Carbon Monoxide Modulates Fas/Fas Ligand, Caspases, and Bcl-2 Family Proteins via the p38α Mitogen-activated Protein Kinase Pathway during Ischemia-Reperfusion Lung Injury

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Abstract

Carbon monoxide is protective in ischemia-reperfusion organ injury but the precise mechanisms remain elusive. We have recently shown that low levels of exogenous carbon monoxide (CO) utilizes p38 MAPK and attenuates caspase 3 activity to exert an anti-apoptotic effect during lung ischemia-reperfusion (I-R) injury. Our current data demonstrate that CO activates the p38α MAPK isoform and the upstream MAPK kinase, MKK3, in order to modulate Fas/Fas ligand expression, caspases 3, 8, and 9, mitochondrial cytochrome c release, Bcl-2 proteins, and poly (ADP-ribose) polymerase (PARP) cleavage. We correlate our in vitro findings with in vivo studies using MKK3-deficient and Fas-deficient mice. Taken together, our data are the first to demonstrate that CO has an anti-apoptotic effect by inhibiting Fas/Fas ligand, caspases, pro-apoptotic Bcl-2 proteins, and cytochrome c release via the MKK3/p38α MAPK pathway.

Key Words: Apoptosis, Carbon monoxide, Mitogen-activated protein kinase, p38, Fas, Caspases, Bcl-2 proteins.
Introduction

Ischemia-reperfusion (I-R) lung injury occurs during transplantation, surgical procedures involving lung vascular clamping, and severe shock. There is increasing evidence that apoptosis plays an important role in the pathogenesis of I-R injury in a variety of organs such as brain (1), heart (2), kidney (3), liver (4) and lung (5). More importantly, inhibiting apoptosis during I-R injury is associated with improved survival and organ function (6,7). Unlike necrosis, apoptosis is a regulated cell death process involving specific pathways and cellular components. Therefore, delineating the precise apoptotic mechanisms involved during I-R injury may help optimize future therapies designed to abrogate I-R injury-induced apoptosis.

The heme oxygenase-1 (HO-1)/carbon monoxide (CO) system has been shown to provide significant protection against hyperoxic lung injury (8), transplant rejection (9), vascular injury (10), and most recently, arteriosclerotic lesions associated with chronic graft rejection (11). CO, a reaction product of HO-1 activity, has been shown to have potent anti-inflammatory, anti-proliferative, and anti-apoptotic effects and thereby confers, at least in part, the cytoprotective effects of HO-1. Furthermore, the mitogen-activated protein kinase pathway (MAPK), specifically p38 MAPK, appears to mediate the biologic effects of CO (12,13). We have recently shown that low-levels of exogenous CO can suppress I-R-induced apoptosis in pulmonary endothelial cells and mouse lungs through p38 MAPK activation and caspase 3 activity inhibition (12).

However, if CO is to have potential as a therapeutic agent, more precise identification of CO-modulated targets will be necessary. Given our previous data showing that the anti-apoptotic effects of CO in I-R lung injury is likely through caspase modulation as well as p38 MAPK activation (12), we extended our investigations to characterize the precise anti-apoptotic pathways and the specific p38 MAPK isoform modulated by CO. We show that CO inhibits Fas/Fas ligand (FasL) expression and
subsequent activation of caspases 3, 8, and 9, poly (ADP-ribose) polymerase (PARP) cleavage, and mitochondrial cytochrome c release. In addition, CO differentially modulates the pro- and anti-apoptotic members of the Bcl-2 family proteins. Furthermore, all these effects of CO depend upon p38 MAPK activation, specifically p38α MAPK and the upstream MAPK kinase, MKK3. We correlate our endothelial cell findings to mouse lungs subjected to I-R by using MKK3-deficient (MKK3-/-) and Fas receptor-deficient (Fas-/-) mice. Taken together, our data are the first to demonstrate in cell and mouse models that the anti-apoptotic effects of CO are dependent on the downregulation of Fas/FasL expression, caspase activity, and modulation of Bcl-2 proteins via the MKK3/p38 α MAPK pathway.

Experimental Procedures

Reagents. The caspase inhibitors Z-D (O-Me)-M-Q-D (O-Me)-fluoromethyl ketone (Z-DQMD-FMK), Z-I-E (OMe)-T-D (OMe)-fluoromethyl ketone (Z-IETD-FMK), Z-L-E (Ome)-H-D (OMe)-fluoromethyl ketone (Z-LEHD-FMK), and p38-specific inhibitor SB203580 were purchased from Calbiochem (San Diego, CA). The phospho-MKK3/6, phospho-p38, and cleaved-PARP antibodies were purchased from Cell Signaling Technology (Beverly, MA). The β-tubulin, Bcl-2, Bcl-XL, Bax, Bid, phospho-p38α, rat IgG-FITC, Fas, and Fas Ligand antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The FasL-blocking antibody (MFL4) was purchased from BD Pharmingen (San Diego, CA).

Cell Culture and CO exposure. Rat primary pulmonary artery endothelial cells (PAEC) were generously provided by Dr. Troy Stevens (University of Alabama, Birmingham, AL) and were exposed to anoxia-reoxygenation (A-R) in the presence or absence of CO according to our previous methods (12).

Murine lung ischemia-reperfusion model and CO exposure. Adult 6 to 8 week old C57BL/6J and Fas receptor mutant mice (B6.MRL-tnfrsf6<sup>lpr</sup> which will be designated as Fas-/-) were obtained from Jackson
Laboratories (Bar Harbor, ME). MKK3-deficient mice (MKK3−/−) have been previously described (14). Mice were exposed to CO 500 ppm during lung I-R as previously described (12).

**Apoptosis Assays.** Annexin V-FITC kit (BD Pharmingen, San Diego, CA) was used to detect the apoptosis of PAEC and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay was used to detect the apoptosis of lung tissues by using the *in situ* cell death detection kit (Roche Molecular Biochemicals, Indianapolis, IN) as detailed previously (12).

