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Mitogen Activated Protein Kinase (MAPK) Pathway Regulates Heme Oxygenase-1 Gene Expression by Hypoxia in Vascular Cells

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ABSTRACT

Hypoxia induces the stress protein heme oxygenase-1 (HO-1), which participates in cellular adaptation. The molecular pathways that regulate *ho-1* gene expression under hypoxia may involve mitogen activated protein kinase (MAPK) signaling and reactive oxygen. Hypoxia (8 h) increased HO-1 mRNA in rat pulmonary aortic endothelial cells (PAEC), and also activated both extracellular signal-regulated kinase 1 (ERK1)/ERK2 and p38 MAPK pathways. The role of these kinases in hypoxia-induced *ho-1* gene expression was examined using chemical inhibitors of these pathways. Surprisingly, SB203580, an inhibitor of p38 MAPK, and PD98059, an inhibitor of mitogen-activated protein kinase kinase (MEK1), strongly enhanced hypoxia-induced HO-1 mRNA expression in PAEC. UO126, a MEK1/2 inhibitor, enhanced HO-1 expression in PAEC under normoxia, but not hypoxia. Diphenylene iodonium, an inhibitor of NADPH oxidase, also induced the expression of HO-1 in PAEC under both normoxia and hypoxia. Similar results were observed in aortic vascular smooth muscle cells. Furthermore, hypoxia induced activator protein (AP-1) DNA-binding activity in PAEC. Pretreatment with SB203580 and PD98059 enhanced AP-1 binding activity under hypoxia in PAEC; UO126 stimulated AP-1 binding under normoxia, whereas diphenylene iodonium stimulated AP-1 binding under normoxia and hypoxia. These results suggest a relationship between MAPK and hypoxic regulation of *ho-1* in vascular cells, involving AP-1. *Antioxid. Redox Signal.* 4, 587–592.

INTRODUCTION

HYPOXIA (lowered pO_2 in blood and tissue) may arise as a consequence of decreased oxygen availability, impaired blood flow (ischemia), or insufficient vascularization, and may occur in disease states such as myocardial infarction, stroke, arteriosclerosis, fibrosis, and cancer (12). In the cardiovascular system, hypoxia dilates the systemic vasculature and constricts the pulmonary vasculature after acute exposure, and may cause pulmonary hypertension and pulmonary vascular remodeling after chronic exposure (18, 22). The mechanisms underlying these tissue-specific responses to hypoxia are still unclear.

Exposure of mammalian cells to hypoxia *in vitro* modulates cell type-specific expression of a number of genes, including those encoding stress proteins, drug detoxification, and glycolytic enzymes, nitric oxide synthases, cytokines, growth factors, matrix metalloproteinases, early response gene products, and growth arrest and DNA-damage-inducible proteins (for reviews, see 12, 30). The 32-kDa stress protein heme oxygenase-1 (HO-1) represents a major hypoxia-inducible protein in rodent cells (11, 19, 26). Unlike its constitutively expressed isozyme HO-2, HO-1 expression responds to diverse chemical and physical stimuli, including oxidants, heavy metals, thiol-reactive substances, and ultraviolet-A radiation (16, 17, 20). HO enzymatic activity catalyzes

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the oxidative catabolism of heme to form biliverdin-IXα, carbon monoxide (CO), and ferrous iron (36, 37). Many recent reports support the hypothesis that HO-1 confers cellular and systemic protection against oxidative stress (23, 28, 29, 39; for review, see 31).

Recent studies have demonstrated the *ho-1* response to hypoxia in vascular cell systems, including bovine aortic endothelial cells (24, 25, 32), rat aortic vascular smooth muscle cells (VSMC), and pulmonary aortic endothelial cells (PAEC) (11, 19). Activation of *ho-1* in PAEC occurred in the context of increased DNA-binding activity of activator protein 1 (AP-1) factors (homo- or heterodimeric Jun and/or Jun/Fos proteins) (8, 11). HO-1 induction in VSMC involved the hypoxia-inducible factor (Hif-1) (11, 19), a global regulator of hypoxia-responsive genes (33).

