dC)-poly(dI-dC), 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM DTT, 50 mM Tris-HCl; pH 7.5]. For each transcription factor, a 32P-end-labeled double-stranded oligonucleotide containing the NF-κB binding consensus sequence (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) or the PPAR-γ consensus sequence (5′-CAA AAC TAG GTC AAA GGT CA-3′) was added to the reaction mixture for an additional 20 min at room temperature. Reaction products were separated on a nondenaturing 4% polyacrylamide gel. Blots were dried on a gel dryer and analyzed by exposure to radiographic film. The specificity of NF-κB and PPAR-γ binding was established by performing a competition assay in which an excess of cold oligonucleotide competitor was preincubated with nuclear extracts from each sample and binding buffer for 10 min at room temperature.

**RT-PCR analysis.** Specific quantitative assays for rat iNOS and COX2 mRNA were developed by using Primer Express software (PE Biosystems, Foster City, CA) as previously published (15). The primers and probes used in this study are listed in Table 1. Briefly, total RNA was extracted from gastric mucosa by homogenizing the tissue in RNAsol B (Tel-test, Houston, TX), and a two-phase solution was made by the addition of CHCl3 followed by shaking and centrifugation as recommended. The aqueous phase was added directly to an iScript (Qiagen, Valencia, CA) spin column by following the manufacturer’s protocol. cDNA was synthesized as previously described (17). Each plate contained an assay-specific synthetic RNA (sRNA) standard spanning a 5-log range in triplicate and a no-template control. The resulting data were analyzed by using SDS software (PE Biosystems, Foster City, CA) with ROX (Invitrogen, Carlsbad, CA) as the reference dye. The final data were normalized to β-actin and 36B4 and are presented as %β-actin transcripts: (molecules of transcript/molecules of β-actin transcript) × 100.

**Statistics.** All values are expressed as means ± SE of n observations where n represents the number of animals. For all experimental groups (n ≥ 5), statistical significance was determined by analysis of variance followed by Tukey’s post hoc test. A P value less than 0.05 was considered statistically significant.

**RESULTS**

**SnPPN inhibits hepatic heme oxygenase activity.** As shown in Table 2, liver from control rats receiving only saline exhibited an endogenous level of heme oxygenase activity, which increased with the addition of LPS. Heme oxygenase inhibition with SnPPN suppressed heme oxygenase activity by more than 50% both in the presence and absence of LPS and in the presence of ketamine.

**Table 3. Western blot: heme oxygenase-1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Immunoactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>Saline</td>
</tr>
<tr>
<td>Saline</td>
<td>Saline</td>
</tr>
<tr>
<td>Saline</td>
<td>Ketamine</td>
</tr>
<tr>
<td>Saline</td>
<td>Ketamine</td>
</tr>
<tr>
<td>SnPPN</td>
<td>Saline</td>
</tr>
<tr>
<td>SnPPN</td>
<td>Saline</td>
</tr>
<tr>
<td>SnPPN</td>
<td>Ketamine</td>
</tr>
<tr>
<td>SnPPN</td>
<td>Ketamine</td>
</tr>
</tbody>
</table>

Values are mean densitometric units ± SE; n ≥ 5 per group. *P < 0.05. Ketamine: 70 mg/kg intraperitoneal. **P < 0.05 vs. Saline-Saline-Saline. ***P < 0.05 vs. Saline-Ketamine/LPS.
Heme oxygenase inhibition prevents ketamine-induced hepatoprotection. As shown in Fig. 1, animals treated with LPS had significantly increased serum levels of ALT compared with control animals receiving saline, consistent with hepatic injury. Pretreatment with ketamine almost completely abolished the effects of LPS on ALT release, consistent with hepatoprotection. In contrast, heme oxygenase inhibition with SnPPN given in combination with ketamine reversed the ability of ketamine to attenuate LPS-induced increases in serum ALT. SnPPN administration by itself or in combination with LPS did not affect ALT release.

