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The Antiapoptotic Effect of Heme Oxygenase-1 in Endothelial Cells Involves the Degradation of p38α MAPK Isoform

Gabriela Silva,1 Andreia Cunha, Isabel Pombo Grégoire, Mark P. Seldon, and Miguel P. Soares2

Heme oxygenase-1 (HO-1) protects endothelial cells (EC) from undergoing apoptosis. This effect is mimicked by CO, generated via the catabolism of heme by HO-1. The antiapoptotic effect of CO in EC was abrogated when activation of the p38α and p38β MAPKs was inhibited by the pyridinyl imidazole SB202190. Using small interfering RNA, p38β was found to be cytoprotective in EC, whereas p38α was not. When overexpressed in EC, HO-1 targeted specifically the p38α but not the p38β MAPK isoform for degradation by the 26S proteasome, an effect reversed by the 26S proteasome inhibitors MG-132 or lactacystin. Inhibition of p38α expression was also observed when HO-1 was induced physiologically by iron protoporphyrin IX (hemin). Inhibition of p38α no longer occurred when HO activity was inhibited by tin protoporphyrin IX, suggesting that p38α degradation was mediated by an end product of heme catabolism. Exogenous CO inhibited p38α expression in EC, suggesting that CO is the end product that mediates this effect. The antiapoptotic effect of HO-1 was impaired when p38α expression was restored ectopically or when its degradation by the 26S proteasome was inhibited by MG-132. Furthermore, the antiapoptotic effect of HO-1 was lost when p38β expression was targeted by a specific p38β small interfering RNA. In conclusion, the antiapoptotic effect of HO-1 in EC is dependent on the degradation of p38α by the 26S proteasome and on the expression of p38β.

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Expression of the stress-responsive gene heme oxygenase-1 (HO-1)1 is part of a vascular response to injury that prevents the development of chronic inflammatory lesions such as those involved in the pathogenesis of atherosclerosis or the rejection of transplanted organs (reviewed in Refs. 1 and 2). HO-1 cleaves the mesocarbon of heme, yielding equimolar amounts of CO, iron (Fe), and biliverdin (3), the latter being reduced into bilirubin by biliverdin reductase (reviewed in Ref. 4). Heme-derived iron induces the expression of H chain ferritin, by which it is subsequently sequestered (5).

HO-1 exerts salutary actions in endothelial cells (EC) where it acts in an antiapoptotic manner (6, 7). The antiapoptotic effect of HO-1 can be mimicked by exogenously applied CO (7, 8), suggesting that heme-derived CO mediates to a large extent this effect (7–10). The antiapoptotic effect of CO is abrogated by SB203580, a pyridinyl imidazole that targets the ATP binding site on p38 MAPK, blocking their kinase activity (8, 10). This suggested that the antiapoptotic effect of CO depends on the activation of the p38 MAPK signal transduction pathway (8, 10).

The p38 MAPK family of proteins regroups four distinct kinases, encoded by different genes, i.e., p38α (p38α/CSBP-1, CSBP-2, Mxi2, and Exip; 38 kDa) (11), p38β (p38β/p38β1 and p38β2; 39 kDa) (12), p38γ (ERK6/SAPK3; 43 kDa) (13), and p38δ (SAPK4; 40 kDa) (Ref. 14 and reviewed in Ref. 15). These share a sequence homology ranging from 74% (p38α vs p38β) to 98% (p38β vs p38β2) and a canonical dual phosphorylation site (Thr-Gly-Tyr) (reviewed in Ref. 15). Pyridinyl imidazoles, such as SB203580, inhibit the activity of p38α and p38β but spare p38γ and p38δ (16), thus suggesting that the antiapoptotic effect of CO, abrogated by SB203580, acts via the p38α and/or the p38β MAPK isoforms.

Mice genetically deficient in p38α (17) but not p38β (18) are embryonic lethal, showing unequivocally that the biological actions of p38α and p38β are not overlapping. Cardiomyocytes and fibroblasts derived from p38α-deficient mice are less susceptible to undergo apoptosis, suggesting that p38α is proapoptotic (19). In support of this notion, p38α activation promotes apoptosis (20) in L929 fibroblasts (21), myocytes (22), HeLa cells (23), and Jurkat T cells (24). In contrast, p38β activation is antiapoptotic in these cells (21–24). This suggests that p38α and p38β have antagonistic effects in controlling apoptosis, i.e., p38α being pro- and p38β antiapoptotic (reviewed in Ref. 25). Based on these studies, as well as the growing body of evidence that CO acts via p38 MAPK to prevent EC from undergoing apoptosis, we hypothesized that HO-1/CO might avoid signaling via the cytotoxic p38α isoform, signaling preferentially via the cytoprotective p38β isoform. Our present data provide a mechanism to explain how this occurs, i.e., HO-1 targets specifically p38α for degradation by the 26S proteasome sparing p38β. This finding is in keeping with the recent observation that the antiapoptotic effect of CO in EC requires the expression of p38β (26).
Materials and Methods

Cell culture

Primary HUVEC (Cambrex) and bovine aortic EC (BAEC; Cell Systems) were cultured as previously described (8). HeLa cells were obtained from American Type Culture Collection.

