Carbon Monoxide Induces Heme Oxygenase-1 via Activation of Protein Kinase R–Like Endoplasmic Reticulum Kinase and Inhibits Endothelial Cell Apoptosis Triggered by Endoplasmic Reticulum Stress

Ki Mo Kim, Hyun-Ock Pae, Min Zheng, Raekil Park, Young-Myeong Kim, Hun-Taeg Chung

Abstract—Carbon monoxide (CO), a reaction product of the cytoprotective heme oxygenase (HO)-1, is antiapoptotic in a variety of models of cellular injury, but the precise mechanisms remain to be established. In human umbilical vein endothelial cells, exogenous CO activated Nrf2 through the phosphorylation of protein kinase R–like endoplasmic reticulum kinase (PERK), resulting in HO-1 expression. CO-induced activation of PERK was followed by the phosphorylation of eukaryotic translation initiation factor 2α and the expression of activating transcription factor 4. However, CO fails to induce X-box binding protein-1 expression and activating transcription factor 6 cleavage. CO had no significant effect on synthesis of endoplasmic reticulum (ER) chaperone proteins such as the 78-kDa glucose-regulated proteins 78 and 94. Instead, CO prevented X-box binding protein-1 expression and activating transcription factor 6 cleavage induced by ER-stress inducers such as thapsigargin, tunicamycin and homocysteine. CO also prevented endothelial apoptosis triggered by these ER inducers through suppression of C/EBP homologous protein expression, which was associated with its activation of p38 mitogen-activated protein kinase. Similarly, endogenous CO produced from endothelial HO-1 induced by either exogenous CO or a pharmacological inducer was also cytoprotecive against ER stress through C/EBP homologous protein suppression. Our findings suggest that CO renders endothelial cells resistant to ER stress not only by downregulating C/EBP homologous protein expression via p38 mitogen-activated protein kinase activation but also by upregulating Nrf2-dependent HO-1 expression via PERK activation. Thus, the HO-1/CO system might be potential therapeutics in vascular diseases associated with ER stress. (Circ Res. 2007;101:919-927.)

Key Words: carbon monoxide ■ endoplasmic reticulum stress ■ apoptosis ■ heme oxygenase-1

Heme oxygenase (HO) is a rate-limiting and microsomal enzyme that catalyzes the oxidative degradation of free heme to biliverdin, free iron, and carbon monoxide (CO). There are 3 distinct isofoms of HO: HO-1, HO-2, and HO-3. The HO-2 isoform is constitutively expressed and is present in high concentrations in the brain and testes. In contrast, HO-1 is ubiquitously distributed and strongly induced by oxidative, nitrosative, osmotic, and hemodynamic stresses.1–5 Endoplasmic reticulum (ER) stress triggers unfolded protein response (UPR).6–8 In the mammalian cells, UPR is a signaling network consisting of 3 ER-resident sensors: the kinase and endoribonuclease (IRE1), the protein kinase R (PKR)-like ER kinase (PERK), and the basic leucine–zipper activating transcription factor 6 (ATF6).6–9 On ER stress, IRE1 is dimerized, activated, and allowed to alternatively splice X-box binding protein (Xbp)-1 mRNA by removing a 26-bp intron. This transcription frameshift permits Xbp-1 to act as a transcription activator of genes containing upstream ER-stress response elements. Activation of both IRE-1 and ATF6 increases the expression of ER-resident chaperones such as the 78-kDa glucose-regulated protein (GRP) 78 and GRP94, which facilitate the restoration of proper protein folding within the ER.10 UPR-mediated PERK activation impedes protein translation via phosphorylation-dependent inhibition of eukaryotic translation initiation factor (eIF)2α.11 Independent of its translational regulatory capacity, PERK-dependent signals elicit the activation of the survival transcription factor NF-E2–related factor-2 (Nrf2) via site specific phosphorylation.12 PERK belongs to an eIF2α kinase family that is responsive to distinct stress signals and includes the interferon-inducible PKR, the hemin-regulated inhibitor kinase (HRI), and the general control of amino acid biosynthesis kinase (GCN2), which responds to uncharged transfer RNAs and adapts cells

