Protection of transplant-induced hepatic ischemia/reperfusion injury with carbon monoxide via MEK/ERK1/2 pathway downregulation

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Although carbon monoxide (CO) is commonly known to be toxic at high concentrations due to its ability to interfere with oxygen delivery, CO is endogenously generated in the body through heme degradation by heme oxygenases (HO) and considered lately as a gaseous messenger molecule, which is necessary to maintain the cellular processes of the body, mainly via the activation of the soluble guanylate cyclase (sGC)/cGMP pathway (15, 22, 29, 39). In the liver, under physiological conditions, mRNA and protein of the constitutive HO isozyme HO-2 are abundantly expressed on the hepatocytes (16). Endogenously produced CO by the hepatocytes could readily reach hepatic stellate cells and sinusoidal endothelial cells and regulate sinusoidal tone, intrahepatic vascular resistance, and blood flow distribution as well as contractility of bile canaliculi (28, 32, 36). Indeed, biological function of endogenously produced CO in the liver was substantiated when the inhibition of CO production with zinc protoporphyrin IX, a competitive inhibitor of HO, increased hepatic vascular resistance in a steady-state condition (37).

The liver often suffers from ischemia/reperfusion (I/R) injury during diverse clinical situations, including hemorrhagic shock, septic shock, abdominal surgeries, and liver transplantation. Hepatic I/R injury is known to generate an inducible HO isoform, HO-1, which provides cytoprotection via the generation of bioactive byproducts, such as CO. Accordingly, activation of the HO system by an HO-1 inducer or by HO-1 gene therapy has been shown to confer hepatoprotection against warm and cold I/R injury in experimental animals (3, 10, 19).

Likewise, exogenously provided gaseous CO also protects cold hepatic I/R injury in the ex vivo isolated liver perfusion model (4). Using the rat liver transplantation model, we (17) have recently reported that brief recipient CO inhalation during the peritransplant period ameliorates liver graft damage and inhibits proinflammatory responses during transplant-induced cold hepatic I/R injury. NF-κB, a critical transcription factor for inflammatory cytokines, quickly nuclear translocates after transplantation and is thought to play roles in upregulating proinflammatory cytokines in hepatic I/R injury (7, 38). Interestingly, however, inhaled CO does not inhibit NF-κB activation, yet it is capable of downregulating proinflammatory responses of hepatic I/R injury (17), suggesting that CO inhibits its inflammatory responses and protects liver grafts independently of the NF-κB signaling pathway.

Several previous studies (26, 27) have suggested the involvement of MAPK signaling pathways during cytoprotection of CO; in LPS-induced endotoxia models, CO selectively inhibits proinflammatory cytokines and increases anti-inflammatory cytokines (e.g., IL-10) through an upregulation of p38 MAPK. MAPK signal transduction pathways represent one of the most widespread mechanisms of eukaryotic cell regulation in response to oxidative and other environmental stress, leading to an upregulation of proinflammatory mediators (e.g., TNF-α, IL-1β, IL-6, and inducible nitric oxide synthase (iNOS)). Accordingly, this study investigates the efficacy and possible mechanisms of inhaled CO in mediating protection against hepatic I/R injury after liver transplantation. The study focuses on the ability of inhaled CO in regulating proinflammatory cytokines; heme oxygenase-1; inducible nitric oxide synthase; extracellular signal-regulated kinase; hepatic nonparenchymal cells.
matory responses and in modulating intracellular signaling pathways with particular attention to the involvement of MAPK signaling pathways.

MATERIALS AND METHODS

Orthotopic liver transplantation. Male Lewis (LEW, RT1) rats weighing 200–300 g (Harlan Sprague Dawley, Indianapolis, IN) were used for both donors and recipients in this study. Animals were maintained in a laminar flow, specific pathogen-free atmosphere at the University of Pittsburgh. The basic techniques of liver harvesting and orthotopic transplantation without hepatic arterial reconstruction were according to the method previously described by Kamada and Calne (18). After harvest, all liver grafts were kept in University of Wisconsin solution at 4°C for 18 h and orthotopically transplanted into syngeneic LEW recipients. Cold preservation for 18 h induced reproducible and significant cold I/R injury in liver grafts without mortality (38, 40, 43). All procedures in this study were performed according to the guidelines of the Council on Animal Care at the University of Pittsburgh and the National Research Council’s Guide for the Care and Use of Laboratory Animals.