Plasmid constructs

Wild-type (WT) hemagglutinin (HA)-tagged human p38α (HA-CSBP2) cDNA (provided by Dr. J. Anrather, Cornell University, Ithaca, NY) was expressed in pcDNA3 (Invitrogen Life Technologies) (8). WTp38α (FLAG-CSBP2) expression plasmid was generated by ligating BamHI/XhoI-excised CSBP2 with the FLAG epitope sequence and cloned into HindIII/XbaI-digested pcDNA3 (Invitrogen Life Technologies). Expression plasmids encoding the WT human FLAG-tagged p38β, p38γ, and p38δ cDNAs (a gift from Dr. R. J. Davis, University of Massachusetts, Worcester, MA) were expressed in pcDNA3 (13, 14, 27). Rat HO-1 cDNA was expressed under the control of the β-actin (β-actin/HO-1) or the CMV (pcDNA3/HO-1) enhancers/promoters (8). The pcDNA3/HO-1 vector contains 97 bp from the rat HO-1 promoter region. LucZ cDNA was expressed under the simian virus 40 early promoter and enhancer (pRSV-β-galactosidase; Promega). Firefly luciferase CDNA was expressed under the control of the simian virus 40 promoter (pGL3-Control; Promega). PCAGGS-AFP, a vector containing the gene coding for the firefly GFP, was expressed under the control of the chicken β-actin promoter (a gift from Dr. T. Morose, Himeji Institute of Technology, Hyogo, Japan) (28). Human p38α and p38β small interfering RNA (siRNA) were expressed under the control of the polymerase III promoter H1 using the pSuper expression vector (29). The sequences used to target human p38α and p38β MAPK were: p38α sense, 5'-CCAGTGCGCCATCCCATGTCAAGAGACATAGTC GCACACTG-3'; antisense, 5'-CCAGTGCGCCATCCCATGTCAAGAGACATAGTC GCACACTG-3'; p38β sense, 5'-GTGATGCCTCAGAGACATAGTC GCACACTG-3'; antisense, 5'-GTGATGCCTCAGAGACATAGTC GCACACTG-3'; and antisense, 5'-GTGATGCCTCAGAGACATAGTC GCACACTG-3'; and antisense, 5'-GTGATGCCTCAGAGACATAGTC GCACACTG-3'.

Results were expressed as mean ± SD from three independent experiments, each done in triplicate (*, p < 0.001 vs ActD treated alone). B, Expression of p38α and p38δ mRNA was detected by RT-PCR in quiescent HUVEC. Purified pcDNA3/CSBP2 (p38α) or pcDNA3/p38β (p38β) cDNAs were used as positive controls. Purified pcDNA3 was used as negative control. C, p38α and p38β were IP from quiescent HUVEC and detected by Western blot (WB) using isoform-specific polyclonal Abs. Ig indicates control Ig used in IP. D, Endogenous p38α (a) and p38β (b) were detected in confluent BAEC using isoform-specific polyclonal Abs. Background staining was established using rabbit serum in naive BAEC (c). Overexpressed p38α (d) and p38β (e) were detected in BAEC transiently transfected with FLAG-p38α (d) or FLAG-p38β (e) using an anti-FLAG Ab. Background staining was established using anti-FLAG Ab in BAEC transiently transfected with pcDNA3 (f). In all panels, DNA was stained with DAPI (blue) and F-actin with TRITC-labeled phalloidin (red). Arrows indicate p38α and p38β staining (green).