**Flow cytometric analysis of cell surface Fas and FasL expression.** PAEC cell surface expression of Fas and FasL were performed using flow cytometry (Becton Dickinson, San Jose, CA) and analyzed using Cellquest software. Briefly, cells were detached using trypsin, washed twice in cold PBS, pelleted, suspended in PBS containing Fas (1:100 dilution), FasL (1:100 dilution), or control rat IgG (1:100 dilution) antibody, and incubated on ice for 45 min. The cells were washed twice with cold PBS, resuspended in PBS containing anti-rat-FITC (1:50 dilution) antibody, and incubated on ice for 45 min. After two washes with PBS, cells were fixed in 1% paraformaldehyde and subjected to flow cytometry analysis.

**Western blot analysis.** Protein levels of phospho-p38, phospho-p38α, phospho-MKK3/6, Bel-2, Bel-XL, cleaved Bid, Bax, cleaved PARP, Fas, and FasL were analyzed by Western blot assays. To verify equivalent sample loading, membranes were stripped with Blot Restore Membrane rejuvenation solution (Chemicon International, Inc., Temecula, CA) and re-probed with anti-total p38 or anti-β-tubulin antibody.

**Isolation of cytosolic fraction and release of cytochrome c.** The cytosolic fraction of PAEC was isolated with Cytochrome c Release Apoptosis Assay Kit (Oncogene Research Products, San Diego, CA). Western blot with mouse anti-cytochrome c monoclonal antibody (Oncogene Research Products, San
Diego, CA) was then performed. To verify equivalent sample loading, membranes were stripped with Blot Restore Membrane rejuvenation solution and re-probed with anti-β-tubulin antibody.

**Measurement of caspase 3, 8 and 9 activity.** The activity of caspases 3, 8, and 9 was measured with colorimetric assays using CaspACE Assay System (Promega, Madison, WI), Caspase 8 Colorimetric Activity Assay Kit (Chemicon International, Inc., Temecula, CA), and Caspase 9 Assay Kit (Calbiochem, San Diego, CA), respectively. Briefly, for PAEC, after treatment with A-R, cells were washed twice with ice-cold PBS and resuspended in cell lysis buffer. Lung tissues were homogenized in lysis buffer (312.5mM HEPES pH 7.5), 31.25% sucrose, 0.3125% CHAPS, 0.1% Triton X-100. Cell and tissue lysates were centrifuged and the supernatants were incubated with the colorimetric substrate, Ac-Asp-Glu-Val-Asp- p-nitroanilide (Ac-DEVD-pNA), N-Acetyl-Ile-Glu-Thr-Asp-p-nitroanilide (Ac-IETD-pNA) or Ac-Leu-Glu-His-Asp-p-nitroanilide (Ac-LEHD-pNA), for caspases 3, 8, and 9, respectively. The release of p-nitroanilide (pNA) from Ac-DEVD-pNA, Ac-IETD-pNA or Ac-LEHD-pNA was measured at 405nm using a spectrophotometer.

**Plasmid constructs and transient transfections.** The p38α MAPK constructs have been previously described (12) and the MKK3 and MKK6 plasmids were obtained from Dr. Jawed Alam. Cells were incubated for 6 h with DNA mixtures containing serum-free media, Fugene 6 Reagent (Roche Molecular Biochemicals, Indianapolis, IN), and wildtype or dominant negative mutant plasmids. After incubation, cells were cultured for an additional 16 h in complete medium and then exposed to A-R in the presence or absence of 15ppm CO. We have demonstrated the transfection efficiency of PAEC to exceed 80% using pEGFP transfections and by examining the cells under phase-contrast and fluorescence microscopy as previously described (15).

**Statistics.** Data are expressed as mean ± SE and analyzed by student’s t-test. Significance was accepted at $P<0.05$. 
Results

CO exerts an anti-apoptotic effect through the p38α isoform of p38 MAPK and the upstream MAPK kinase, MKK3, in PAEC during A-R. We have previously shown that CO activates p38 MAPK and that the inhibition of p38 MAPK using SB203580 ablates the anti-apoptotic effect of CO (12). Despite increased p38 MAPK activity during reperfusion, apoptosis still occurs and this is likely due to the fact that apoptosis is initiated during anoxia; unless p38 MAPK is activated to sufficient levels during anoxia, there is no attenuation of apoptosis during the reperfusion phase (12). In Figure 1A we show a time course of when p38 activation occurs in the presence and absence of CO in PAEC. CO increases phospho-p38 levels during anoxia after 8 h but p38 activation is maximal at 24 h. Of note, in the absence of CO, PAEC cell death is maximal after 24 h anoxia and is maintained during 30 min to 8 h reoxygenation (15). Therefore, in subsequent assays we use 24 h anoxia and 24 h anoxia followed by 1 h reoxygenation as the time points of interest in PAEC. There are four known isoforms of p38 MAPK (α, β, γ, and δ). SB203580 specifically inhibits p38α and p38β, but has no effects on p38γ and p38δ (16). Therefore, in Figure 1B we investigated whether CO activated p38α and/or p38β during A-R. CO activated p38 MAPK, the p38α isoform, and the MAPK kinase(s) upstream of p38 MAPK, MKK3/6, but not p38β (data not shown) during A-R. There are no specific antibodies to MKK3 and MKK6 individually, to the best of our knowledge. In order to delineate the specific roles of p38α, MKK3, and MKK6 in mediating the anti-apoptotic effect of CO during A-R, we performed transient transfection experiments with dominant negative mutant (DNM) plasmids of p38α, MKK3, and MKK6. CO was unable to inhibit A-R-induced apoptosis in PAEC transfected with p38α or MKK3 DNM plasmids (Figures 1C and 1D). However, CO still attenuated A-R-induced apoptosis in PAEC transfected with wildtype p38α (12), wildtype MKK3, wildtype MKK6, or MKK6 DNM (data not shown). Of note, the
background level of cell death due to transfection of p38 DNM or MKK3 DNM in room air was similar to room air alone. These data indicate that CO exerts its anti-apoptotic effect by modulating the MKK3/p38α MAPK pathway, and the ability of SB203580 to attenuate the anti-apoptotic effect of CO is likely due to the inhibition of p38α (since p38β is not involved in our model).