Heme oxygenase inhibition prevents the ability of ketamine to downregulate LPS-induced changes in iNOS. As shown in Fig. 2, LPS upregulated hepatic iNOS compared with saline controls, an effect partially reversed by ketamine. Heme oxygenase inhibition with SnPPN given in combination with ketamine reversed the ability of ketamine to attenuate LPS-induced increases in serum ALT. SnPPN administration by itself or in combination with LPS did not affect ALT release.

Heme oxygenase inhibition upregulates hepatic HO-1 expression. As shown in Table 3, LPS increased HO-1 expression in the liver compared with saline controls. Ketamine upregulated hepatic HO-1 expression in the presence and in the absence of LPS compared with saline counterparts. Heme oxygenase inhibition with SnPPN, alone or in combination with ketamine to the saline- or LPS-treated rats resulted in increased hepatic HO-1 expression.

Carbon monoxide attenuates LPS-induced hepatic injury. As shown in Table 4, in animals receiving room air, administration of LPS significantly increased serum levels of ALT compared with control animals receiving saline. Treatment with CO almost completely abolished the effects of LPS on ALT release, consistent with hepatoprotection.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Serum ALT</th>
</tr>
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<tbody>
<tr>
<td>Air</td>
<td>56.6±4.2</td>
</tr>
<tr>
<td>Air</td>
<td>107.5±6.0*</td>
</tr>
<tr>
<td>CO</td>
<td>51.8±1.7</td>
</tr>
<tr>
<td>CO</td>
<td>58.2±4.3**</td>
</tr>
</tbody>
</table>

Mean serum alanine aminotransferase (ALT, IU/l) ± SE; n = 5 per group. CO, carbon monoxide, 250 ppm over 1 h; Air, room air. *P < 0.05 vs. Air-Saline. **P < 0.05 vs. Air-LPS.

Heme oxygenase inhibition prevents the ability of ketamine to downregulate LPS-induced changes in hepatic COX-2. LPS likewise upregulated hepatic COX-2 compared with saline controls, an effect almost completely negated by ketamine (Fig. 3). Heme oxygenase inhibition with SnPPN given concurrently with ketamine not only prevented the ability of ketamine to downregulate LPS-induced changes in hepatic COX-2 but also resulted in COX-2 levels that were significantly more than those seen in rats receiving LPS. Similar effects were seen in rats not receiving ketamine, since LPS given to rats receiving SnPPN and saline had significantly more COX-2 expression in the liver than rats receiving LPS alone. In the absence of LPS, SnPPN did not have this effect.

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Carbon monoxide attenuates LPS-induced hepatic injury. As shown in Table 4, in animals receiving room air, administration of LPS significantly increased serum levels of ALT compared with control animals receiving saline. Treatment with CO almost completely abolished the effects of LPS on ALT release, consistent with hepatoprotection.

**Fig. 4. Effects of inhaled carbon monoxide (CO; 250 ppm) over 1 h or room air, 1 h prior to intraperitoneal saline or LPS (20 mg/kg), on nuclear factor-κB (NF-κB). A: densitometric analysis; data are means ± SE; n = 5 per group. *P < 0.02 vs. Air-Saline, **P < 0.001 vs. Air-LPS. B: representative electrophoretic mobility shift assay.**
Effects of CO on LPS-induced changes in transcription factors NF-κB and PPAR-γ. As shown in Fig. 4, in rats receiving room air, LPS-induced significant increases in NF-κB activity compared with saline-treated controls, an effect further increased by CO compared with LPS-treated controls. In contrast, LPS significantly diminished PPAR-γ activity compared with saline-treated controls (Fig. 5). However, CO reversed the effects of LPS on PPAR-γ activity compared with LPS-treated controls.