FIGURE 1. Inhibition of p38α and p38β MAPK abrogate HO-1-mediated cytotoxicity in EC. A, BAEC were transiently transfected with a RSV-driven luciferase reporter with or without β-actin/HO-1 expression vector. When indicated, p38 MAPK activity was inhibited by SB202190. Apoptosis was induced by TNF-α plus ActD. Cellular viability (percent viability) was assessed using luciferase activity as described in Materials and Methods. Results are expressed as mean ± SD from three independent experiments, each done in triplicate (*, p < 0.001 vs ActD treated alone). B, Expression of p38α and p38δ mRNA was detected by RT-PCR in quiescent HUVEC. Purified pcDNA3/CSBP2 (p38α) or pcDNA3/p38β (p38β) cDNAs were used as positive controls. Purified pcDNA3 was used as negative control. C, p38α and p38β were IP from quiescent HUVEC and detected by Western blot (WB) using isoform-specific polyclonal Abs. Ig indicates control Ig used in IP. D, Endogenous p38α (a) and p38β (b) were detected in confluent BAEC using isoform-specific polyclonal Abs. Background staining was established using rabbit serum in naive BAEC (c). Overexpressed p38α (d) and p38β (e) were detected in BAEC transiently transfected with FLAG-p38α (d) or FLAG-p38β (e) using an anti-FLAG Ab. Background staining was established using anti-FLAG Ab in BAEC transiently transfected with pcDNA3 (f). In all panels, DNA was stained with DAPI (blue) and F-actin with TRITC-labeled phalloidin (red). Arrows indicate p38α and p38β staining (green).
primers for p38α: sense, 5′-AACCTGTCTCCAGTGGGTCTCT-3′ and antisense, 5′-AGCTTCTAATGCGCAACAGC-3′; for p38β: sense, 5′-GGCTCAGCCACTGCT-3′ and antisense, 5′-CGCCTGGCATCGTTGACGATG-3′; for p38γ sense, 5′-CCGCTTGCCCGTGATGTTT-3′ and antisense, 5′-GTTGCGTTGCTCGTGATGATGAG-3′; and for p38δ sense, 5′-CCTGCTGCACCGCATCGACA-3′ and antisense, 5′-CGGTAAGCCGGCTTGGC-3′.

**Figure 2.** p38β but not p38α is cytoprotective in EC. A, HUVEC were transiently transfected with the RSV-driven luciferase reporter plus p38α, p38β, or control (c) siRNA (pSuper) expression vectors. Cellular viability (percent viability) was assessed as described above (1). HO-1 knockdown in HUVEC was confirmed using siRNA (0, 1500, and 3000 ng per 3 × 10^6 cells). B, HUVEC were transiently transfected with p38α siRNA (0, 1500, and 3000 ng per 3 × 10^6 cells). C, HUVEC were transiently transfected with HA-tagged p38α (CSBP2) plus p38α siRNA (0, 1500, and 3000 ng per 3 × 10^6 cells). D, HUVEC were transiently transfected with p38β siRNA (0, 1500, and 3000 ng per 3 × 10^6 cells). E, HUVEC were transiently transfected with p38α siRNA (0, 500, and 1000 ng of DNA per 3 × 10^6 cells). F, p38α and p38β mRNAs were detected by RT-PCR using specific primers. G, HeLa cells were transiently transfected with p38β siRNA (0, 500, and 1500 ng per 3 × 10^6 cells) and the rest of the procedure was conducted as in E.
SFVVPPQLQEMES and SFKFPFPKPGSLEIQ peptides, respectively (provided by Dr. L. Otterbein, Harvard Medical School, Boston, MA). Primary Abs were detected using a FITC-labeled goat anti-rabbit polyclonal Ab (Pierce). Nuclear (i.e., DNA) and cytoplasmic (i.e., F-actin) compartments were stained using 4',6'-diamidino-2-phenylindole (DAPI; 20 ng/ml; Sigma-Aldrich) and tetramethylrhodamine isothiocyanate (TRITC)-labeled phalloidin (50 ng/ml; Sigma-Aldrich), respectively. Cells were analyzed under a fluorescence microscope (Leica DMIR2). Fluorescence was acquired at λ	ext{em} = 480±40 nm and λ	ext{ex} = 527±30 nm for FITC, λ	ext{ex} = 535±50 nm and λ	ext{em} = 610±75 nm for TRITC and λ	ext{ex} = 360±40 nm and λ	ext{em} = 470±40 nm for DAPI using Metamorph v.4.6r5 software (Universal Imaging Corporation) and treated with ImageJ software.

Flow cytometry

Cells were harvested by trypsin/EDTA digestion and resuspended in cold PBS/10% FCS. Fluorescent labeling was evaluated using a FACS equipped with CellQuest software (BD Biosciences). GFP expression was evaluated by comparison of fluorescent labeling of GFP-transfected vs GFP-non-transfected cells.