CO inhalation. CO gas (1%) was mixed with air gas (21% oxygen) in a stainless steel mixing cylinder to adjust CO concentration. A 3.70-ft³ chamber was filled with the CO gas mixture, and CO concentration was continuously monitored by a CO analyzer (Interscan, Chatsworth, CA). Animals were exposed to different concentrations of CO in a CO chamber with rat chow and water ad libitum.

Experimental design. In the first series of experiments, different concentrations of CO (20, 100, and 250 ppm) were given to the recipient rat for 1 h before and 24 h after the transplant surgery to examine the effect of CO inhalation on blood carboxyhemoglobin (HbCO) levels and hepatic cold I/R injury. Air gas was used as the control (0 ppm of CO). Recipient rats were killed at 6, 24, and 48 h after transplantation for blood and liver graft samples (n = 7 for each time point). Hepatic injury was assessed by serum alanine transaminase (ALT) levels and histopathological changes.

In the second series of experiments, we investigated the alteration of proinflammatory and apoptosis-related and intracellular signaling molecules during early reperfusion periods up to 6 h. Based on the result of the initial dose-response experiments, recipient rats were exposed to CO 100 ppm for 1 h before and until death at 1, 3, and 6 h after reperfusion for liver graft samples (n = 4–7 for each time point). In the separate groups of recipients, U0126 (MEK inhibitor, Promega, Madison, WI) was intravenously given two times at 30 min before and 10 min after reperfusion at a dose of 50 µg/kg with and without inhaled CO.

HbCO measurement. A heparinized arterial blood sample (0.2 ml) was taken, and blood HbCO and SaO2 levels were measured using OSM3 Hemoximeter (Radiometer Copenhagen, Copenhagen Denmark).

Serum ALT levels. Hepatic injury was assessed by serum ALT levels using the Opera Clinical Chemistry System (Bayer, Tarry Town, NY).

Histopathology. Liver graft tissues were fixed in 10% formalin, embedded in paraffin, sectioned into 6-µm thickness, and stained with hematoxylin-eosin. The percentage of necrotic area was estimated by random examination of 10 low power fields (×40) of each section. Polymorphonuclear leukocytes (PMN) were stained using a naphthal AS-D chloroacetate esterase staining kit (Sigma Diagnostics, St. Louis, MO) and identified by nuclear morphology and bright red positive staining. Positively stained cells were counted in 20 high-power fields (×200) per each section and expressed as the number of cells per one millimeter squared.

Isolation of cytoplasmic and nuclear proteins. Frozen liver tissues were suspended in buffer containing 10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, and 0.5% Nonidet P-40 and homogenized for 20 s with a Polytron homogenizer (Kinematica, Littau, Switzerland). Nuclei were recovered by microcentrifugation at 7,500 rpm for 5 min. The supernatant containing cytoplasmic protein was collected and stored at −80°C for Western blot analysis. Nuclear proteins were extracted at 4°C by gently resuspending the nuclei pellet in buffer, containing 20 mM HEPES (pH 7.9), 10% glycerol, 1.5 mM MgCl2, 10 mM KCl, and 0.2 mM EDTA followed by 1 h of incubation at 4°C with occasional vortexing. After microcentrifugation at 13,000 rpm for 15 min at 4°C, the supernatant containing nuclear protein was collected and frozen at −80°C. All buffers contained the following additional ingredients: 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, and 0.1 mM Na-panadate and protease inhibitors. Protein concentration was quantitated with Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Western blot analysis. Fifty micrograms of cytosolic, nuclear, or whole protein were separated by electrophoresis on 8–15% acrylamide SDS gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). For the blocking of nonspecific binding, 5% nonfat dry milk in PBS-Tween was added to the membrane for 1 h at room temperature. Membranes were incubated overnight with primary rabbit polyclonal or mouse monoclonal antibodies. After repeat washings with PBS-Tween, membranes were incubated with secondary goat anti-rabbit or anti-mouse antibody (1:10,000, Pierce Chemical, Rockford, IL) for 45 min. After repeat washings with PBS-Tween, membranes were developed with the SuperSignal detection systems (Pierce Chemical) and exposed to film. The band intensities were measured by National Institutes of Health Image analysis software. The following primary antibodies were used: iNOS (N32030, Transduction Laboratories, Lexington, KY); HO-1 (SPA-896, StressGen Biotechnology, Victoria, BC, Canada); phosphorylated (p)-p38 (#9211), p-JNK (#9251), p-ERK1/2 (#9121), p-c-MyC (#9401), p-Elk-1 (#9181), total (t)-p38 (#9212), t-JNK (#9258), Stat1 (Tyr701) (#9171), Stat1 (Ser727) (#9177), Stat3 (Tyr705) (#9131), p-Stat3 (Ser727) (#9134), t-Stat1(#9171), t-Stat3 (#9132), and anti-cleaved caspase-3 antibody (#9661) (all from Cell Signaling Technology, Beverly, MA); p-ERK1/2 (sc-7383); and p-ERK1/1 (sc-94, Santa Cruz Biotechnology, Santa Cruz, CA).