Effects of CO on LPS-induced changes in hepatic iNOS, COX-2, and HO-1. The effects of CO on LPS-induced changes in iNOS and COX-2 mRNA transcripts are shown in Table 5. As depicted, LPS upregulated expression of both iNOS and COX-2 compared with saline-treated controls. Interestingly, CO did not significantly affect the LPS-induced upregulation of iNOS or COX-2 transcripts when normalized to β-actin. When similar assessments were done at the protein level, CO completely prevented LPS from increasing hepatic iNOS immunoreactivity and significantly attenuated the effects of LPS on COX-2 immunoreactivity (Figs. 6 and 7). Lastly, in rats receiving room air, LPS upregulated hepatic HO-1 compared with saline controls, an effect prevented by administration of CO (Table 6).

DISCUSSION

This study confirms that ketamine possesses protective effects against LPS-induced hepatic injury (30, 45). More importantly, our data suggests that the heme oxygenase enzyme system plays an important role in mediating the beneficial effects of ketamine since heme oxygenase inhibition with SnPPN reversed the ability of ketamine to attenuate LPS-induced increases in serum ALT levels and iNOS and COX-2 proteins. Furthermore, when CO, the end product of the HO-1 catalytic reaction, was added to our LPS model, we found decreased levels of iNOS and COX-2 protein as well as a reduction in LPS-induced liver injury.

The heme oxygenase enzyme system represents an important anti-inflammatory pathway that has been shown to exhibit powerful protective effects against a variety of stressors in different organ systems (5, 6, 11, 27, 28, 36). The anti-inflammatory properties of HO-1 are secondary to production of metabolites generated during the catabolism of heme. These include biliverdin, free iron, and CO. Biliverdin and its reduced product bilirubin scavenge oxygen and hydroxyl radicals, as well as lipid hydroperoxides (13, 33, 40). Additionally, free oxygen molecules that are available to be converted into

Table 5. Quantitative real-time RT-PCR

<table>
<thead>
<tr>
<th>Treatment</th>
<th>iNOS</th>
<th>COX-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>0.0017 ± 0.0018</td>
<td>0.432 ± 0.089</td>
</tr>
<tr>
<td>Air</td>
<td>11.09 ± 34.4*</td>
<td>7.37 ± 2.42*</td>
</tr>
<tr>
<td>CO</td>
<td>2.46 ± 0.624</td>
<td>0.732 ± 0.199</td>
</tr>
<tr>
<td>CO</td>
<td>72.2 ± 16.9**</td>
<td>9.54 ± 2.64**</td>
</tr>
</tbody>
</table>

Values are mean %β-actin (molecules of transcript/molecules of β-actin transcript ×100) ± SE; n = 5 per group. iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2. *P < 0.05 vs. Air-Saline. **P < 0.05 vs. CO-Saline.
Cytotoxic free radicals are scavenged by HO-1 in the degradation of heme. HO-1-derived CO has been demonstrated to be an important cellular messenger and regulatory molecule in a variety of physiological functions, similar to those described for NO (8–10, 18, 38, 46). The signaling functions of CO are also similar to NO except that NO, by virtue of its function as a free radical, can react with other free radicals to form peroxynitrite, whose breakdown products are cytotoxic, whereas CO has mostly anti-inflammatory effects (39, 42). CO also regulates cGMP activity through activation of soluble guanylate cyclase, which has been shown to regulate endothelium-dependent vasodilatation and inhibit aggregation of platelets, thereby possibly improving the microvascular circulation (9, 12). In addition, CO contributes to downregulation of iNOS activity in two different ways: first, CO binds to its heme-iron moiety, thereby inhibiting production of NO, and second, heme degradation by HO-1 scavenges any available heme that may be used for iNOS dimer formation and NO production (32, 41). In this study, CO clearly downregulated iNOS expression according to protein assessments. However, CO did not prevent the LPS-induced increase of iNOS mRNA transcripts; thus it appears that the effects of CO on iNOS expression are primarily posttranscriptional. This is consistent with the findings of Kim et al. (22), who have shown that CO reduced cytokine-stimulated increases in NO production, iNOS protein, and iNOS dimerization in hepatocytes but did not prevent the cytokine-induced increases in iNOS mRNA transcripts.