The molecular signaling pathways, including the initiating events, or oxygen-sensing apparatus, regulating hypoxic *ho-1* gene activation remain unclear. Existing models of oxygen sensing implicate the O_2-dependent modulation of reactive oxygen species (ROS) production by a number of hemoprotein complexes (*i.e.,* mitochondrial cytochrome-*c*-oxidase, cytochrome P450, NADPH:oxidase) (4, 14, 34, 41). Increased ROS production in response to hypoxia has been observed in certain cell types (*i.e.,* cardiomyocytes) (5, 38). Recently, Kacimi and colleagues reported the involvement of p38 mitogen-activated protein kinase (MAPK) in regulation of HO-1 expression under hypoxia (15).

The current studies strongly suggest that MAPK signaling pathways may play a tissue-specific role in the regulation of *ho-1* gene expression under hypoxia in PAEC and VSMC. These studies also demonstrate that diphenylene iodonium (DPI), an antioxidant and flavoprotein reductase inhibitor, strongly induces HO-1 expression in vascular cells.

MATERIALS AND METHODS

Chemicals

MAPK pathway inhibitors were from Calbiochem (San Diego, CA, U.S.A.). DPI was from Sigma (St. Louis, MO, U.S.A.). These drugs were prepared as dimethyl sulfoxide (DMSO) stock solutions and sterile-filtered. Poly[d(I-C)] and the Complete Mini protease inhibitor tablets were from Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.). Unless otherwise indicated, all other reagent chemicals were from Sigma.

Cell culture and treatments

Primary cultures of rat VSMC and PAEC were prepared and cultured as previously described (11) and used for experiments as completely confluent monolayers at passages 12–16 (PAEC) and 15–20 (VSMC). Cells were exposed to hypoxic conditions in humidified, tightly sealed modular chambers (Billups–Rothenberg, Del Mar, CA, U.S.A.) filled to 1 atm with a premixed hypoxic gas mixture (1% O_2, 5% CO_2, 94% N_2) (Valley National Gas, Pittsburgh, PA, U.S.A.) and placed at 37°C for the indicated times. Corresponding normoxic controls were maintained for equivalent times in humidified incubators filled with an atmosphere of 95% air/5% CO_2. Drugs were applied from concentrated DMSO stock solutions directly to the culture media either immediately prior or 1 h prior to exposure to the hypoxia as indicated. The concentration of DMSO did not exceed 0.5%.

Northern blot analysis

Total RNA was isolated using the TriZol reagent according to the manufacturer's instructions (GibcoBRL, Grand Island, NY, U.S.A.). HO-1 cDNA was labeled with [α-^{32}P]-dCTP (3000 Ci/mmol; Amersham, Piscataway, NJ, U.S.A.) using a random primer labeling kit (Roche Molecular Biochemicals). Northern blot analyses were performed as previously described (11). Images were quantified using NIH image 1.62 software. HO-1 mRNA induction was reported as fold increase of the ratio of HO-1 signal to 18S mRNA relative to the corresponding normoxic control.