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to amino acid starvation. Of these, PERK is localized at ER membrane and required for the cellular response to ER stress. After the eIF2α/\(e^{\alpha}F2\) kinase activation, eIF2\(e^{\alpha}F2\) phosphorylation inhibits translation of a majority of cellular proteins while paradoxically promoting a cytoprotective gene expression program known as the integrated stress response. Among upregulating genes, activating transcription factor (ATF) 4 plays an important role in activating genes that promote the linked processes of import and metabolism of thiol-containing amino acids and resistance to oxidative stress and the transcription factor C/EBP homologous protein (CHOP), which induces apoptosis by promoting protein synthesis and oxidative stress.

Nrf2 activation has been implicated in the promotion of cell survivals following ER stress. UPR-dependent PERK activation promotes PERK-dependent phosphorylation and nuclear localization of Nrf2, which results in increased transcription of Nrf2 target genes of phase II detoxifying enzymes such as HO-1. We recently showed that CO-releasing molecule or CO gas induces HO-1 expression via Nrf2 activation in rat hepatocytes and human HepG2 cells.

In this study, we tried to further examine whether CO could also induce Nrf2 activation and HO-1 expression in human endothelial cells and, if so, to elucidate the molecular basis of CO-induced Nrf2 activation responsible for HO-1 expression. In addition, we also investigated the effects of CO on endothelial apoptosis triggered by ER stress. We provide evidence that CO induces Nrf2-dependent HO-1 expression via PERK signaling pathway and prevents apoptosis triggered by ER stress via CHOP suppression.

### Materials and Methods

**Reagents**

Tricarbonyl dichlororuthenium (II) dimmer (RuCO), homocysteine (HCys), ruthenium chloride (RuCl\(_2\)), CO gas, arsenite, and hemoglobin (Hb) were purchased from Sigma-Aldrich (St Louis, Mo). Thapsigargin (TG), tunicamycin (TM), and HO-1 antibody were obtained from Calbiochem (La Jolla, Calif). Lipofectamine 2000 was obtained from Invitrogen Life Technology (Grand Island, NY). Antibodies to Nrf-2, phospho (p)-PERK, PERK, p-eIF2\(e^{\alpha}F2\), CHOP, ATF4, HRI, GRP78, GRP94, Lamin A, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif); and antibodies to GCN2, p-GCN2, PKR, p-PKR, p38, p-p38, extracellular signal-regulated (ERK), p-ERK, c-Jun N-terminal kinase (JNK), and p-JNK were from Cell Signaling Technology (Beverly, Mass). Poly(ADP-ribose) polymerase (PARP) and ATF6 antibodies were purchased from Biomol (Plymouth Meeting, Pa) and Imgenex (San Diego Calif), respectively. Xbp-1 antibody was purchased from Biolegend (San Diego, Calif). The small interfering (si)RNAs against PERK, eIF2α, CHOP, and HO-1 were obtained from Santa Cruz Biotechnology. An in situ cell death detection kit was obtained from Roche (Penzberg, Germany). All other chemicals were obtained from Sigma-Aldrich.

**Detection of CO Release**

The release of CO from RuCO was assessed spectrophotometrically by measuring the conversion of deoxymyoglobin to carbonmonoxy myoglobin, as described previously. The amount of carbonmonoxy myoglobin measured after the reaction revealed that 0.72 mol of CO was liberated per 1 mol of RuCO.

**Treatment With CO Gas**

Saturated stock solution of CO was prepared in buffer containing 140 mmol/L NaCl, 5 mmol/L KCl, and 20 mmol/L HEPES, pH 7.3, as described previously. CO stock solution was freshly prepared for every experiment.