**Inhibition of Fas/FasL or modulation of the MKK3/p38 MAPK pathway by CO attenuates I-R-induced apoptosis *in vitro* (PAEC) and *in vivo* (mouse lung) during I-R.** The binding of FasL to the Fas receptor is a prototypic signal for apoptosis, and therefore we investigated whether FasL inhibition can attenuate A-R-induced apoptosis in PAEC. Pretreatment of PAEC with a blocking antibody to FasL decreased apoptosis to similar levels as exogenous CO (Figures 2A and 2B). In addition, 1 h pretreatment with 10µM of SB203580, a specific inhibitor of p38 MAPK, attenuated the anti-apoptotic effect of CO. Pretreatment with SB203580 or anti-FasL in room air showed basal levels of cell death similar to room air alone. Lung I-R injury is an *in vivo* correlate of A-R injury in pulmonary cells. Similar to our *in vitro* data, our *in vivo* data confirm that CO has an anti-apoptotic effect during I-R that is mediated by MKK3/p38 MAPK. In Figure 2C Panel b, we show that wildtype mice subjected to lung I-R exhibited increased TUNEL staining throughout the lung compared to naïve mice (Panel a). Exogenous CO significantly attenuated I-R-induced TUNEL staining (Panel d). In the presence of a specific p38 MAPK inhibitor, SB203580, or in the genetic absence of MKK3, CO had little effect (Panels e and f, respectively). Of note, similar to our cell data, CO retained its anti-apoptotic effect in MKK6-deficient mice (data not shown). Furthermore, we confirm our *in vitro* data by showing that Fas−/− mice do not exhibit I-R-induced lung apoptosis, suggesting that the Fas pathway may potentially be a mechanism of I-R-induced apoptosis (Panel c).
CO decreases Fas and FasL expression through the MKK3/p38 MAPK pathway \textit{in vitro} and \textit{in vivo} during I-R. In PAEC and mouse lung, we illustrated that the anti-apoptotic effect of CO is dependent upon MKK3/p38 MAPK and that Fas/FasL inhibition also has a profound anti-apoptotic effect. Therefore, we hypothesized that CO exerts an anti-apoptotic effect during I-R by modulating the Fas/FasL pathway through MKK3/p38 MAPK. Anoxia alone or A-R increased Fas expression, which was significantly decreased in the presence of CO (Figure 3A lanes 2-5). Furthermore, CO-mediated attenuation of Fas expression was ablated in the presence of a specific p38 MAPK inhibitor, SB203580 (lanes 7 and 8). Similar results were obtained for FasL expression in PAEC (Figure 3B). Cells treated with SB203580 in room air (lane 6) showed basal levels of Fas and FasL expression similar to room air alone. In Figure 3C lane 3 we confirmed our data \textit{in vivo} by showing that CO decreased I-R-induced Fas/FasL expression in lung tissue. However, CO could not decrease Fas/FasL expression in wildtype mice treated with a specific p38 MAPK inhibitor SB203580 or MKK3\(^{-/-}\) mice subjected to lung I-R (Figure 3C lanes 4 and 7, respectively). Naïve MKK3\(^{-/-}\) mice exhibited basal levels of Fas and FasL expression (Figure 3C lane 5). These data indicate that CO can inhibit the expression of Fas/FasL and that this effect depends upon MKK3/p38 MAPK in both cells and mouse lung during I-R injury.

CO inhibits the activity of caspase 3, 8, and 9 through the MKK3/p38 MAPK pathway \textit{in vitro} and \textit{in vivo} during I-R. We have previously shown that CO inhibits caspase 3 activity via p38 MAPK and that this contributes to the anti-apoptotic effect of CO in PAEC during A-R (12). Our current studies investigate the roles of other caspases and potential downstream targets in the anti-apoptotic effects of CO. Caspase 3 activation is regulated by at least two pathways, the “mitochondrial pathway,” which involves the release of cytochrome \textit{c} from the mitochondria into the cytosol, and subsequent caspase 9 and caspase 3 activation (17) and/or receptor-mediated pathways, such as Fas/FasL binding which leads to caspase 8 and then caspase 3 activation. Activated caspase 3 then cleaves substrates, such as PARP,
which leads to DNA fragmentation and apoptosis. Therefore, in the next series of studies we determined whether CO modulates caspases 3, 8, and 9, PARP, and cytochrome c release. In Figure 4A lanes 4 and 5, we first show that CO can effectively attenuate A-R-induced caspase 3, 8, and 9 activation in PAEC during A-R. In addition, pretreatment with SB203580 ablated the ability of CO to inhibit A-R-induced caspase activation, suggesting that CO depends upon p38 MAPK in order to modulate caspases during A-R in PAEC (lanes 6 and 7). We have already shown that CO decreases Fas/FasL expression through MKK3/p38 MAPK (Figure 3) and given that blocking FasL also effectively diminishes A-R-induced caspase activities (Figure 4A lanes 8 and 9), we postulated a potential sequence of events, namely, that CO activates MKK3/p38α MAPK, leading to decreased Fas/FasL expression and a subsequent decrease in the activity of caspases 3, 8, and 9. Although it appears that CO retains some ability to decrease caspase 3 activity despite treatment with SB203580 (Figure 4A lanes 6 and 7), there was no statistical difference between lanes 2/3 and lanes 6/7. We certainly recognize that there may be other pathways aside from p38 MAPK that are involved, however, at this juncture it is beyond the scope of our studies. Our in vivo data corroborates that CO attenuates caspase 3, 8, and 9 activity through MKK3/p38 MAPK during lung I-R. CO was unable to decrease caspase activity in mouse lungs that were pretreated with SB203580, a p38 MAPK inhibitor, or that were MKK3-deficient (Figure 4B).