Nuclear protein extraction

All procedures were performed at 4°C with precooled solutions and equipment. Extraction solutions contained freshly added protease inhibitor tablets. Cell monolayers were rinsed twice with phosphate-buffered saline, scraped in 2 ml of TEN (40 m*M* Tris-Cl, 1 m*M* EDTA, 150 m*M* NaCl, pH 7.4), and pelleted at 500 *g*. The cell pellets were rinsed in hypotonic buffer A [10 m*M* HEPES, pH 7.9, 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 5 m*M* NaF, 2 m*M* $NaVO_4$, 0.5 m*M* dithiothreitol (DTT)] for 5 min and then pelleted at 500 *g*. The cell pellets were resuspended in 2 volumes of hypotonic buffer A and swelled for 15 min at 4°C. Suspensions were homogenized for 10 strokes in a Dounce-type homogenizer with a type B pestle (Wheaton Science Products, Millville, NJ, U.S.A.). Homogenates were centrifuged for 15 min at 3,300 *g*. The nuclear pellets were recovered and resuspended in 1 volume of low-salt buffer (20 m*M* HEPES, pH 7.9, 25% glycerol, 1.5 m*M* $MgCl_2$, 20 m*M* KCl, 0.2 m*M* EDTA, 0.5 m*M* DTT), followed by the dropwise addition of 1 volume of high-salt buffer (20 m*M* HEPES, pH 7.9, 25% glycerol, 1.5 m*M* $MgCl_2$, 1.2 *M* KCl, 0.2 m*M* EDTA). Nuclei were extracted at 4°C on a tilt board for 30 min. Extracts were centrifuged for 30 min at 12,000 *g*. The supernatant was dialyzed for 1 h at 4°C in dialysis buffer (20 m*M* HEPES, pH 7.9, 20% glycerol, 100 m*M* KCl, 0.2 m*M* EDTA, 5 m*M* NaF, 2 m*M* $NaVO_4$, 0.5 m*M* DTT) and centrifuged at 12,000 *g* for 20 min at 4°C. The recovered protein was flash-frozen in liquid N_2 and stored at −80°C. Protein concentrations were determined using dye reagent (Bio-Rad, Hercules, CA, U.S.A.), with bovine serum albumin as the standard.

Electrophoretic mobility shift gels

An AP-1 specific double-stranded oligonucleotide (top strand: 5′-CGCTTGATGAGTCAGCCGGAA-3′) (Promega, Madison, WI, U.S.A.) was ^{32}P-labeled at the 5′-OH by T4 polynucleotide kinase (NEBiolabs, Beverly, MA, U.S.A.) and 5′[γ-^{32}P]ATP (5,000 Ci/mmol; Amersham). Labeled oligonucleotides were separated from unincorporated label using Sephadex G-25 chromatography (Roche Molecular Biochemicals). DNA-binding reactions were assembled by combining 5 μg of nuclear protein with ^{32}P-labeled double-stranded oligonucleotide probe (50,000–250,000 cpm) in a reaction containing 10 m*M* Tris, pH 7.4, 50 m*M* NaCl, 1 m*M* DTT, 1

m*M* EDTA, 10% glycerol, 0.3 mg/ml bovine serum albumin, and 2 μg of poly[d(I-C)]. For competition experiments, 87.5 fmol of cold oligonucleotide was also added. The reactions were incubated for 15 min at 30°C. Samples were electrophoresed on a 4% acrylamide (80:1 crosslinker ratio) gel. Gels were dried at 80°C and exposed to Biomax MR film (Eastman Kodak Co., Rochester, NY, U.S.A.).