Cell viability assays

HUVEC were either cotransfected with p38α or p38β siRNA expression vectors (1.5 μg/well of a 6-well plate) plus luciferase (pGL3-Control) or β-galactosidase (pRSV-β-galactosidase) reporters (0.25 μg/well of a 6-well plate). Viability was assessed 72 h after transfection according to luciferase or β-galactosidase activities as assayed using a luciferase assay system (Promega) or Galacto-Light (Applied Biosystems), respectively. Cells were washed once in cold PBS under agitation (5 min; 200 rpm at room temperature) in a basic orbital shaker (Ks 260; IKA-Works) and once in cold PBS without further agitation before lysis (Promega). Luciferase and β-galactosidase activities were measured using a MicroIlumat Plus luminometer (LB96V; Berthold Technologies). When indicated, apoptosis was induced by TNF-α (50 ng/ml) in the presence of the transcription inhibitor ActD (10 μg/ml) for 8 h. To assess the effect of HO-1 in TNF-α-induced apoptosis, BAEC were cotransfected with β-actin/HO-1 (0.5–0.7 μg/well of a 6-well plate). To evaluate whether reconstitution of the cellular pool of p38α reverted the protective effect of HO-1, BAEC were cotransfected with HO-1 plus FLAG-p38α. FLAG-p38β was used as a control. To evaluate the effect of p38β on HO-1 cytoprotection, HUVEC were cotransfected with HO-1 alone or HO-1 plus p38β siRNA vectors. HUVEC were serum-starved (1% FBS) 24 h after transfection and exposed to cycloheximide (CHX; 10 μg/ml) alone or CHX plus TNF-α (50 ng/ml) for 8 h. Transfections were done in triplicate or in quadruplicate in at least three independent experiments. Relative percentage of cell viability were normalized for each transfection to control EC treated with ActD or CHX.

Apoptosis assays

HUVEC were either cotransfected with p38α siRNA or p38β siRNA vectors (1.5 μg/well) plus pCAGGS-AFP reporter (50 ng/well). Alexa Fluor 470-conjugated annexin V (Molecular Probes) was used to detect apoptosis in GFP-positive cells by flow cytometry, according to the manufacturer’s instructions.

CO exposure

BAEC were exposed to synthetic air or to 10,000 parts per million (ppm) CO in synthetic air, both supplemented with 5% CO2 for 24 h, as previously described (31).

Statistical analysis

The standard experimental design used consisted of having at least three independent assays for every condition tested. Each assay was taken as a unique experiment independent of other assays. For viability/apoptosis assays, each experiment was performed in triplicate or quadruplicate. Statistical analysis was performed using Student’s t test. Significance was inferred after the Bonferroni correction for α = 0.05/κ, where κ is the number of tests performed within the same set of experiments. Data are shown relative to control conditions as mean ± SD.

Results

The ant apoptotic effect of HO-1 is abrogated under inhibition of the p38α and p38β activity

When transiently overexpressed in EC, HO-1 prevented TNF-α plus ActD-mediated apoptosis, as assessed by monitoring the expression of a coexpressed luciferase reporter (Fig. 1A) (8, 10). Similar results were obtained when GFP or β-galactosidase reporters were used to monitor EC viability (data not shown). The protective effect of HO-1 was abrogated by SB202190 (Fig. 1A) (8, 10), a pyridinyl imidazole that inhibits the activation of both the p38α and p38β, but not that of the p38γ or p38δ MAPK isoforms (16). Expression of mRNA encoding p38α and p38β was detected in quiescent EC by RT-PCR (Fig. 1B). Specificity of the oligonucleotides used was confirmed using human p38α, p38β, p38γ, or p38δ cDNA expression vectors (data not shown). Expression of p38α and p38β in EC was further assessed at the protein level by Western blot (Fig. 1C) and immunofluorescence (Fig. 1D). Specificity of the Abs used was confirmed by Western blot using whole cell lysates from EC transiently transfected with human p38α, p38β, p38γ, or p38δ cDNA expression vectors (data not shown).

Both endogenous and transiently overexpressed p38α and p38β localized in the nuclei and only to a lesser extent in the cytoplasm of quiescent EC, as assessed by immunostaining (Fig. 1D). These data show that quiescent EC express both the p38α and p38β MAPK isoforms (32) and that when overexpressed these p38 isoforms localize in similar cellular compartments to those of the endogenous isoforms.

**FIGURE 3.** Activation of p38α or p38β by TNF-α is not modulated by HO-1. A, BAEC were transiently transfected with HA-p38α with or without pcDNA3/HO-1 and exposed to TNF-α (50 ng/ml, 5 min). HA-p38α was IP with anti-HA Ab. Phosphorylated or total HA-p38α (p-p38α and p38α, respectively) was detected by Western blot. HO-1 and α-tubulin were detected in whole cell lysates (WCL) by Western blot. B, BAEC were transiently transfected with FLAG-p38β with or without pcDNA3/HO-1 and exposed to TNF-α as in A. FLAG-p38β was IP with anti-FLAG Ab. Phosphorylated or total FLAG-p38β (p-p38β and p38β, respectively) was detected by Western blot. HO-1 and α-tubulin were detected in whole cell lysates by Western blot. C, Relative levels of p38α and p38β phosphorylation were quantified and normalized to total p38α or p38β as described in Materials and Methods. Relative values shown as arbitrary units (AU) were normalized to EC, not transfected with HO-1 and exposed to TNF-α (1). Values from four independent experiments are shown as mean ± SD.
The p38\(\beta\) but not p38\(\alpha\) isoform is cytoprotective in EC