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**Figure 1.** Effects of RuCO on Nrf2 activation and HO-1 expression in HUVECs. A, Cells were incubated for 2 hours (Nrf2 activation) or 6 hours (HO-1 expression) with indicated concentrations of RuCO or RuCl\(_2\), in the presence or absence of 50 μg/mL Hb. CF indicates cytosolic fraction; NF, nuclear fraction. CO gas (20 μmol/L) was used as a positive control. Western blot analysis (top) for Nrf2 and HO-1 and densitometry analysis (bottom) of all bands were performed as described in Materials and Methods. Blots shown are representative of 3 independent experiments. B, Cells transfected with HO-1-Luci or control vector were exposed for 3 hours to 20 μmol/L RuCO, 20 μmol/L CO gas, or 20 μmol/L RuCl\(_2\) in the presence or absence of Hb. Cell lysates were assayed for luciferase activity as the fold induction by normalizing the transfection efficiency and dividing values of each experiment relative to the untreated control. Values are means ± SD from 3 independent experiments. *P<0.05 with respect to each untreated group, **P<0.05.
Cell Culture

Human umbilical vein endothelial cells (HUVECs) from human umbilical cord veins were isolated as described previously and used for experiments in passages 3 to 8. HUVECs were maintained in EGM-2 medium (Cambrex BioScience Inc, Walkersville, Md) in a humidified chamber containing 5% CO2 at 37°C.

Cell Viability Assay

Cell viability was determined by a modified MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction assay, as described previously. Absorbance was measured in an ELISA reader at 570 nm, with the absorbance at 690 nm to correct for background, and viability was expressed as percentage of untreated controls.

Plasmid Constructions

HO-1, the original clone of which was a kind gift from Dr A. M. K. Choi (University of Pittsburgh, Pa), was subcloned into pcDNA3 vector. Human HO-1 promoter construct was generated by PCR amplification of the target sequence, followed by cloning it into plasmid containing reporter gene. Briefly, an ~4.4-kb fragment of the 5'-flanking region of the human HO-1 gene including the transcription initiation site (spanning region of 4384 bp to 24 bp) was amplified from HeLa cell genomic DNA using PCR primer containing proper restriction enzyme sites for the cloning (5'-GCTGAGCTCCAGCCTGTCACACAGCAGTTAGGC-3' and 5'-ACGCTCGAGAGGAGGCAGGCGTTGACTGCC-3'). Enzyme-digested fragment was cloned into sacI and XhoI site of the pGL3 basic vector containing the firefly luciferase cDNA (Promega, Madison, Wis) to obtain pGL3 HO-1/4384-Luci construct. All sequences of pGL3 HO-1/4384-Luci were confirmed and verified the presence of the correct and the absence of any other nucleotide changes by DNA sequencing.

TUNEL Assay

Cells were plated in slide chambers. After treatment, cells were fixed with 70% ethanol in PBS. Cells were washed once, permeabilized by...
incubating with 100 μL of 0.1% Triton X-100/0.1% sodium citrate and then washed twice in PBS. The TUNEL reaction was performed at 37°C for 1 hour with 0.3 nmol of fluorescein isothiocyanate-12-dUTP, 3 nmol of dATP, 2 μL of CoCl₂, 25 U of terminal deoxynucleotidyl transferase, and TdT buffer (30 mmol/L Tris, pH 7.2, 140 mmol/L sodium cacodylate) in a total reaction volume of 50 μL. The reaction was stopped with 2 μL of 0.5 mol/L EDTA. Cells were observed under a fluorescence microscope.

Preparation of Nuclear Fraction and Cytosolic Fraction

HUVECs were incubated with or without reagents. They were harvested, washed in ice-cold PBS buffer, and kept on ice for 1 minute. The suspension was mixed with buffer A (10 mmol/L HEPES, pH 7.5/10 mmol/L KCl/0.1 mmol/L EGTA/0.1 mmol/L EDTA/1 mmol/L dithiothreitol/0.5 mmol/L phenylmethylsulfonyl fluoride/5 μg/mL aprotinin/5 μg/mL pepstatin/10 μg/mL leupeptin) and lysed by 3 freeze–thaw cycles. Cytosolic fractions were obtained by centrifugation at 12 000 g for 20 minutes at 4°C. The pellets were resuspended in buffer C (20 mmol/L HEPES, pH 7.5/0.4 mol/L NaCl/1 mmol/L EDTA/1 mmol/L dithiothreitol/1 mmol/L phenylmethylsulfonyl fluoride/5 μg/mL aprotinin/5 μg/mL pepstatin/10 μg/mL leupeptin) ice for 40 minutes and centrifuged at 14 000 g for 20 minutes at 4°C. The resulting supernatant was used as soluble nuclear fractions. Protein content was determined with BCA protein assay reagent (Pierce, Rockford, Ill).