RESULTS

A sustained hypoxia (1% O_2) of 8-h duration induced HO-1 mRNA steady-state levels in PAEC (three- to fourfold induction in the ratio of HO-1 to 18S mRNA, relative to the normoxic control) (Fig. 1). To test the hypothesis that MAPK(s) participate in this response in either PAEC or VSMC cells, chemical inhibitors of MAPKs were administered in combination with hypoxic or normoxic culture conditions. Concurrent treatment with the inhibitor of p38 MAPK, SB203580 (10 μ*M*), during hypoxia dramatically enhanced HO-1 mRNA levels in PAEC (7.3 fold vs. normoxic control) (Fig. 1). Similar results were also observed when the drug was applied 1 h prior to the onset of hypoxia (data not shown). Hypoxia induced HO-1 mRNA in VSMC after 8 h of treatment (3.2-fold), which reached higher levels after 16–24 h of exposure (4.6–4.9 fold) (Fig. 2). SB203580 also increased HO-1 mRNA levels when included during the hypoxic period (8 h) in VSMC (6.7-fold) (Fig. 2). The inhibitor of mitogen-activated protein kinase kinase (MEK1), PD98059 (10 μ*M*), when included during the hypoxic period (8 h), also enhanced hypoxia-dependent HO-1 mRNA accumulation in PAEC (5.0-fold) (Fig. 1B). Both SB203580 and PD98059 increased HO-1 mRNA levels under normoxic conditions in PAEC (1.6- and 4.2-fold, respectively) (Fig. 1), but only SB203580 induced HO-1 expression under normoxia in VSMC (2.1-fold) (Fig. 2). Interestingly, UO126 (10 μ*M*), an inhibitor with specificity for both MEK1 and MEK2, strongly induced HO-1 mRNA expression under normoxic conditions (16.6-fold) and slightly under hypoxic conditions (4.5-fold) in PAEC (Fig 1B). UO126 had little effect under normoxia or hypoxia in VSMC (Fig. 2). DMSO (used as a vehicle for the drug delivery) did not affect HO-1 mRNA levels under normoxic or hypoxic conditions.

DPI, an antioxidant and flavoprotein reductase inhibitor (7, 27, 35), was used to test the hypothesis that ROS generated during the hypoxia may be responsible for hypoxic HO-1 induction. Interestingly, DPI strongly induced HO-1 mRNA under both normoxic and hypoxic conditions in PAEC

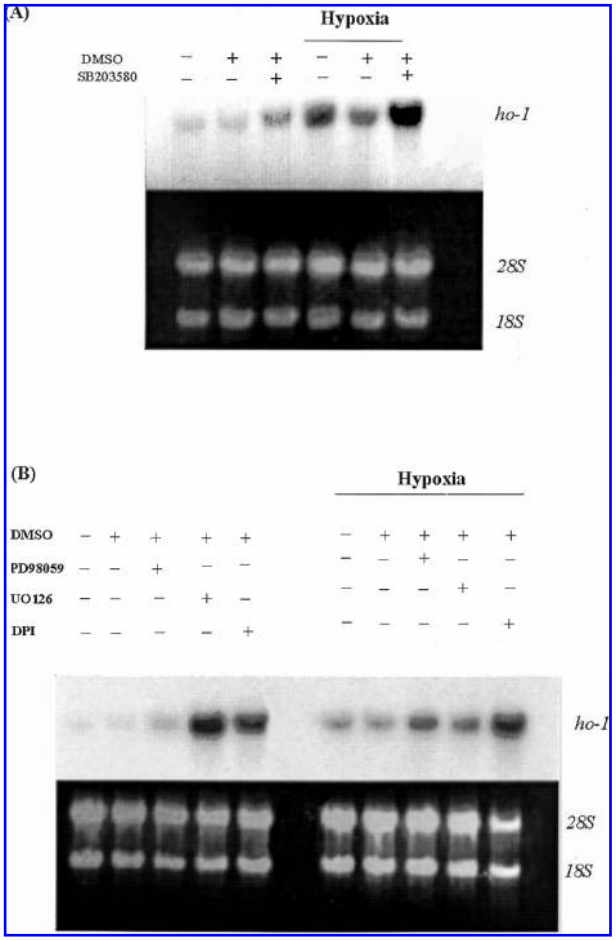

FIG. 1. Effect of MAPK inhibitors and DPI on hypoxia-inducible HO-1 mRNA expression in PAEC. Confluent monolayers of PAEC cells were treated with *in vitro* hypoxia (1% O_2) or normoxia (20% O_2) for 8 h in the absence or presence of SB203580 (10 μ*M*) **(A),** PD98059, UO126, or DPI (10 μ*M each*) **(B).** Total RNA was isolated, electrophoresed on 1% agarose gels, transferred to nitrocellulose, and probed with a ^{32}P-labeled HO-1 cDNA probe. Ethidium bromide staining of 18S and 28S rRNA is presented to show the relative loading of the lanes. A representative autoradiogram of three experiments is shown.