Real-time RT-PCR. Total RNA was extracted from the liver tissues using the TRIzol reagent (Life Technologies) according to the manufacturer’s instructions. RNA content was measured using 260/280 UV spectrophotometry. mRNA expression was quantified by SYBR Green two-step, real-time RT-PCR. Total RNA pellets were suspended in RNase-free water, followed by removal of potentially contaminating DNA by treatment with DNase I (Life Technologies). One microgram of total RNA from each sample was used for reverse transcription with an oligo dT (Life Technologies) and a Superscript II (Life Technologies) to generate first-strand cDNA. The PCR reaction mixture was prepared using SYBR Green PCR Master Mix (PE Applied Biosystems, Foster City, CA) using the previously published primers (23, 25). Each sample was analyzed in duplicate using the conditions recommended by the manufacturer. Thermal cycling conditions were 10 min at 95°C to activate the AmpliTaq Gold DNA polymerase, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min on an ABI PRISM 7000 Sequence Detection System (PE Applied Biosystems). With the use of the manufacturer’s software, real-time PCR data were plotted as the ΔRn, fluorescence signal vs. the cycle number. The threshold cycle was defined as the cycle number at which the ΔRn crosses this threshold. The expression of each gene was normalized to GAPDH mRNA content and calculated relative to naïve control using the comparative cycle threshold method (23).

Serum NO measurement. Serum nitrite (NO2−) and nitrate (NO3−) were measured by using a commercially available kit (Cayman Chemical, Ann Arbor, MI).

Statistical analysis. Data are means ± SE. Comparisons between the groups at different time points were performed using the Student’s t-test or ANOVA using the Statview program (Abacus Concepts, Berkeley, CA). Differences were considered significant at a P value <0.05.
RESULTS

**CO inhalation increases blood HbCO levels in a dose-dependent manner.** HbCO levels quickly increased and reached a peak level by 1 h after initiation of CO inhalation. The peak HbCO levels were CO concentration dependent and were 3.5 ± 0.1, 5.4 ± 0.5, 13.5 ± 0.1, and 24.9 ± 0.7% with 0, 20, 100, and 250 ppm, respectively (Fig. 1). Recipient HbCO levels were maintained at the peak level for the remaining period of CO exposure and then decreased to the baseline level within 6 h after the termination of CO treatment (17, 23). Methemoglobin levels were maintained at <1%. Arterial oxygen contents were reduced due to an increase of HbCO in CO-treated animals. However, no apparent adverse effects were noticed with transient increases in HbCO levels to ~25%, and the animals functioned normally, as we previously reported (17, 23).

**CO treatment attenuates hepatic cold I/R injury.** Effects of different concentrations of CO on hepatic preservation injury were assessed by serum ALT levels (6 and 24 h) and severities of hepatic necrosis and PMN accumulation in liver grafts (48 h). As we have previously shown, the serum ALT levels in the air-treated control group gradually increased and peaked at 24 h after reperfusion (4,209 ± 448 IU/l), associating with massive hepatic necrosis and accumulation of PMN by 48 h (38, 40, 43). Inhaled CO at 100 and 250 ppm, but not 20 ppm, significantly decreased serum ALT levels to <50% of air-controls (100 ppm: 1,830 ± 260; 250 ppm: 1,875 ± 300 IU/l; Fig. 2A). Sinusoidal congestion and necrosis in liver grafts in the air-treated group were markedly reduced with CO inhalation at 100 and 250 ppm (Fig. 2, B and C). Consistent with the improvement in liver function tests and the area of liver necrosis, numbers of PMN significantly decreased with 100 and 250 ppm CO (Fig. 2C). These results indicate a concentration-dependent hepatoprotection mediated by CO inhalation. Accordingly, CO 100 ppm inhalation was used in the subsequent experiments.