**Caspase 8 activation is upstream of caspases 9 and 3 during anoxia.** We next attempted to delineate a general order of the caspases using caspase-specific inhibitors. The specificity of the caspase inhibitors have been previously validated (18). When PAEC were pretreated with Z-DQMD-FMK, a caspase 3-specific inhibitor, during anoxia, only caspase 3 activity was attenuated, indicating that caspase 3 was downstream of caspases 8 and 9 (Figure 4C lane 3). When PAEC were pretreated with Z-IETD-FMK, a caspase 8-specific inhibitor, during anoxia, all three caspases were inhibited (Figure 4C lane 4). When PAEC were pretreated with Z-LEHD-FMK, a caspase 9-specific inhibitor, caspase 8 activity was not
affected, whereas caspases 3 and 9 where inhibited (Figure 4C lane 5). The data indicated that the sequence of caspase activation was 8, 9, and then 3 in PAEC during anoxia. Of note, inhibiting any of the caspases (8, 9, or 3) significantly attenuated A-R-induced apoptosis in PAEC (data not shown).

**CO inhibits PARP cleavage through MKK3/p38 MAPK in vitro and in vivo during I-R.** Caspase 3 activation results in PARP cleavage and subsequent DNA fragmentation and apoptosis (19). In Figure 5A we show that CO inhibits PARP cleavage via MKK3/p38 MAPK in PAEC and mouse lung. We confirmed that PARP cleavage is downstream of caspases 8, 9, and 3 by showing that pretreatment with either caspase 3, 8 or 9-specific inhibitors all diminished PARP cleavage in PAEC during anoxia (Figure 5B).

**CO inhibits Bid cleavage through the MKK3/p38 MAPK pathway in vitro and in vivo during I-R.** Caspase 8 cleaves Bid, a BH3 domain-containing pro-apoptotic Bcl-2 family protein, into its active, truncated form with subsequent translocation to the mitochondria where it induces the release of cytochrome c in a manner that is 500-fold more potent than Bax, another pro-apoptotic Bcl-2 protein (20,21). We demonstrated that caspase 8 activity was significantly increased during I-R and that CO attenuated caspase 8 activity via MKK3/p38 MAPK in Figure 4. We then illustrated that A-R can induce the cleavage of the precursor form of Bid (Figure 6A upper bands) to the active, truncated form tBid (lower bands) and that CO can inhibit Bid cleavage via p38 MAPK in PAEC (Figure 6A top panel lanes 4-7). Our *in vivo* data in Figure 6A bottom panel correlate with our *in vitro* data. We next confirmed that caspase 8, rather than caspase 3 or 9, is responsible for Bid cleavage during anoxia in PAEC (Figure 6B). Inhibition of caspase 8 activity with Z-IETD-FMK inhibited anoxia-induced Bid cleavage (Figure 6B lane 4), whereas inhibiting caspase 3 or 9 had no effect (Figure 6B lanes 3 and 5, respectively).

**CO inhibits A-R-induced cytochrome c release and this precedes caspase 9 and 3 inhibition.** Cleaved Bid can translocate to the mitochondria and induce cytochrome c release, which then leads to
caspase 9 and 3 activation (21). We found that CO can attenuate A-R-induced cytochrome c release (Figure 7A lanes 4 and 5) and that this is through p38 MAPK activity (Figure 7B lane 7). FasL inhibition also attenuates cytochrome c release (Figure 7A lanes 6 and 7). When we pretreated PAEC with Z-IETD-FMK, a caspase 8-specific inhibitor, cytosolic cytochrome c levels were decreased during anoxia (Figure 7B lane 4) whereas caspase 3 and 9 inhibition had no effect (Figure 7B lanes 3 and 5, respectively). These data in conjunction with our data showing that CO modulates caspase 8 via MKK3/p38 MAPK (Figure 4) indicate that CO inhibits cytochrome c through p38 MAPK activation and caspase 8 inhibition, which precedes caspase 9 and 3 inhibition. Of note, CO does not modulate Bax (see below).

**CO increases anti-apoptotic Bcl-2 family protein expression through the MKK3/p38 MAPK pathway in vitro and in vivo during I-R.** In Figure 8 top panel, we show that A-R decreases the endogenous levels of the anti-apoptotic proteins Bcl-2 and Bcl-X_L in PAEC. CO inhibits the A-R-induced decrease in Bcl-2 and Bcl-X_L expression but depends upon p38 MAPK activity, as shown by the effects of SB203580 pretreatment (Figure 8 top panel lanes 6 and 7). A-R and CO had no effects on the expression of the pro-apoptotic Bax. Our in vivo data also strongly support our in vitro results by showing that CO can increase the expression of Bcl-2 and Bcl-X_L during lung I-R and that the effect of CO is dependent upon the MKK3/p38 pathway (Figure 8 bottom panel). CO was unable to increase Bcl-2 and Bcl-X_L expression in mice that were pretreated with a specific p38 MAPK inhibitor SB203580 (lane 4) or that were deficient in MKK3 (lane 6) during I-R. Of note, inhibiting FasL with anti-FasL antibody had the same effect on cleaved Bid, Bcl-2 and Bcl-X_L expression as CO in PAEC during A-R (data not shown).