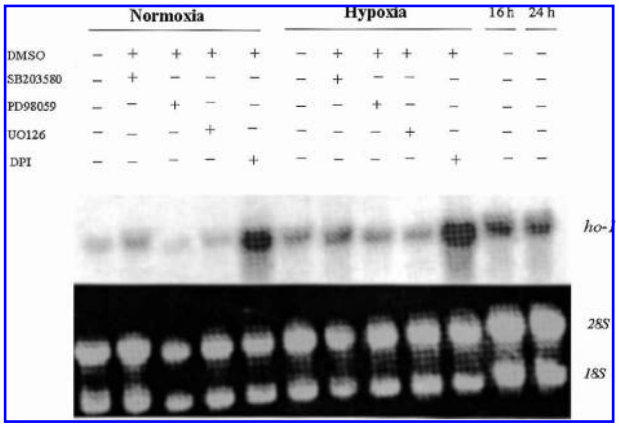

FIG. 2. Effect of MAPK inhibitors and DPI on hypoxia-inducible HO-1 mRNA expression in VSMC. Confluent monolayers of VSMC cells were treated with *in vitro* hypoxia (1% O_2) or normoxia (20% O_2) for 8 h in the absence or presence of SB203580, PD98059, UO126, or DPI (10 μ*M* each). Total RNA was isolated, electrophoresed on 1% agarose gels, transferred to nitrocellulose, and probed with a ^{32}P-labeled HO-1 cDNA probe. Ethidium bromide staining of 18S and 28S rRNA is presented to show the relative loading of the lanes. A representative autoradiogram of three experiments is shown.

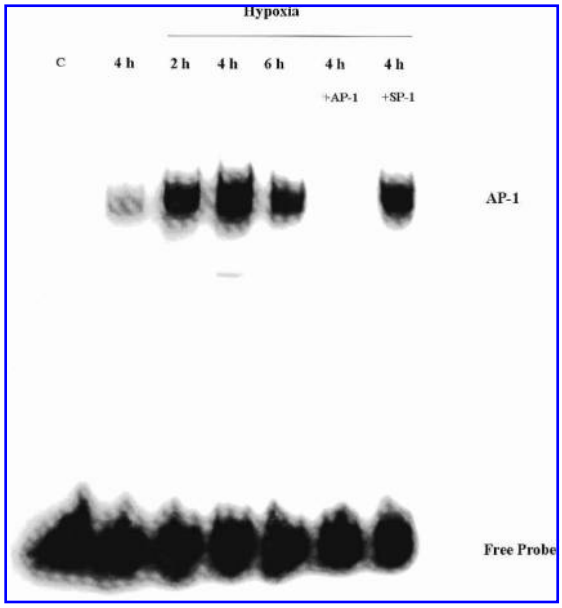

FIG. 3. Kinetics of AP-1 DNA-binding activity following hypoxia treatment in PAEC. Confluent monolayers of PAEC cells were treated with *in vitro* hypoxia (1% O_2) for 2, 4, or 6 h, or normoxia (20% O_2) for 4 h. Nuclear protein was extracted from isolated nuclei, incubated with a ^{32}P-labeled oligonucleotide containing the AP-1 consensus sequence, and electrophoresed on a 4% polyacrylamide gel. Control lanes received either no protein (lane 1), an excess of cold specific competitor (AP-1) or cold nonspecific competitor (SP-1). A representative autoradiogram of three experiments is shown.

(9.3- and 11.4-fold, respectively) and in VSMC (12.4- and 14.4-fold, respectively) (Figs. 1B and 2).