Western Blot and Densitometry Analyses

After treatment, cells were harvested and washed twice with ice-cold PBS. Cells were lysed with 1× Laemmli lysis buffer (2.4 mol/L glycerol/0.14 mol/L Tris, pH 6.8/0.21 mol/L sodium dodecyl sulfate/0.3 mmol/L bromophenol blue) and boiled for 10 minutes. Protein content was measured with BCA protein assay reagent (Pierce). The samples were diluted with 1× lysis buffer containing 1.28 mol/L β-mercaptoethanol, and equal amounts of protein (20 μg of protein) were separated on 7.5% to 12% SDS-PAGE and transferred to poly(vinylidene difluoride) membranes. The membranes were blocked with 5% nonfat milk in PBS containing 0.1% Tween 20 (PBS-T) for 10 minutes and incubated with antibodies to HO-1 (1:1000), p-PERK (1:500), p-eIF-2α (1:1000), Xbp-1 (1:500), GRP78 (1:1000), GRP94 (1:1000), ATF-6 (1:1000), CHOP (1:500), p-ERK (1:1000), p-p38 (1:1000), p-JNK (1:1000), HRI (1:500), p-GCN2 (1:500), or p-PKR (1:500) in PBS-T containing 1% nonfat milk for 3 hours. After washing 3 times with PBS-T, the membranes were hybridized with horseradish peroxidase–conjugated secondary antibodies for 40 minutes. Following 5 washes with PBS-T, they were incubated with chemiluminescent solution for 5 minutes and protein bands were visualized on x-ray film. For the densitometry analysis, optical density (the gray-scale value of pixels: 0 to 255) was measured on the inverted digital images using Scion Image (Scion Corp, Frederick, Md).

Transfection of siRNAs

Predisigned siRNAs against human HO-1 (catalog no. SC-35554), PERK (catalog no. SC-36213), eIF2α (catalog no. SC-35272), CHOP (catalog no. SC-35437), and control scrambled siRNA (catalog no. SC-37007) were purchased from Santa Cruz Biotechnology. The sense strands of siRNAs against HO-1, PERK, eIF2α, and CHOP are as follows: HO-1, UGCUAAACAUCAGCGCUCUU, UCCAGCUUUUUGAGGAGU, CGUGGGCACUGAAGGCUUU,
and AAGCCCUGAGUUUCAAGUA; PERK, CGAGAGCCGG-
GAUUUAUUGA, GGAUGAAAUUUGGCUGAAA, and CAGA-
CACACACAGGACAAGUA; eIF2/H9251, GGCUUGUUAUGGUUAUGAA,
CCUCGGUAUGUAAUGACUA, and GAGAGGCUUGAAAGAG-
AAA; CHOP, GAAGGCUUGGAGUAGACAA, GGAAAGGUCU-
CAGCUUGUA, and GUCUCAGCUUGUAUAAGA. Cells were
transfected with double-stranded siRNAs (40 nmol/mL) for 12 hours
by the Lipofectamine method according to the protocol of the
manufacturer (Invitrogen Life) and recovered in fresh media con-
taining 10% FBS for 24 hours. The interference of HO-1, PERK,
CHOP, or eIF2/H9251 expressions was confirmed by immunoblot using
anti-HO-1, PERK, CHOP, or eIF2α antibodies; scrambled siRNA
was used as a control.

Measurement of Promoter Activity
Cells were transiently transfected with the promoter constructs using
the transfection reagent Lipofectamine 2000. After harvest, cells
were lysed in reporter lysis buffer (Promega). Cell extract (20 μL)
was mixed with 100 μL of the luciferase assay reagent, and the
emitted light intensity was measured using the luminometer AutoLu-
mat LB953 (EG and G Berthold, Bad Wildbad, Germany). Fold
induction was calculated as intensity value from each experimental
group divided by value from control group after normalization of
transfection efficiency by β-galactosidase assay.