Fig. 6. Effects of inhaled CO (250 ppm) over 1 h or room air, 1 h prior to intraperitoneal saline or LPS (20 mg/kg), on LPS-induced inducible nitric oxide synthase (iNOS). A: densitometric analysis; data are means ± SE; n = 5 per group. *P < 0.05 vs. Air-Saline, **P < 0.005 vs. Air-LPS. B: representative Western immunoblot.

Fig. 7. Effects of inhaled CO (250 ppm) over 1 h or room air, 1 h prior to intraperitoneal saline or LPS (20 mg/kg), on LPS-induced cyclooxygenase-2 (COX-2) expression. A: densitometric analysis; data are means ± SE; n = 5 per group. *P < 0.01 vs. Air-Saline, **P < 0.005 vs. Air-LPS. B: representative Western immunoblot.
HO-1 has also been shown to modulate the cyclooxygenase system through production of CO. In our study, inhibition of heme oxygenase activity by use of SnPPN resulted in an increase in LPS-stimulated production of COX-2 and exogenous CO diminished hepatic COX-2 protein immunoreactivity in response to LPS. This is in agreement with the findings of Nakao et al. (34), who reported that pretreatment of rodents with CO inhalation decreased levels of COX-2 protein and improved gastrointestinal ileus. However, as was the case with iNOS, it would appear that the effects of CO on hepatic COX-2 immunoreactivity are not due to changes in COX-2 mRNA transcripts since we did not detect any changes in COX-2 mRNA transcripts in rats receiving CO and LPS. Thus, although Suh et al. (43) found that CO binds to promoter elements of the COX-2 gene to decrease its transcription, our study suggests that another mechanism is operational to explain the effects of CO on LPS-induced changes in hepatic COX-2 protein.

Upregulation of both iNOS and COX-2 have been shown to play a role in hepatic injury that results from LPS (45). In the present study we found that ketamine upregulated HO-1 and attenuated LPS-induced hepatic injury and upregulation of iNOS and COX-2, whereas HO-1 inhibition prevented these effects. Furthermore, administration of CO prevented LPS-induced hepatic injury and upregulation of iNOS and COX-2. These data suggest that the heme oxygenase system, through its production of CO, is capable of modulating the NO synthase and cyclooxygenase systems in the liver, rendering it less susceptible to injury from LPS, and that HO-1 is required for ketamine to exert its effects.

Interestingly, CO had different effects on LPS-induced changes in transcription factor activity. We found that CO further increased LPS-induced enhancement of NF-κB DNA binding activity but prevented the ability of LPS to diminish PPAR-γ activity. We have previously shown that ketamine attenuates LPS-induced changes in hepatic NF-κB and PPAR-γ transcription factor activity (1, 45). At the time, we postulated that ketamine downregulates hepatic iNOS and COX-2 expression in response to LPS through a reduction in NF-κB activity. However, our present study would appear to refute this hypothesis because, although CO diminished the ability of LPS to increase iNOS and COX-2 immunoreactivity, it enhanced NF-κB activity and did not affect the LPS-induced increases in iNOS and COX-2 mRNA transcripts. Our findings with respect to the effects of CO on NF-κB are consistent with the literature given that Sarady et al. (38) demonstrated that the ability of CO to alter LPS-induced changes in NF-κB are tissue and situation specific, whereas Kim et al. (23) found that CO enhances NF-κB activity in primary hepatocytes through reactive oxygen species generation, Akt phosphorylation, and IkB phosphorylation and degradation. In contrast to NF-κB, LPS attenuated hepatic PPAR-γ activity, a transcription factor generally thought to exert anti-inflammatory effects. CO prevented the ability of LPS to diminish PPAR-γ activity, an effect we also observed with ketamine (1). Thus it is conceivable that ketamine may exert some of its effects through enhanced heme oxygenase activity and increased CO production, which in turn restore PPAR-γ activity, similar to the actions of ketamine. However, changes in PPAR-γ activity alone do not seem to fully explain changes in iNOS and COX-2 protein immunoreactivity, since iNOS and COX-2 mRNA transcripts did not change.