Consistent with previous reports (11), short-term hypoxia (1% O_2, 2–6 h) strongly induced AP-1 DNA-binding activity in PAEC, with an apparent maximum at 4 h (Fig. 3). Although the magnitude of the hypoxia response was variable in subsequent experiments, the addition of MAPK inhibitors 1 h prior to the hypoxia treatment, and their subsequent presence during hypoxia, had distinct effects on AP-1 binding activity. Specifically, SB203580 (10 μM) enhanced hypoxia-inducible AP-1 binding activity and, to a lesser extent, AP-1 binding activity under normoxic conditions (Fig. 4A). PD98059 stimulated AP-1 DNA-binding activity when applied in combination with hypoxia, but not under normoxic conditions (Fig. 4B). Interestingly, UO126, which stimulated HO-1 mRNA levels only under normoxia in PAEC (Fig. 1B), also stimulated AP-1 DNA-binding activity under normoxia (Fig. 4C) but slightly inhibited such activity under hypoxia. Consistent with its effects on HO-1 expression in PAEC, DPI stimulated AP-1 DNA-binding activity in PAEC under both normoxic and hypoxic conditions (Fig. 4D).

DISCUSSION

This study demonstrates that inhibitors of MAPK pathways dependent on p38 MAPK and MEK1 activate HO-1 mRNA expression in two vascular cell types, PAEC and VSMC, under hypoxic conditions and, to a lesser extent, under normoxia. Interestingly, the inhibitor of MEK1 and MEK2, UO126, produced the strongest normoxic induction of HO-1 mRNA levels.

Previous studies with cardiomyocytes have also implicated a role for p38 MAPK in the hypoxic *ho-1* activation pathway (15). In contrast to the current studies, Kacimi and colleagues observed that SB203580 inhibited hypoxia-inducible HO-1 expression in cardiomyocytes, whereas PD98059 or tyrosine kinase inhibitors had no effect (15). Previous studies using diverse chemical inducers of *ho-1* activation have also implied intermediary roles for MAPK activation. For example, induction of *ho-1* transcription in MCF-7 cells by the heavy metal salt $CdCl_2$ was blocked by

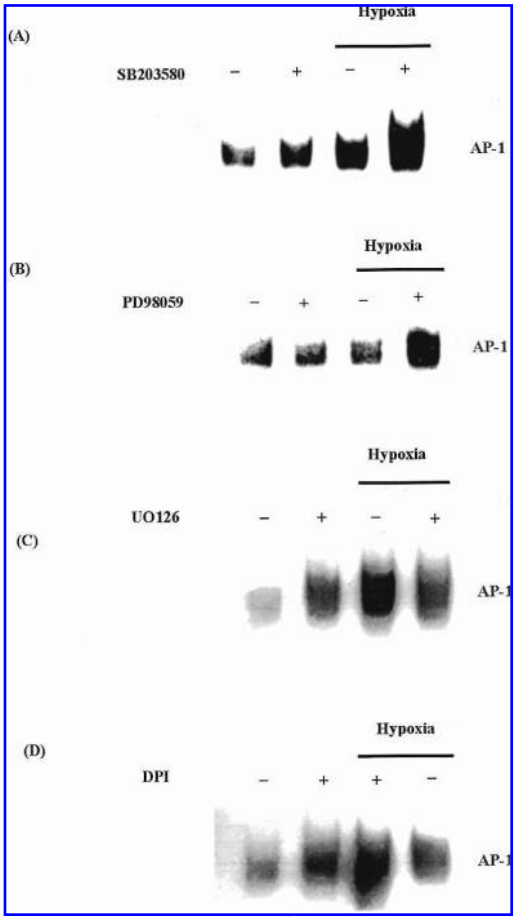

FIG. 4. Effect of MAPK inhibitors and DPI on hypoxia-inducible AP-1 DNA-binding activity in PAEC. Confluent monolayers of PAEC cells were treated with either SB203580 **(A),** PD98059 **(B),** UO126 **(C),** or DPI **(D)** (10 μM each, 1-h pretreatment) followed by *in vitro* hypoxia (1% O_2) for 4 h or normoxia (20% O_2) for 4 h. Nuclear protein was extracted from isolated nuclei, incubated with a ^{32}P-labeled oligonucleotide containing the AP-1 consensus sequence, and electrophoresed on a 4% polyacrylamide gel. Control lanes received either no protein (lane 1), an excess of cold specific competitor (AP-1), or cold nonspecific competitor (SP-1). Data are representative of two or three experiments each. Only the shifted regions of the autoradiograms are shown.