Statistical Analyses
Data are expressed as means±SD. Statistical analysis in this study
includes ANOVA and the post hoc group comparisons after Bonfer-
roni and Scheffé procedures. Probability values of <0.05 were
considered as significant.

Results
CO Induces Nrf2 Activation and HO-1 Expression
In HUVECs, the CO donor RuCO induced Nrf2 nuclear
translocation and HO-1 expression in a dose-dependent man-
ner (Figure 1A). This was further confirmed by our observa-
tion that RuCO also enhanced HO-1 promoter activity (Fig-
ure 1B). The effects of RuCO on Nrf2 activation and HO-1
expression were abolished when CO gas spontaneously re-
leased from RuCO was scavenged by Hb. Moreover, treat-
ment of cells with equivalent molar concentrations of ruthen-
ium (RuCl2) did not induce Nrf2 activation and HO-1
expression (Figure 1A). Our observations, therefore, suggest
that CO is only an effector molecule capable of inducing
HO-1 expression. In agreement with these findings, CO gas
induced Nrf2 activation and HO-1 expression (Figure 1A)
and also enhanced HO-1 promoter activity (Figure 1B).

CO Induces Nrf2-Dependent HO-1 Expression via
PERK Activation
Because PERK signaling activates Nrf2, leading to increased
expression of Nrf2 target genes including HO-1,17,18 we
examined whether RuCO could phosphorylate PERK in
HUVECs. As shown in Figure 2A, RuCO itself phosphory-
lated PERK and its downstream eIF2α in a time-dependent
manner, which was similar to the effects of TG or TM. Unlike
its effect on PERK phosphorylation, RuCO did not phosphor-
ylate HIR, GCN2, or PKR (Figure 2B). Moreover, RuCO could not induce eIF2α/H9251 phosphorylation when the cellular PERK expression was knocked down with a PERK-specific siRNA (Figure 2C), suggesting that eIF2α/H9251 phosphorylation by RuCO is mediated mainly via a PERK pathway. In addition, RuCO induced ATF4 expression, presumably via PERK-mediated eIF2α pathway (Figure 2D). We next examined whether PERK activation by RuCO could result in Nrf2-dependent HO-1 expression. RuCO could not induce Nrf2 nuclear translocation and HO-1 expression in the cells transfected with siRNA against PERK (Figure 2E). Blockage of eIF2α downstream by an eIF2α-specific siRNA had no effect on Nrf2 activation and HO-1 expression by RuCO (Figure 2E). Thus, our data clearly indicate that Nrf2 is rapidly mobilized from the cytosol to the nucleus in response to RuCO via PERK activation, thereby resulting in HO-1 expression.

CO Has No Effect on Xbp1 and ATF6 but Suppresses Xbp1 Expression, ATF6 Cleavage, and Apoptosis by ER Stress

Given that RuCO induces PERK activation in HUVECs, we reasoned that RuCO might also induce other ER-resident transmembrane proteins: IRE1-mediated Xbp1 expression and protease-mediated ATF6 cleavage. Surprisingly, RuCO had no significant effect on Xbp1 expression and ATF6 cleavage (Figure 3A). In addition, the expression of GRP78 and GRP94, 2 known chaperones in the ER, was not affected by RuCO (Figure 3B). Of note was that CO inhibited TG-induced Xbp1 expression and ATF6 cleavage (Figure 3C).