Interestingly, in our model of LPS-induced hepatic injury, inhibition of HO-1 with SnPPN enhanced the ability of LPS to increase hepatic iNOS and COX-2 immunoreactivity; however, it did not exacerbate hepatic injury (Figs. 1–3). Although we found that HO-1 activity appears to be required for ketamine to exert its hepatoprotective effects, it also appears that other factors likely play a role in limiting the degree of hepatic injury sustained as a result of iNOS and COX-2 upregulation. These factors are likely to be independent of HO-1, since the degree of hepatic injury from LPS was equivalent both in the presence and absence of HO-1 inhibition with SnPPN (Fig. 1). Additionally, these factors appear to be independent of ketamine, as the degree of injury from LPS when HO-1 was inhibited was equivalent in the absence and presence of ketamine. Taken together, these data indicate that mechanisms intrinsic to the organism function to limit the degree of hepatic injury despite further increases in iNOS and COX-2 expression. Furthermore, HO-1 expression itself appears to be regulated through a mechanism of feedback inhibition, as administration of exogenous CO prevented the upregulation of HO-1 in response to LPS, similar to the results found by Srisko et al. (39).

This study extends our previous findings with ketamine by demonstrating a potential mechanism to account for its beneficial effects. We previously reported that LPS upregulates hepatic iNOS and COX-2 and that selective iNOS or selective COX-2 inhibition prevented LPS-induced liver injury suggesting both iNOS and COX-2 play a role in mediating the deleterious effects of LPS (30, 45). Thus it is noteworthy that in the present study, HO-1 inhibition not only negated the hepatoprotective effects of ketamine but also attenuated the ability of ketamine to downregulate hepatic iNOS and COX-2. Administration of CO, the end product of the HO-1 catalyzed reaction, prevented hepatic injury from LPS and the upregulation of hepatic iNOS and COX-2. Consequently, these data suggest that heme oxygenase plays a key role in mediating the hepatoprotective effects of ketamine and, taking them together, we postulate that ketamine exerts its hepatoprotective effects against LPS-induced liver injury by upregulating HO-1, which in turn downregulates hepatic iNOS and COX-2.

The mechanism by which ketamine induces upregulation of HO-1 remains to be elucidated. Others have shown that, in a variety of tissues, the anti-inflammatory effects of exogenous 15-deoxy-Δ-12,14-prostaglandin J2 (15-DPGJ2), a ligand for the intranuclear receptor PPAR-γ, are mediated through up-regulation of HO-1 (19, 29). Ketamine may upregulate HO-1 through PPAR-γ. This receptor and other known factors involved in the upregulation of HO-1 such as Nrf-2 are clear targets for future studies. We have previously shown that
inhibition of HO-1 reverses the effects of ketamine on LPS-induced gastric injury and that changes in gastric iNOS immuno-reactivity in response to ketamine mirror the LPS-induced changes in iNOS mRNA transcripts (16). Future investigations involving the effects of ketamine on LPS-induced changes in iNOS and COX-2 mRNA transcripts in the liver and the effects of ketamine when given after LPS are warranted. Additionally, investigations involving the effects of CO on LPS-induced gastric injury would extend our previous findings exploring the role of the heme oxygenase system in the gastroprotective effects of ketamine.

Death due to multiple organ failure remains a major problem in the critically ill patient population. Future improvements in the care of these patients will focus on modulation of the complex redundant pathways that govern the inflammatory response in order that the incidence of multiple organ failure is reduced and patient survival improved. Moreover, critically ill patients are usually managed in the intensive care unit, frequently require multiple surgical procedures, and as a result often receive a variety of anesthetics and sedatives. Ketamine, an agent that is already FDA approved for clinical use, could be a valuable adjunct since it may attenuate organ dysfunction that can occur as a result of inflammatory insults encountered during a prolonged hospital course.

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