**Discussion**

There is significant evidence for the pivotal role of apoptosis in the pathogenesis of I-R organ
injury (6,7,22). Strategies to attenuate I-R-induced apoptosis will expand the currently limited therapeutic options. We have found that CO acts as a potent regulator of apoptosis and may be one mechanism whereby the induction of HO-1, the enzyme responsible for more than 90% of endogenous CO generation, affords significant cytoprotection against a variety of noxious stimuli such as lung I-R injury. We previously demonstrated that exogenous administration of 15ppm CO, an extremely low level significantly below accepted safety standards, dramatically inhibited A-R-induced apoptosis and that the anti-apoptotic effect of CO is mediated via p38 MAPK and involves caspase 3 (12). Our current studies more precisely delineate potential mechanisms utilized by CO. We show exogenous CO activates MKK3/6 and p38α MAPK, but not p38β MAPK, during A-R in PAEC. The upstream MAPK kinases, MKK3 and MKK6, are thought to be the predominant kinases responsible for activating p38 MAPK. Differences in the relative contribution of these protein kinases in activating p38 MAPK are attributed to the p38 MAPK isoform involved, the stimuli, and cell type. For instance, MKK3 is the major activator of p38 MAPK in PC-12 cells exposed to osmotic stress, while MKK6 is the dominant activator of p38 MAPK in epithelial cells exposed to osmotic stress, TNF-α, and IL-1 and in monocytes stimulated by bacterial lipopolysaccharide (23-25). Furthermore, it also has been reported in transformed HeLa and COS-7 cells that MKK3 activates p38α and p38γ, while MKK6 activates p38α, p38β, and p38γ (26). The different signal transduction pathways initiated by various stimuli leading to the activation of different p38 MAPK isoforms by one or more specific MAPK kinases may account for stimulus-specific and cell-specific responses.

We show in our I-R model that in the presence of p38α DNM or MKK3 DNM (in PAEC) or MKK3 deficiency (in mice), CO can no longer attenuate I-R-induced apoptosis. Notably, MKK6 DNM transfection in cells or MKK6 deficiency in mice had no effect on the anti-apoptotic effect of CO. The literature presents both pro-apoptotic and anti-apoptotic effects of p38 MAPK activation that are likely a
reflection of cell type, different inducers, and, potentially, the differential modulation of each of the different p38 MAPK isoforms. Although p38α MAPK is generally thought to be pro-apoptotic, there are recent reports that p38α MAPK, but not p38β MAPK, can inhibit the apoptotic death of differentiating neurons (27). The p38 MAPK isoforms are likely coupled to distinct upstream signal transduction pathways. This would enable activation of specific p38 MAPK isoforms in response to a variety of stimuli. Alternatively, the p38 MAPK isoforms may have different downstream targets, which would allow coupling of the various p38 MAPK isoforms to specific biologic responses. Thus, the differential activation of the p38 MAPK isoforms can facilitate cell type- and stimulus-specific cellular responses and may account for the multiple actions of p38 MAPK, thereby highlighting the importance of precisely identifying the p38 MAPK isoforms and upstream modulators involved. Our data indicate that the anti-apoptotic effect of CO, in our models of endothelial cell and lung I-R, is dependent upon MKK3/p38α MAPK pathways.

The Fas (CD95)/Fas ligand (CD95L) system is a key regulator of apoptosis. Binding of Fas by its ligand FasL can induce caspase 8 activation and lead to the activation of downstream caspases, followed by cleavage of key regulatory proteins, such as PARP, and ultimately result in apoptosis (28-30). The Fas/FasL system was upregulated in myocytes during hypoxia, ischemia and I-R (31,32). Kitamura et al. found that increased Fas/FasL expression in lung tissues after lipopolysaccharide injury played a critical role in lung injury and that the proper regulation of the Fas/FasL system was important for the potential treatment of acute lung injury or acute respiratory distress syndrome (33). Ke et al. showed that HO-1 gene transfer prevented Fas/FasL-mediated apoptosis and significantly prolonged allogeneic orthotopic liver transplantation survival (34). We show that I-R lung injury increases Fas/FasL expression in PAEC and lung tissues, which can then be modulated by CO. The administration of a blocking FasL antibody in cells or Fas deficiency in mice decreased apoptosis to levels found in CO-treated cells and animals.
Moreover, CO modulates Fas/FasL and subsequent downstream effectors via p38α MAPK and MKK3 in lung I-R. CO could not attenuate Fas and FasL expression and the downstream effectors in the presence of p38 MAPK inhibition or MKK3-deficiency.

We also show that CO differentially modulates pro- and anti-apoptotic Bcl-2 family members through the MKK3/p38 MAPK pathway. I-R injury decreases anti-apoptotic protein levels (Bcl-2 and Bcl-XL), but CO maintains the levels of Bcl-2 and Bcl-XL while decreasing levels of the pro-apoptotic cleaved Bid during I-R. Bid has been implicated in the TNF and Fas death signal pathways (21,35). The precise mechanism through which Bid is proteolytically activated in PAEC and lung during I-R is unclear at the present time. However, in other cells and tissues, several intracellular molecules have been proposed to be the activators of Bid, including caspase 8, granzyme B, and caspase 3 (20,21,36,37). The Fas/caspase 8 pathway has been reported to be the most efficient mechanism for Bid cleavage in various cell types and could be the major pathway for Bid cleavage in our model. We show that Fas/FasL expression and caspase 8 activity are significantly increased during I-R, and that the blockade of Fas/FasL during A-R also attenuated Bid cleavage. Furthermore, caspases 9 and 3 have no effect on Bid cleavage. CO exposure can inhibit cleavage of Bid during I-R but is dependent upon p38 MAPK/MKK3 activity in cells and mouse lung. There is no change in the pro-apoptotic protein level of Bax during PAEC A-R with or without CO exposure. We also demonstrate that CO attenuates the apoptotic events downstream of Bid cleavage, namely cytochrome c release, caspase 9 and 3 activation, and finally PARP cleavage. We confirm that the aforementioned events are mediated by CO through MKK3/p38α MAPK and Fas/FasL modulation.