Having shown that CO inhibited Xbp1 expression and ATF6 cleavage following ER stress by TG (Figure 3C), we thus asked whether CO could prevent apoptosis triggered by ER stress. Treatment of HUVECs with TG resulted in a significant increase in apoptosis that was prevented by the exogenous administration of RuCO (Figure 3D). Consistent with this, RuCO greatly diminished TG-induced cell death (Figure 3D). Similarly, RuCO also prevented Xbp1 expression, ATF6 cleavage, and apoptosis induced by other ER-stress inducers, TM (Figure 4) and HCys (Figure 5). These findings prompted us to examine whether CO could inhibit proapoptotic CHOP expression by ER stress. The ER-stress inducers markedly increased CHOP expression, which was reversed by RuCO treatment (Figure 6A). We next investigated whether PERK activation by RuCO could contribute to its inhibition of TG-induced CHOP expression and found that RuCO-mediated inhibition of CHOP expression was slightly reversed by PERK siRNA but not by eIF2α siRNA (Figure 6B). This could be explained by a possibility that there would be endogenous CO that was produced by HO-1 induction through PERK activation by RuCO. In line with this, CoPP, which can induce cellular HO-1 expression to produce endogenous CO, also inhibited TG-induced CHOP expression (Figure 6C), which was further supported by our observation that inhibition of cellular HO-1 synthesis by a HO-1-specific siRNA reversed the inhibitory effects of RuCO and CoPP on TG-induced CHOP expression (Figure 6C) and cell death (Figure 6D). These data suggest that both exogenous and endogenous CO may have a capacity of inhibiting CHOP expression by ER stress.

**Figure 5.** Effects of RuCO on HCys-induced Xbp-1 and ATF6 and cell death in HUVECs. A, Cells were preincubated for 6 hours with 20 μmol/L RuCO or 1% ethanol in the presence or absence of 50 μg/mL Hb and then were exposed for 6 hours to 50 μmol/L HCys. B, Cells were preincubated for 6 hours with RuCO or ethanol in the presence or absence of Hb and then were exposed to HCys for 12 hours (apoptosis) or 18 hours (cell viability). Western blot analysis for ATF6 and Xbp-1, TUNEL assay for apoptosis, and MTT assay for cell viability were performed as described in Materials and Methods. Blots shown are representative of three independent experiments. Each bar represents mean ± SD from 3 independent experiments. *P<0.05.
CO Inhibits Proapoptotic CHOP Expression via a p38 Mitogen-Activated Protein Kinase–Dependent Pathway

To further determine the importance of CHOP in TG-induced apoptosis, we used siRNA methodology to silence the CHOP gene. The siRNA specific for CHOP was transiently transfected into HUVECs, which were then subjected to TG treatment. Transfection of CHOP siRNA inhibited the apoptotic PARP cleavage even in the presence of TG (Figure 7A, bottom), along with increased cell viability (Figure 7A, top). Notably, a CHOP-specific siRNA mimicked the cytoprotective/antiapoptotic action of RuCO and, in combination with RuCO, did not further enhance cytoprotective effects of RuCO (Figure 7A), thus supporting that CO may prevent TG-induced apoptosis by suppressing CHOP expression.

Because CO suppresses endothelial apoptosis mainly through a mechanism that is dependent on the activation of p38 mitogen-activated protein kinase (MAPK) pathway,23 we finally examined the role(s) of p38 MAPK in the effects of CO with respect to CHOP expression. RuCO increased p38 MAPK activation in dose- and time-dependent manner but not the activation of ERK or JNK (Figure 7B). SB203580, a specific inhibitor of p38 MAPK, abrogated the inhibitory effects of CO on TG-induced CHOP expression and cell death (Figure 7C), suggesting that CO inhibits CHOP expression via p38 MAPK–dependent manner.

Discussion

The HO-1/CO system has been shown to provide significant protection against vascular injury, transplant rejection, hypoxic lung injury, and atherosclerotic lesions.3–5 CO, a reaction product of HO-1 activity, has been shown to have potent antiinflammatory, antiproliferative, and antiapoptotic effects and thereby mimics the cytoprotective effects of HO-1. In our previous study,19 we revealed reciprocal feedback relationships between HO-1 and CO: HO-1 produces CO and CO induces HO-1 expression in Nrf2-dependent pathway in the liver cells. In the present study, we confirmed that CO is
capable of inducing Nrf2 activation and HO-1 expression in human endothelial cells as well. These findings implicate a novel endothelial protective function of CO. Indeed, our results showed that both exogenous and endogenous CO prevents endothelial apoptosis triggered by ER stress.