The p38\(\alpha\) and p38\(\beta\) MAPK isoforms have antagonistic effects in controlling cellular viability in a variety of cell types, i.e., p38\(\alpha\) is proapoptotic, whereas p38\(\beta\) is antiapoptotic (21–24). We asked whether this would also be the case in EC. Inhibition of p38\(\alpha\) using a p38\(\alpha\) siRNA expression vector increased EC viability, as compared with EC transfected with a control siRNA (Fig. 2A). This observation suggests that endogenous p38\(\alpha\) is cytotoxic in EC. On the contrary, when the expression of p38\(\beta\) was targeted using a p38\(\beta\) siRNA, EC viability was decreased, as compared with EC transfected with a control siRNA (Fig. 2A). This observation suggests that endogenous p38\(\beta\) is cytoprotective in EC.

Transient overexpression of p38\(\beta\) siRNA with a GFP reporter revealed that transfected EC expressed the early apoptotic marker phosphatidylserine V at the cell surface (−40–50% of GFP+ EC), as detected by flow cytometry (Fig. 2B). This suggests that inhibition of endogenous p38\(\beta\) expression triggers EC to undergo apoptosis.

Transient cotransfection of EC with p38\(\alpha\) plus p38\(\alpha\) siRNAs or p38\(\beta\) plus p38\(\beta\) siRNA expression vectors resulted in suppression of the targeted p38 isoforms. Namely, expression of HA-p38\(\alpha\) (Fig. 2C) or FLAG-p38\(\beta\) (Fig. 2D) was decreased in a dose-response manner by the p38\(\alpha\) and p38\(\beta\) siRNAs, respectively. Specificity of the siRNA for the targeted p38 isoforms was confirmed in transiently transfected HeLa cells (Fig. 2. E and F). The p38\(\alpha\) siRNA inhibited endogenous p38\(\alpha\) but not p38\(\beta\) mRNA expression (Fig. 2E). The p38\(\beta\) siRNA inhibited endogenous p38\(\beta\) but not p38\(\alpha\) mRNA expression (Fig. 2F). This data demonstrate that the p38\(\alpha\) and p38\(\beta\) siRNAs are specific for the targeted p38 isoforms. Expression of p38\(\alpha\) or p38\(\beta\) mRNA was not decreased to undetectable levels (Fig. 2, E and F), probably because HeLa transfection efficiency was ~50%, as assessed by flow cytometry using a GFP reporter (data not shown).

Effect of HO-1 on p38\(\alpha\) and p38\(\beta\) activation

Given that in EC p38\(\beta\) is cytoprotective whereas p38\(\alpha\) is not (Fig. 2, A and B), we asked whether HO-1 would inhibit specifically the activation of p38\(\alpha\) and/or induce that of p38\(\beta\). EC were transiently cotransfected with p38\(\alpha\) or p38\(\beta\) with or without HO-1 expression vectors and exposed to TNF-\(\alpha\) (50 ng/ml, 5 min). Activation of p38\(\alpha\) and p38\(\beta\) was monitored by the relative level of phosphorylation, as assessed by Western blot after isofrom-specific immunoprecipitation. Overexpression of HO-1 resulted in a relative decrease of TNF-\(\alpha\)-mediated p38\(\alpha\) phosphorylation, as compared with control EC that did not express HO-1 (Fig. 3A). However, this effect was due to a decrease in p38\(\alpha\) expression, because when normalized to the total level of p38\(\alpha\), the relative level of phosphorylated p38\(\alpha\) remained unchanged in EC overexpressing HO-1, as compared with control EC (Fig. 3, A and C). Overexpression of HO-1 failed to modulate p38\(\beta\) phosphorylation (Fig. 3, B and C). These data suggest that HO-1 does not interfere with the signal transduction pathway triggered by TNF-\(\alpha\) and leading to p38\(\alpha\) or p38\(\beta\) MAPK activation.