In summary, our observations that FasL inhibition can reproduce the effects of CO on Bcl-2 proteins, cytochrome c release, and PARP in conjunction with our data showing that CO-modulated
Fas/FasL expression is downstream of MKK3/p38α MAPK suggest that a likely progression of CO-mediated anti-apoptotic signaling is first MKK3/p38α MAPK activation, then Fas/FasL downregulation, followed by decreased caspase 8 activity, then differential modulation of pro- and anti-apoptotic Bcl-2 proteins, and finally, decreased cytochrome c release and cleaved PARP expression. Furthermore, we are the first to validate these cellular mechanisms of CO in vivo when we demonstrate that Fas-deficient mice do not exhibit I-R-induced apoptosis and that p38 MAPK inhibition or MKK3 deficiency in mice ablates the ability of CO to modulate Fas/FasL and downstream apoptosis effectors.
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Figure legends

Figure 1. The anti-apoptotic effect of CO is mediated by MKK3/p38α MAPK in PAEC during A-R. 

A, Immunoblots of the time course of phosphorylated (and therefore, activated) p38 MAPK during anoxia in the presence or absence of CO. Panel a, Anoxia alone. Panel b, Anoxia in the presence of CO. Lane 1, room air control (RA), Lane 2, 0.5 h anoxia in the absence of CO (panel a) or presence of CO (panel b), Lane 3, 1 h anoxia in the presence or absence of CO, Lane 4, 4 h anoxia in the presence or absence of CO, Lane 5, 8 h anoxia in the presence or absence of CO, Lane 6, 16 h anoxia in the presence or absence of CO, Lane 7, 24 h anoxia in the presence or absence of CO. Total p38 was used as a loading control. The data are representative of three independent experiments. 

B, Immunoblots of MKK3/6, p38 MAPK, and p38α MAPK isoform in the presence of CO in PAEC during A-R. Lane 1, room air control (RA); Lane 2, 24 h anoxia (24A); Lane 3, 24 h anoxia followed by 1 h reoxygenation (24A/1R); Lane 4, 24 h anoxia in the presence of CO (24A+CO); Lane 5, 24 h anoxia followed by 1 h reoxygenation in the presence of CO (24A+CO/1R+CO). β-tubulin was used as a loading control. The data are representative of three independent experiments. 

C, Flow cytometry analysis of apoptosis after PAEC were transfected with p38α DNM or MKK3 DNM and then exposed to room air, 24A or 24A/1R in the presence or absence of CO. The data are representative of three independent experiments. 

D, Graphical quantitation of the mean flow cytometry result from three independent experiments +/- S.E. in PAEC during A-R. *P<0.05 compared to RA.

Figure 2. The anti-apoptotic effect of CO is through MKK3/p38 MAPK and Fas/FasL inhibition has similar effects in vitro (PAEC) and in vivo (mouse lung). 

A, Flow cytometry analysis of apoptosis after PAEC were exposed to RA, 24A or 24A/1R in the presence or absence of CO, SB203580 (a specific p38 MAPK inhibitor), or a FasL-blocking antibody (anti-FasL). The data are representative of three independent experiments. 

B, Graphical quantitation of the mean flow cytometry result +/- S.E. in PAEC during A-R.
P<0.05 compared to RA. C, Lung apoptosis was detected with TUNEL staining after wildtype, Fas−/−, and MKK3−/− mice were subjected to lung I-R in the presence or absence of CO. Panel a, untreated mice (Naïve); Panel b, 30 min ischemia followed by 2 h reperfusion (Wildtype I/R); Panel c, Fas−/− mice subjected to I-R (Fas−/− I/R); Panel d, I-R in the presence of CO (CO/I/R); Panel e, Wildtype mice pretreated with SB203580, then subjected to I-R in the presence of CO (SB/CO/I/R); Panel f, MKK3−/− mice subjected to I-R in the presence of CO (MKK3−/− CO/I/R). Arrows denote TUNEL positive (dark purple) cells. The data are representative of three independent experiments.

Figure 3. CO decreases Fas and FasL expression through the MKK3/p38 MAPK pathway during I-R in vitro and in vivo. A, PAEC were stained with anti-Fas antibody or control rat-IgG (negative control) during A-R in the presence or absence of CO and with or without 1 h pretreatment with SB203580, a specific p38 MAPK inhibitor, during A-R. The mean flow cytometry result from three independent experiments +/- S.E. is shown. *P<0.05 compared to RA. B, PAEC were stained with anti-FasL antibody or control rat-IgG (negative control) during A-R in the presence or absence of CO and with or without 1 h pretreatment with SB203580, a specific p38 MAPK inhibitor. The mean flow cytometry result from three independent experiments +/- S.E. is shown. *P<0.05 compared to RA. C, Lung lysates from wildtype and MKK3−/− mice were analyzed for Fas and FasL expression by immunoblotting with anti-Fas and anti-FasL antibodies, respectively, according to “Experimental Procedures.” β-tubulin was used to control for loading. The data are representative of three independent experiments.

Figure 4. CO inhibits the activity of caspase 3, 8, and 9 through MKK3/p38 MAPK during I-R in vitro and in vivo. A, Caspase activity in PAEC was detected according to “Experimental Procedures” after cells were exposed to CO in the presence or absence of SB203580, a specific inhibitor of p38 MAPK, or in the presence of a FasL-blocking antibody (anti-FasL) during A-R. The mean caspase activity from three independent experiments +/- S.E. is shown. *P<0.05 compared to RA. B, Caspase activity in lung lysates
from wildtype and MKK3−/− mice was detected after mice were subjected to lung I-R in the presence or absence of CO or SB203580. **P<0.05 compared to naïve. C, Caspase activity in PAEC was detected after pretreatment with a caspase 3-specific inhibitor (Z-DQMD), a caspase 8-specific inhibitor (Z-IETD), or a caspase 9-specific inhibitor (Z-LEHD) during 24A. *P<0.05 compared to 24A.