SB203580, but not by PD98059. Furthermore, transfection with the dominant-negative mutants of p38α and transcription factor Nrf2 blocked the response (3). Activation of the chicken *ho-1* promoter by sodium arsenite required both extracellular signal-regulated kinase (ERK) and p38 MAPK because it could be suppressed by both PD98059 and SB203580 (9). Overexpression of the dominant-negative forms of Ras, ERK kinase (MEK1), and p38 MAPK suppressed sodium arsenite activation of the chicken *ho-1* promoter (9). Furthermore both PD98059 and SB203580 blocked HO-1 mRNA expression in HeLA cells following exposure to nitric oxide donor compounds (6). In contrast, Masuya and colleagues reported that the activation of HO-1 expression by sodium arsenite, heme, or $CdCl_2$ in HeLa cells was suppressed by chemical inhibitors of tyrosine kinases (*i.e.,* herbimycin and genistein), but not by MAPK inhibitors (21). The artificial overexpression of the mitogen-activated protein kinase kinase kinases (MEKK1, TAK1, and ASK1) induced *ho-1* in HEPG2 cells in the absence of chemical inducing agents (40).

The paradigm from previous studies implies a general positive correlation between p38 MAPK and *ho-1* activation, which is contrary to the current observations. It is apparent, however, that the regulatory function and relative importance of p38 MAPK and MEK1/2-dependent pathways in the regulation of *ho-1* gene expression may vary significantly in a tissue- and inducer-specific manner.

We have also shown that the antioxidant compound DPI induces both HO-1 mRNA expression and AP-1 DNA-binding activity under both normoxia and hypoxia in PAEC. It was previously shown that the turmeric-derived antioxidant curcumin, a natural phenolic antioxidant, induced *ho-1* under normoxic and hypoxic conditions in bovine aortic endothelial cells (25). The antioxidant compound pyrrolidine dithiocarbamate also dramatically up-regulated the rat *ho-1* gene in VSMC (10). DPI inhibits hypoxia-inducible ROS generation and p42/p44 MAPK activation in PAEC cultures (Sasidhar and Choi, unpublished observation), and it is possible that DPI activates HO-1 expression under hypoxia through inhibition of NADPH oxidase-derived ROS generation and MAPK signaling, thus mimicking the effects of PD98059. DPI may share a common pathway with other antioxidant compounds in stimulating HO-1 expression.

The current experiments show that AP-1 activation mirrors HO-1 expression in response to three stimuli (*i.e.,* hypoxia, MAPK inhibitors, and DPI). Composite stress-response elements containing AP-1 consensus sequences overlapping with antioxidant response element sequences are located within two different enhancer regions, E1 and E2, of the mouse *ho-1* gene (1, 2, 13). The activation of *ho-1* by the antioxidant pyrrolidine dithiocarbamate was previously shown to be mediated through the E2 enhancer and involves activation of AP-1 DNA binding, and it is likely that the response to DPI occurs via a similar mechanism (10).

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ABBREVIATIONS

AP-1, activator protein 1; DMSO, dimethyl sulfoxide; DPI, diphenylene iodonium; DTT, dithiothreitol; ERK, extracellular signal-regulated kinase; HO, heme oxygenase; MAPK, mitogen activated protein kinase; MEK, mitogen-activated protein kinase kinase; PAEC, pulmonary aortic endothelial cells; PD98059, MEK1 inhibitor; ROS, reactive oxygen species; SB203580, p38 MAPK inhibitor; UO126, MEK1/MEK2 inhibitor; VSMC, vascular smooth muscle cells.

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