HO-1 inhibits specifically the expression of the p38\(\beta\) isoform in EC

Given that the amount of IP p38\(\alpha\) was decreased in EC overexpressing HO-1, as compared with control EC (Fig. 3A), we asked whether HO-1 would decrease p38\(\alpha\) expression in EC. Transient coexpression of HO-1 with HA-p38\(\alpha\) plus FLAG-p38\(\beta\) in EC resulted in significant inhibition of p38\(\beta\) but not of p38\(\beta\) protein expression, as compared with control EC that did not express HO-1 (Fig. 4, A and B). The ability of HO-1 to inhibit p38\(\alpha\) expression was dose dependent in that increasing levels of HO-1 resulted in decreasing levels of p38\(\alpha\) protein expression, as assessed by Western blot (Fig. 4, C and D). This effect was specific to p38\(\alpha\), since HO-1 did not modulate p38\(\beta\) expression (Fig. 4, E and F). Transient coexpression of HO-1 with FLAG-p38\(\alpha\) also resulted in inhibition of p38\(\alpha\) protein expression, as compared with control EC that did not overexpress HO-1 (data not shown). This data demonstrate that the ability of HO-1 to suppress p38\(\alpha\) expression is not linked to the epitope used to detect p38\(\alpha\) in this assay, i.e., HA vs FLAG.

To ascertain whether inhibition of p38\(\alpha\) can occur under physiological conditions, we tested whether hemin, an inducer of endogenous HO-1 expression, would inhibit p38\(\alpha\) in EC. As expected (30), hemin induced high levels of HO-1 expression in EC (Fig. 5, A, C, and D). This was associated with reduced expression of transiently overexpressed p38\(\alpha\), i.e., 40–80% reduction, as compared with control EC not exposed to hemin (Fig. 5, A and B). Hemin had no significant effect on transiently overexpressed p38\(\beta\), as assessed by Western blot (Fig. 5, A and B). We then asked whether hemin would also inhibit endogenous p38\(\alpha\) and/or p38\(\beta\)
expression. Endogenous p38α was reduced by 70–80% in EC exposed to hemin, as compared with control EC (Fig. 5, C and E). Hemin failed to reduce the expression of endogenous p38β, suggesting that its effect is specific to p38α (Fig. 5, D and E). To ascertain that HO-1 mediated the inhibition of p38α expression observed in EC exposed to hemin, we assessed whether this effect was ablated when HO-1 expression was targeted by a HO-1 siRNA (Fig. 5, F). HUVEC were either not transfected or transfected with HA-p38α, FLAG-p38β, HO-1, and α-tubulin were detected by Western blot in whole EC lysates. B. Relative levels of p38α and p38β proteins were quantified and normalized to 100% of p38α or p38β expression in EC not exposed to hemin. Shown is the mean ± SD of three independent experiments (*, p = 0.01 vs untreated cells). C and D, HUVEC were exposed to hemin (2.5 μM), endogenous p38α (C), and p38β (D) were IP and detected by Western blot. HO-1 and α-tubulin were detected in whole cell lysates (WCL) as in A. E. Relative levels of endogenous p38α and p38β were quantified as in B and are shown as mean ± SD from three independent experiments (*, p = 0.0003 vs untreated cells). F, HUVEC were either not transfected or transfected with HO-1 siRNA and exposed 48 h thereafter to hemin (2.5 μM). Endogenous p38α was IP and detected by Western blot. HO-1 and α-tubulin were detected in whole cell lysates as in A. A, C, and D, Immunoblots are representative of three independent experiments used for quantification.

**CO inhibits p38α expression in EC**

We have previously shown that CO is antiapoptotic in EC (8). We reasoned that if inhibition of p38α was required to sustain this antiapoptotic effect then CO should inhibit p38α expression. We first tested whether HO-1 enzymatic activity, which generates CO, was required to inhibit p38α expression. We found this to be the case as the ability of transiently transfected HO-1 to inhibit the expression of cotransfected p38α was impaired when HO activity was repressed by tin protoporphyrin IX (SnPPIX) (33) (Fig. 6, A and B). In contrast, the inhibition of p38α expression was enhanced when EC were exposed to hemin (Fig. 6, A and B), the natural substrate of HO-1 enzymatic activity (30). In the absence of detectable HO-1 expression, exogenous CO (10,000 ppm) mimicked the effect of transiently overexpressed HO-1, inhibiting p38α expression (Fig. 6, C and D). These observations suggest that HO-1 inhibits p38α expression in EC via the generation of CO.

The antiapoptotic effect of HO-1 is impaired upon reconstitution of the cellular pool of p38α

To determine whether the antiapoptotic effect of HO-1 was functionally linked to its ability to inhibit specifically the expression of p38α (Figs. 4–6), we assessed whether the antiapoptotic effect of HO-1 was impaired when p38α expression was restored ectopically. We found that this is the case. Transient overexpression of p38α with HO-1 impaired the antiapoptotic effect of HO-1 (Fig. 7A). This did not occur when p38β was transiently overexpressed with HO-1 (Fig. 7A). To ensure that p38α and p38β were coexpressed at similar levels, their relative levels of expression were compared by Western blot using an anti-FLAG Ab recognizing both tagged isoforms. This proved to be the case (Fig. 7B).