In an effort to understand the possible mechanism(s) responsible for Nrf2 activation and HO-1 expression by CO, we tested whether CO could induce PERK phosphorylation that has been already reported to activate Nrf2 leading to HO-1 expression. Interestingly, CO phosphorylated PERK, and CO-induced PERK activation was followed by eIF2\alpha phosphorylation and then ATF4 expression. Four mammalian eIF2\alpha kinases have been identified so far. PKR is activated by double-stranded RNA of viral or synthetic origin. HRI inhibits protein synthesis in heme-deprived lysates and stressed cells. A third eIF2\alpha kinase, GCN2, is specifically activated in response to amino acid deprivation. Finally, PERK is activated in response to accumulation of misfolded protein in the ER. In our study, we found that CO activates only PERK among four known eIF2\alpha kinases (Figure 2B). This was further confirmed by our observation that inhibition of cellular PERK synthesis by siRNA completely abolished eIF2\alpha phosphorylation by CO (Figure 2C). The downstream target of PERK for cell survival was recently identified as Nrf2. PERK-dependent phosphorylation leads to the nuclear accumulation of Nrf2 and increases transcription of Nrf2 target genes. In this study, we found that CO induces Nrf2-dependent HO-1 expression via PERK activation.

The abovementioned findings raised a question as to whether CO might also activate other ER-bound sensor proteins: Xbp-1 and ATF6. Unlike its PERK activation, CO did not activate Xbp-1 or ATF6 (Figure 3A). Instead, CO blocked Xbp-1 and ATF6 activation caused by the ER stress response (Figure 3C). This prompted us to study the effects of CO on ER-stress-induced apoptosis. Indeed, CO inhibited endothelial apoptosis triggered by three well-known ER stress inducers: TG, TM, and HCs. These results suggest that the HO-1/CO system upregulates the cell survival pathways and/or downregulates the cell death pathways, leading to rescue the apoptotic cell from excessive/prolonged UPR by ER stress.

To decrease ER protein accumulation and maintain ER function, the UPR attenuates translation via PERK-eIF2\alpha pathway, increases the folding capacity of the ER by upregulation of ER-chaperones via the transcription factors ATF6 and Xbp-1, and degrades misfolded proteins via the ER-associated degradation pathway. However, when ER stress is prolonged or excessive, the proapoptotic transcription factor CHOP is activated. CHOP expression is also upregu-
lated in response to a variety of cellular stresses. Several mechanistic links between CHOP expression and cell fate have been reported.\textsuperscript{14} By activating GADD34, which dephosphorylates p-Ser51 of the eIF2α, CHOP promotes reversal of translational repression caused by the activated eIF2α kinase family.\textsuperscript{14} Thus, CHOP poises cells for death by increasing ER client protein load and oxidative stress. Our study revealed a surprising role of the HO-1/CO system in attenuating CHOP expression both in homeostatic and UPR conditions. CO blocked the CHOP expression in TG-, TM-, and HCystreated cells. Cells depleted of HO-1 by using siRNA showed that both exogenous and endogenous CO contributed to suppress the CHOP expression. CO-induced suppression of CHOP expression in our study corresponds with previous work demonstrating that CHOP expression correlates negatively with the presence of Nrf2; CHOP expression in Nrf2\textsuperscript{--/--} cells is constitutively higher than that in wild-type cells, and Nrf2 overexpression attenuates CHOP accumulation during the UPR.\textsuperscript{29} Because Nrf2 upregulates HO-1 expression, HO-1/CO system could be the effector molecule for downregulating CHOP expression. In an effort to understand the molecular mechanism(s) by which CO suppresses CHOP expression for the survival of cells from UPR, we tested the effects of SB203580, an inhibitor for p38 MAPK. Our results show that SB203580 totally abrogates the CO-mediated suppression of CHOP expression induced by ER stress.

In conclusion, we have demonstrated that CO induces Nrf2-dependent HO-1 expression via the PERK pathway and prevents apoptosis triggered by ER stress via p38 MAPK-dependent inhibition of CHOP expression. The cytoprotective effects of CO may be relied on the upregulation of HO-1 expression via PERK activation as well as the downregulation of proapoptotic CHOP expression via p38 MAPK activation.

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Disclosures
None.

References
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