**Figure 5. CO inhibits PARP cleavage through MKK3/p38 MAPK during I-R in vitro and in vivo.** A top, PAEC were exposed to A-R in the presence or absence of CO or SB203580, a specific p38 MAPK inhibitor, and cell lysates were analyzed for PARP cleavage by immunoblotting with anti-cleaved PARP antibody as described in “Experimental Procedures.” A bottom, Wildtype or MKK3−/− mice were subjected to lung I-R in the presence or absence of CO or SB203580 and lung lysates were analyzed for PARP cleavage by immunoblotting with anti-cleaved antibody. B, PAEC were exposed to 24 h anoxia in the presence or absence of caspase 3 (Z-DQMD), 8 (Z-IETD), or 9 (Z-LEHD)-specific inhibitors and then analyzed for PARP cleavage by immunoblotting with anti-cleaved PARP antibody. β-tubulin was used as a loading control for all immunoblots. The data are representative of three independent experiments.

**Figure 6. CO attenuates pro-apoptotic Bid cleavage through MKK3/p38 MAPK activation during I-R in vitro and in vivo.** A top, PAEC were exposed to A-R in the presence or absence of CO or SB203580, a specific p38 MAPK inhibitor, and cell lysates were analyzed for Bid cleavage (tBid) by immunoblotting with anti-Bid antibody, as described in “Experimental Procedures.” A bottom, Wildtype or MKK3−/− mice were subjected to lung I-R in the presence or absence of CO or SB203580, and lung lysates were analyzed for Bid cleavage by immunoblotting with anti-Bid antibody. B, PAEC were exposed to 24 h anoxia in the presence or absence of caspase 3 (Z-DQMD)-, 8 (Z-IETD)-, or 9 (Z-LEHD)-specific inhibitors and then analyzed for Bid cleavage by immunoblotting with anti-Bid antibody. β-tubulin was used as a loading control for all immunoblots. The data are representative of three independent experiments.
Figure 7. **CO attenuates cytochrome c release during A-R in PAEC.** The cytosolic fraction of PAEC was isolated and analyzed for cytochrome c release by immunoblotting with anti-cytochrome c antibody as described in “Experimental Procedures.” **A,** PAEC were exposed to A-R in the presence or absence of CO and then analyzed for cytochrome c release. **B,** PAEC were exposed to 24A in the presence or absence of SB203580, a specific p38 MAPK inhibitor, or caspase 3 (Z-DQMD)-, 8 (Z-IETD)-, or 9 (Z-LEHD)-specific inhibitors and then analyzed for cytochrome c release. β-tubulin was used as a loading control for all immunoblots. The data are representative of three independent experiments.

Figure 8. **CO increases anti-apoptotic Bcl-2 family proteins through MKK3/p38 MAPK activation during I-R in vitro and in vivo.** **Top,** PAEC were exposed to A-R in the presence or absence of CO or SB203580, a specific p38 MAPK inhibitor, and then analyzed for Bax, Bcl-XL, and Bcl-2 by immunoblotting with the respective antibodies as described in “Experimental Procedures.” **Bottom,** Wildtype or MKK3−/− mice were subjected to lung I-R in the presence or absence of CO or SB203580 and lung lysates were analyzed for Bax, Bcl-XL, and Bcl-2 by immunoblotting with the respective antibodies. β-tubulin was used as a loading control for all immunoblots. The data are representative of three independent experiments.
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Figure 1A

Anoxia

| RA | 0.5h | 1h | 4h | 8h | 16h | 24h |
|----|------|----|----|----|-----|-----|
| 1  | 2    | 3  | 4  | 5  | 6   | 7   |

Phospho-p38
p38

Anoxia/CO

| RA | 0.5h | 1h | 4h | 8h | 16h | 24h |
|----|------|----|----|----|-----|-----|
| 1  | 2    | 3  | 4  | 5  | 6   | 7   |

Phospho-p38
p38
Figure 1B

| RA  | 24A | 1R  | 24A+CO | 24A+CO/1R+CO |
|-----|-----|-----|--------|--------------|
| 1   | 2   | 3   | 4      | 5            |

Phospho-MKK3/6
Phospho-p38
Phospho-p38α
β-tubulin
Figure 1C

- 24A
- 24A+CO
- p38αDNM/24A+CO
- MKK3DNM/24A+CO
- 24A/rh
- 24A+CO/1R+CO
- p38αDNM/24A+CO/1R+CO
- MKK3DNM/24A+CO/1R+CO
- RA
- p38αDNM/RA
- MKK3DNM/RA

FL1-Annexin
Figure 2A

- 24A
  - FL1-Annexin: 8.4%
  - FL1-Annexin: 14.4%

- 24A+CO
  - FL1-Annexin: 4.2%
  - FL1-Annexin: 2.7%

- SB/24A+CO
  - FL1-Annexin: 7.0%
  - FL1-Annexin: 9.5%

- anti-FasL/24A
  - FL1-Annexin: 4.0%
  - FL1-Annexin: 5.7%

- 24A/1hR
  - FL1-Annexin: 14.5%
  - FL1-Annexin: 12.0%

- 24A+CO/1R+CO
  - FL1-Annexin: 1.5%
  - FL1-Annexin: 5.8%

- SB/24A+CO/1R+CO
  - FL1-Annexin: 4.3%
  - FL1-Annexin: 17.7%

- anti-FasL/24A/1R
  - FL1-Annexin: 1.4%
  - FL1-Annexin: 4.4%

- RA
  - FL1-Annexin: 1.8%
  - FL1-Annexin: 2.6%

- SB/RA
  - FL1-Annexin: 3.5%
  - FL1-Annexin: 2.2%

- anti-FasL/RA
  - FL1-Annexin: 1.1%
  - FL1-Annexin: 4.2%
Figure 2B

Graph showing the percentage of apoptotic cells in different treatments:
- RA
- 24A
- 24A/1R
- 24A+CO
- 24A+CO/1R+CO
- SB/RA
- SB/24A+CO
- SB/24A+CO/1R+CO
- anti-Fas/RA
- anti-FasL/24A
- anti-FasL/24A/1R