To assess whether the cytoprotection afforded by HO-1 was dependent on the expression of p38β, EC were transiently cotransfected with HO-1 with or without a p38β siRNA expression vector. When coexpressed with HO-1, p38β siRNA decreased EC viability, as compared with control EC that overexpressed HO-1 but not the p38β siRNA (Fig. 7C). TNF-α plus CHX failed to induce significant EC apoptosis when HO-1 was overexpressed (Fig. 7 C) (8). However, this was no longer the case when EC overexpressed HO-1 plus p38β siRNA (Fig. 7 C), indicating that p38β expression is required to sustain the cytoprotective effect of HO-1 against TNF-α-mediated apoptosis.
FIGURE 6. CO inhibits p38α expression in EC. A, BAEC were transiently transfected with HA-p38α with or without pcDNA3/HO-1 and exposed to either hemin or SnPPIX. Proteins in whole cell extracts were detected by Western blot. B, Relative levels of p38α were quantified, normalized to α-tubulin, and normalized again to the level of p38α expressed in EC not transfected with HO-1 (100%). Results shown are the mean ± SD from three independent experiments using 10 μM hemin or SnPPIX (*, p = 0.01 vs control). C, BAEC were transiently transfected with HA-p38α with or without pcDNA3/HO-1 and when indicated exposed to CO (10,000 ppm). Proteins were detected by Western blot as in A. D, Relative levels of p38α were quantified as in B and values expressed relative to control EC that did not express HO-1 and not exposed to CO (100%). Values shown are the mean ± SD from three independent experiments (*, p = 0.01 vs control, **, p = 0.008 vs control). Immunoblots are representative of the three independent experiments.

HO-1 targets p38α for degradation by the 26S proteasome

Because HO-1 suppressed the expression of p38α but not p38β when their cDNAs were overexpressed under the control of the same promoter, i.e., minimal CMV (Fig. 4A), HO-1 should not target the transcription of these p38 MAPK isoforms. Therefore, we tested whether HO-1 would act posttranscriptionally, i.e., targeting mRNA or protein stability, to inhibit specifically the expression of p38α. When transiently p38α or p38β were coexpressed with HO-1, the expression of p38α or p38β mRNAs was not modulated, as compared with control EC that did not express HO-1 (Fig. 8, A and B). This excluded not only the possibility that HO-1 would target the transcription of these p38 isoforms but also that it would modulate the stability (half-life) of their mRNA. We then asked whether HO-1 would act directly on the p38α protein to inhibit its expression, such as by targeting it for proteolytic degradation by the 26S proteasome. We found this to be the case, as suppression of 26S proteasome activity by MG-132 impaired the decrease of p38α protein expression observed in EC that overexpress HO-1 (Fig. 8, C and D). This effect was dose dependent in that increasing concentrations of MG-132, i.e., 0.5–20 μM, resulted in increasing levels of p38α protein expression (Fig. 8, C and D). The involvement of the 26S proteasome pathway in p38α degradation was confirmed using lactacystin, an inhibitor of the 26S proteasome pathway. This data suggests that the ability of HO-1 to inhibit the expression of p38α is strictly dependent on the presence of a fully active 26S proteasome pathway.

Inhibition of p38α degradation impairs the antiapoptotic effect of HO-1

If the ability of HO-1 to trigger p38α degradation via the 26S proteasome was required to support its antiapoptotic effect, then inhibition of the 26S proteasome activity should impair the antiapoptotic effect of HO-1. Indeed, the 26S proteasome inhibitor MG-132 reverted the antiapoptotic effect of HO-1 in EC (Fig. 8F). This was associated with complete restoration of p38α expression, despite the overexpression of HO-1 (Fig. 8C), suggesting that degradation of p38α by the 26S proteasome is a prerequisite for the antiapoptotic effect of HO-1.

Discussion

Expression of HO-1 plays a critical role in the control of inflammatory reactions such as those involved in the pathogenesis of several inflammatory diseases (34), including atherosclerosis (reviewed in Refs. 1 and 35). The salutary effects of HO-1 were originally associated with its ability to confer cytoprotection against a broad spectrum of pro-oxidant stimuli. In keeping with this notion, we found that HO-1-derived CO is antiapoptotic in EC (6, 8), an effect that should contribute to the overall salutary effects of HO-1, since EC apoptosis is a highly proinflammatory event perse (36, 37). The finding that HO-1-derived CO modulates the proinflammatory response of activated monocyte/macrophages (38) suggests that its protective effects might also be exerted via this action (39).