**CO treatment suppresses hepatic I/R injury-induced mRNA expression of inflammatory cytokines.** In the air-treated group, TNF-α mRNA expression rapidly increased during hepatic cold I/R injury and peaked at 1 h after reperfusion. CO inhalation (100 ppm) significantly suppressed TNF-α mRNA expression.
expression at 1 h to 28% of that in the air-treated group (Fig. 3A). A similar tendency was observed for IL-6 mRNA expression, but the difference did not reach statistical significance (Fig. 3B). The anti-inflammatory cytokine IL-10 was previously shown to be upregulated with CO (26); however, hepatic I/R injury increased IL-10 mRNA expression and there was no difference between CO and air-treated groups (Fig. 3C). When normal unoperated animals were exposed to 100 ppm CO for 1 h, these cytokine mRNA levels in the liver were not altered.

**CO treatment downregulates hepatic iNOS/NO pathway after I/R injury.** Since HO-1 and iNOS are stress-responding enzymes and have been shown to reciprocally work to induce cytoprotection, the effect of inhaled CO on the hepatic iNOS/NO pathway in I/R injury was examined. In the air-treated group, iNOS mRNA expression peaked at 3 h, followed by protein expression at 6 h after reperfusion. CO inhalation (100 ppm) markedly suppressed hepatic iNOS mRNA expression at 1 and 3 h and protein expression at 6 h after reperfusion (Fig. 4, A and B). Serum NO levels increased in the air-treated group with a peak at 6 h, and CO 100 ppm inhibited NO production (Fig. 4C). CO inhalation (100 ppm for 1 h) to naïve unoperated animals without I/R injury did not affect iNOS mRNA levels (Fig. 4A) or protein expression (data not shown) in the liver.

**CO treatment does not influence hepatic HO-1 expression.** The involvement of endogenous HO-1 in CO-mediated hepatic protection against I/R injury was also examined in both mRNA and protein levels. After cold I/R, HO-1 mRNA expression was upregulated and peaked at 3 h after reperfusion in both air- and CO (100 ppm)-treated groups (Fig. 5A). HO-1 protein expression markedly increased at 6 h after reperfusion in both groups (Fig. 5B), and strong protein expression was maintained for 48 h (data not shown). There was no significant difference in HO-1 protein expression between air- and CO-treated groups during the observation period; however, CO treatment tended to reduce HO-1 upregulation. The results suggest that the protective effect of inhaled CO on hepatic cold I/R injury is independent of endogenous HO-1 induction.
CO treatment suppresses phosphorylation of MAPK ERK1/2 in liver grafts after cold I/R injury. We next investigated the possible involvement of MAPK in the hepatoprotection of CO. Rapid and transient activation of all three MAPK subtypes was seen in air-treated liver grafts at 1 h after reperfusion and subsided within 3 h. CO significantly reduced p-ERK1/2 expression in liver grafts at 1 h to ~50% of that observed in air-treated group (Fig. 6). CO tended to increase p38 phosphorylation at 1 h after transplantation; however, the difference was not statistically significant. It was noted in the liver of naïve unoperated animals that CO treatment (100 ppm for 1 h) induced ~8 fold increase of p-p38 MAPK compared with naïve untreated livers (Fig. 6). JNK phosphorylation was not altered by CO, and p-JNK expression was similar between air- and CO-treated groups.

CO treatment downregulates the upstream and downstream of ERK signaling pathway in graft liver. To further confirm the inhibition of ERK1/2 pathway with inhaled CO in this study, we next examined phosphorylation of MEK1/2, an upstream of ERK1/2, and downstream transcriptional factors such as c-Myc and Elk-1, in liver grafts. In addition, p-ERK1/2 expression in nuclear extract was also investigated. CO inhibition significantly reduced phosphorylation of cytosolic MEK1/2, and nuclear ERK1/2 and c-Myc at 1 h after reperfusion, confirming the inhibition of MEK/ERK1/2 pathway with inhaled CO in this model (Fig. 7). However, no difference was observed in p-Elk-1 levels. Since the activation of JNK and p38 MAPK pathways also induces Elk-1 upregulation, Elk-1 phosphorylation in CO-treated hepatic grafts may be through these unaffected signaling pathways. The inhibition of MEK with U0126 (50 μg/kg at 30 min before and 10 min after reperfusion) resulted in a significant reduction of serum ALT levels, indicating the involvement of MEK/ERK pathways in hepatic I/R injury. Combination treatment of U0126 plus inhaled CO 100 ppm did not induce additional protection, and serum ALT levels were similarly decreased among inhaled CO alone, U0126 alone, and CO plus U0126 treatment groups (Fig. 7C).