The graph includes error bars indicating the standard error of the mean.
Figure 2C

a) Naïve
b) Wildtype I/R
c) Fas−/− I/R
d) CO/I/R
e) SB/CO/I/R
f) MKK3−/− CO/I/R
Figure 3B

Fas Ligand Expression (%)

1 2 3 4 5 6 7 8

RA 24A 24A/1R 24A+CO 24A+CO/1R+CO SB/RA SB/24A+CO SB/24A+CO/1R+CO

* * *
### Figure 3C

|       | Wildtype |         |       |       |         |       |         |       |       |
|-------|----------|---------|-------|-------|---------|-------|---------|-------|-------|
|       | Naive    | I/R     | CO/I/R| CO/I/R| Naive   | I/R   | CO/I/R  |       |       |
| 1     |          |         |       |       | 5       |       |         |       |       |
| 2     |          |         |       |       | 6       |       |         |       |       |
| 3     |          |         |       |       | 7       |       |         |       |       |
| 4     |          |         |       |       |         |       |         |       |       |

|       | SB       |         |       |       |         |       |         |       |       |
|-------|----------|---------|-------|-------|---------|-------|---------|-------|-------|
|       |          |         |       |       | 1       |       |         |       |       |
|       |          |         |       |       | 2       |       |         |       |       |
|       |          |         |       |       | 3       |       |         |       |       |
|       |          |         |       |       | 4       |       |         |       |       |

- **Fas**
- **FasL**
- **β-tubulin**
Figure 4A

Caspase Activities (Fold Induction)

- Caspase 3
- Caspase 8
- Caspase 9

RA

24A

24A/1R

24A+CO

24A+CO/1R+CO

SB/24A+CO

SB/24A+CO/1R+CO

anti-Fasl/24A

anti-Fasl/24A/1R

Caspase Activities (Fold Induction)

1 2 3 4 5 6 7 8 9
Figure 4B

Caspase Activity (Fold induction)

- caspase 3
- caspase 8
- caspase 9

Legend:
- MKK3-/- CO/I/R
- MKK3-/- I/R

Experiments include:
- Naive
- I/R
- CO/I/R
- SB/CO/I/R
- MKK3-/- I/R
- MKK3-/- CO/I/R
Figure 4C

Caspase Activities (Fold Induction)

RA  24A  Z-DQMD/24A  Z-IETD/24A  Z-LEHD/24A

Caspase 3  Caspase 8  Caspase 9

* * *

1                      2                         3        4                     5
Figure 5A

PAEC

| RA | 24A | 1R | CO | 24A | 1R | SB+CO |
|----|-----|----|----|-----|----|-------|
| 1  | 2   | 3  | 4  | 5   | 6  | 7     |

Cleaved PARP
β-tubulin

Mouse lung

| Wildtype | MKK3−/− |
|----------|---------|
| Naive    | I/R     | CO/I/R | SB   | CO/I/R |
| 1        | 2       | 3      | 4    | 5      |

Cleaved PARP
β-tubulin
### Figure 5B

|    | RA | 24A | Z-DQMD | Z-IETD | Z-LEHD |
|----|----|-----|--------|--------|--------|
| 1  | 1  | 2   | 3      | 4      | 5      |

- **Cleaved PARP**
- **β-tubulin**
Figure 6A

PAEC

|       | RA | 24A | 1R |       | CO  | 24A | 1R |       | SB+CO | 24A | 1R |
|-------|----|-----|----|-------|-----|-----|----|-------|-------|-----|----|
| 1     | 1  | 2   | 3  |       | 4   | 5   |    |       | 6     | 7   |

Bid  
tBid  
β-tubulin

Mouse lung

|       | Wildtype |       | MKK3-/ |       |
|-------|----------|-------|--------|-------|
|       | Naive    | I/R   | CO/I/R | SB    | CO/I/R |
|       | 1        | 2     | 3      | 4     | 5      |

Bid  
tBid  
β-tubulin
Figure 6B

|       | RA | 24A | Z-DQMD | Z-IETD | Z-LEHD |
|-------|----|-----|--------|--------|--------|
| 1     |    |     |        |        |        |
| 2     |    |     |        |        |        |
| 3     |    |     |        |        |        |
| 4     |    |     |        |        |        |
| 5     |    |     |        |        |        |

Bid

tBid

β-tubulin
Figure 7A

|     | RA  | 24A | 1R  |     | 24A | 1R  |     |
|-----|-----|-----|-----|-----|-----|-----|-----|
| 1   |     |     |     |     |     |     |     |
| 2   |     |     |     |     |     |     |     |
| 3   |     |     |     |     |     |     |     |

CO  

|     |     |     | 24A |     | 1R  |     |
|-----|-----|-----|-----|-----|-----|-----|
| 4   |     |     |     |     |     |     |
| 5   |     |     |     |     |     |     |

anti-FasL  

|     | 24A | 1R  |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|
| 6   |     |     |     |     |     |     |
| 7   |     |     |     |     |     |     |

Cytochrome c

β-tubulin
Figure 7B

Cytochrome c

β-tubulin
### PAEC

|     | RA 24A 1R | CO 24A 1R | SB+CO 24A 1R |
|-----|-----------|-----------|--------------|
| 1   | 2         | 3         | 4            |

- **Bax**
- **Bcl-X<sub>L</sub>**
- **Bcl-2**
- **β-tubulin**

### Mouse lung

|       | Naive 1 | I/R 2 | CO/I/R 3 | SB 4 | I/R 5 | CO/I/R 6 |
|-------|---------|-------|----------|------|-------|----------|

- **Bax**
- **Bcl-X<sub>L</sub>**
- **Bcl-2**
- **β-tubulin**

*Figure 8*
Carbon monoxide modulates Fas/Fas ligand, caspases, and Bcl-2 family proteins via the p38alpha mitogen-activated protein kinase pathway during ischemia-reperfusion lung injury

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