Both the anti-inflammatory (38) and antiapoptotic (8, 10, 40) effects of HO-1-derived CO are exerted via the p38 MAPK transduction pathway (reviewed in Ref. 1). At first, this seemed paradoxical, since p38 MAPK can promote the expression of proinflammatory cytokines in monocyte/macrophages, e.g., TNF-α (11), as well as apoptosis (25). This was, therefore, difficult to conciliate with the observation that CO exerts the exact opposite effects via the same signal transduction pathway. We reasoned that CO might signal via a specific subset of p38 MAPK isoforms that would act essentially in an anti-inflammatory and/or antiapoptotic manner. We tested this hypothesis in the context of EC apoptosis, restricting our analysis to the p38α and p38β isoforms, because SB203580, a compound which inhibits only the activity of these two isoforms (16) suppresses the antiapoptotic effects of HO-1 and/or CO in EC (8).

We have confirmed that both p38α and p38β MAPK isoforms were expressed in EC and that the antiapoptotic effect of HO-1 was impaired when the activation of these p38 isoforms was inhibited
isoforms (Fig. 3), we reasoned that there must be another mechanism via which this can be accomplished. The observation that HO-1 decreased the cellular pool of p38α but not that of p38β (Figs. 4 and 5) suggested that this might be the mechanism via which HO-1 avoids the cytotoxic action of p38α, thus favoring signaling via the cytoprotective p38β isoform. This effect was found to be strictly dependent on the enzymatic activity of HO-1 (Fig. 6, A and B) and to be mimicked in the absence of detectable HO-1 expression by exogenously applied CO (Fig. 6, C and D), suggesting that it is CO that inhibits p38α expression in EC.

Given the above, it appears that the ability of HO-1 to modulate signaling via the p38 MAPK does not involve upstream kinases or phosphatases targeting specifically one or the other of these p38 isoforms. Instead, HO-1 acts in a cytoprotective manner by inhibiting specifically the expression of p38α, thus directing signals emanating from upstream kinases toward the cytoprotective p38β isoform. That p38β sustains the ant apoptotic effect of HO-1 was demonstrated by the observation that the cytoprotective effect of HO-1 was ablated when p38β expression was suppressed (Fig. 7C). This finding is in keeping with the recent observation that the cytoprotective effect of one of the products of heme degradation by HO-1, i.e., CO, is also lost when EC fail to express p38β, such as in EC isolated from p38β-deficient mice (26). Our present data add significantly to these findings in that we demonstrate not only that the ant apoptotic effect of HO-1 requires the expression of p38β (Fig. 7C), but also that it requires the inhibition of p38α expression. This notion is supported by the observation that reconstitution of p38α expression negated the ant apoptotic effect of HO-1 in EC (Fig. 7A). This set of observations should solve the somewhat paradoxical finding that HO-1-derived CO signals via the p38 MAPK signal transduction pathway to exert its ant apoptotic effects (8).

The inhibition of p38α expression by HO-1 occurred under physiological conditions such as when endogenous HO-1 expression was induced by hemin (Fig. 5, A–C). This effect was lost when HO-1 expression was suppressed by a HO-1 siRNA, confirming that the ability of hemin to reduce p38α expression was mediated by HO-1 (Fig. 4F). Based on these observations, it is reasonable to assume that under the experimental conditions used overexpressed HO-1 mimics the physiological effect of endogenous HO-1 in inhibiting specifically the expression of p38α in EC.

Our present data also reveal that HO-1 targets specifically the p38α protein for degradation by the 26S proteasome, an effect that was blocked by MG-132 and lactacystin, two specific inhibitors of the 26S proteasome (Fig. 8, C, D, and E). That degradation of p38α by the 26S proteasome is required to sustain the ant apoptotic effect of HO-1 is suggested by the observation that MG-132 negated the ant apoptotic effect of HO-1 (Fig. 8F).

Based on the data reported here, there are at least two possible explanations as to the mechanism underlying the involvement of p38α and p38β in the ant apoptotic effect of HO-1. One is that p38α degradation is sufficient per se to mediate this effect. This possibility should probably be disregarded based on the observation that p38β expression is required to sustain the ant apoptotic effect of HO-1 (Fig. 7C) (26). An alternative explanation would be that HO-1 alters the ratio of cytotoxic p38α vs cytoprotective p38β, simply allowing in this manner the action of p38β to predominate over p38α. As argued above, our data support this as being the mechanism involved in the ant apoptotic effect of HO-1 in EC.

In conclusion, HO-1-derived CO modulates signaling via the p38 MAPK signal transduction pathway in a manner that is essentially ant apoptotic in EC. CO inhibits the cytotoxic action of p38α, probably promoting that of the cytoprotective p38β isoform.
This occurs via specific degradation of p38α by the 26S proteasome, an effect shown hereby to be critical for the antiapoptotic action of HO-1 in EC. Whereas it is likely that a similar mechanism may be involved in other biological activities of CO, such as its anti-inflammatory effects in monocyte/macrophages, this remains to be demonstrated.

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Disclosures

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