CO reduces phosphorylation of STATs in graft liver. Previous in vitro studies have shown that the activation of p38 MAPK pathway with CO attenuates oxidative injury-induced apoptosis via STAT3 activation (46). Accordingly, we investigated the influence of CO inhalation on phosphorylations of STAT1 and STAT3 at 1 h after reperfusion by Western blot using nuclear protein. Both 701 tyrosine and 727 serine phosphorylated STAT1 and STAT3 were detected during hepatic I/R injury. CO inhalation significantly decreased tyrosine phosphorylation of STAT1 and both tyrosine and serine phosphorylations of STAT3, compared with the air-treated group (Fig. 8).

CO does not affect apoptosis-related genes in graft liver. We next investigated whether CO inhalation could affect apoptosis in liver grafts. There was no significant increase of mRNA levels for proapoptotic Bax or antiapoptotic Bcl-2 during early (1–3 h) posttransplant period in both groups. However, protein levels of cleaved caspase-3 gradually elevated in the liver after reperfusion in both air- and CO-treated groups (Fig. 9).

DISCUSSION

With the use of in vivo rat liver transplantation model, this study provides evidence that inhaled CO attenuates cold hepatic I/R injury associated with 18-h cold preservation and subsequent transplantation. Recipient treatment with inhaled CO significantly reduced hepatic enzyme elevation and necrosis with a downregulation of proinflammatory mediators, including TNF-α, IL-6, iNOS, and HO-1. CO concentrations of 100 and 250, but not 20 ppm, showed similar levels of protection, and the CO effects appeared to be concentration dependent. The majority of previous in vivo studies have demonstrated the cytoprotective effects of CO using considerably high concentrations between 250 and 1,000 ppm (14, 33). Recently, however, an extremely low CO concentration of 10 ppm was shown to be sufficient in LPS-induced rodent inflammation models in attenuating serum TNF-α elevation and improving animal survival (26, 30). The failure to treat hepatic I/R injury with 20 ppm CO in this study may suggest that relatively higher concentrations of CO are required to combat severe I/R injury associated with liver transplantation.

In this study, we focused on the actions of CO in downregulating inflammatory mediators and analyzed the molecular signaling pathways involved in the hepatic protective function of CO. In agreement with previous studies (6, 7, 44, 47), transplant-induced hepatic I/R injury resulted in a prompt and brief activation of all three subtypes of MAPK. Inhaled CO in this study showed significant inhibition of hepatic ERK1/2 phosphorylation during the early postreperfusion period. Both upstream (MEK1/2) and downstream (c-Myc) signals were
also inhibited, confirming the involvement of ERK1/2 pathway in CO-mediated hepatic protection. Several previous in vitro studies have shown the inhibition of ERK MAPK pathway with CO; with the use of human airway smooth muscle cells, CO markedly inhibited IL-1β-induced granulocyte-macrophage colony-stimulating factor synthesis via ERK1/2 inhibition without affecting the activation of JNK or p38 MAPK (34). When rat primary pulmonary artery endothelial cells were

![Fig. 6. Phosphorylation of MAPKs in graft liver after ischemia/reperfusion injury.](image)

**A**: Phosphorylated (p)-p38, p-ERK1/2, and p-JNK in graft liver at 1 and 3 h after reperfusion were evaluated in Western blot using hepatic cytoplasmic protein in CO (100 ppm) and air-treated groups. Each lane represents a different animal. **B**: band intensity was expressed compared with total (t)-p38, (t)-ERK1/2, and (t)-JNK. *P < 0.05, #P < 0.001 vs. air-treated group (n = 4 for unoperated normal liver; n = 7 for each group at 1 h; and n = 5 for each group at 3 h). NM, normal animals.

**Fig. 7. Downregulation of ERK1/2 signaling pathway in graft liver by CO inhalation.** **A**: phosphorylation of upstream of ERK1/2 (MEK1/2) and downstreams of ERK1/2 (c-Myc and Elk-1) in liver graft was investigated at 1 h after reperfusion by Western blot using cytoplasmic protein (for MEK1/2) or nuclear protein (ERK1/2, c-Myc, and Elk-1). Each lane represents a different animal. **B**: band intensity was expressed as a ratio to normal rat liver. *P < 0.05, #P < 0.001 vs. air-treated group (n = 4 for each group). NM, normal animals. **C**: serum ALT levels at 6 and 24 h after reperfusion. Recipient animals were treated with the MEK inhibitor U0126 (50 μg/kg iv at 30 min before and 10 min after reperfusion), inhaled CO (100 ppm for 1 h before and 24 h after reperfusion), or combination of both. DMSO (vehicle) was injected at the same time points for the control. *P < 0.05 vs. DMSO and air-treated group (n = 3–5).
treated with TNF-α, ERK1/2, JNK, and p38 MAPK were activated and CO exhibited marked attenuation of ERK1/2 MAPK phosphorylation but accentuated TNF-α-induced p38 MAPK phosphorylation (31). Further, CO protected BNL CL.2 mouse liver cells and primary rat hepatocytes from glucose deprivation-induced cytotoxicity through inhibition of ERK MAPK (8). In correlation with these in vitro studies, downregulation of ERK1/2 signaling pathway with CO could be one of the important mechanisms to mediate cytoprotection in vivo. Indeed, the blockade of MEK/ERK1/2 pathway with specific inhibitors is shown to be beneficial by reducing proinflammatory mediators in a variety of in vivo injury models (2, 13, 24, 41, 42).

Both HO-1 and iNOS are stress-responding enzymes, and previous studies have shown the interaction of HO-1/CO and iNOS/NO pathways during tissue/cell injury. NO has been shown to induce HO-1 expression (8, 45), while others show that HO-1/CO inhibits iNOS gene expression and/or its activity (11, 12, 21). In this model of hepatic I/R injury, both HO-1/CO and iNOS/NO pathways were activated; however, the activation patterns were different. iNOS protein expression was transiently seen during the early posttransplant period of 3–12 h, and it was restricted on the hepatocytes, as we previously reported (43). On the other hand, HO-1 protein was expressed in more extended periods of 3-48 h. Multiple liver cells could express HO-1, and several studies (5, 35) have localized HO-1 on the hepatic nonparenchymal cells (most likely Kupffer cells) in the early period after injury with later expression on the hepatocytes. In the present study, CO markedly blocked iNOS expression and attenuated NO production in the liver graft with I/R injury, while hepatic I/R injury-induced HO-1 upregulation was less affected by CO treatment. Although a previous study in TNF-α/d-galactosamine-induced mouse liver injury model has shown the requirement of iNOS upregulation for the protective effects of CO (48), the current study did not clarify the relationship between CO and NO. Transplant-induced-hepatic I/R injury is a cascade of events involving numerous signals for various target molecules on multiple cells, each of which augments injurious and repairing processes. Thus, the relationship between these stress-responding enzymes could be more complicated in this model.

CO is shown to exert an antiapoptotic effect by downregulating proapoptotic signals such as Bax and caspase-3 and by upregulating antiapoptotic signals of the Bcl-2 family (23, 25). CO inhalation attenuated apoptotic cell death in vivo I/R injury models of the heart (1, 20), lung, kidney (25), and small intestine (23). In this study, however, CO inhalation did not affect apoptotic Bax, Bcl-2, or caspase-3 expression in liver grafts. Since antiapoptotic signaling mechanisms of CO are shown to involve STAT3 activation via p38 MAPK pathway, inhibition of STAT3 with CO in this study could explain the lack of antiapoptotic action of CO in this model. STAT3 activation has been shown to be in part dependent on ERK signaling (9), and suppression of ERK1/2 with CO could account for the decreased activation of STAT3.
In conclusion, recipient treatment with CO inhalation results in amelioration of cold preservation injury after liver transplant with a marked decrease in hepatic expression of proinflammatory cytokines/mediator, such as TNF-α, IL-6, and iNOS. CO-mediated downregulation of ERK1/2 signaling pathway might be involved in the mechanisms by which CO inhalation confers the protection against cold I/R injury. Although further experiments will be necessary to identify specific protective mechanisms, our data strongly support the possibility that CO inhalation therapy would be a novel strategy to combat cold I/R injury.

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