Hypoxia-inducible Factor-1 Mediates Transcriptional Activation of the Heme Oxygenase-1 Gene in Response to Hypoxia*

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Exposure of rats to hypoxia (7% O₂) markedly increased the level of heme oxygenase-1 (HO-1) mRNA in several tissues. Accumulation of HO-1 transcripts was also observed after exposure of rat aortic vascular smooth muscle (VSM) cells to 1% O₂, and this induction was dependent on gene transcription. Activation of the mouse HO-1 gene by all agents thus far tested is mediated by two 5'-enhancer sequences, SX2 and AB1, but neither fragment was responsive to hypoxia in VSM cells. Hypoxia-dependent induction of the chloramphenicol acetyltransferase (CAT) reporter gene was mediated by a 163-bp fragment located approximately 9.5 kilobases upstream of the transcription start site. This fragment contains two potential binding sites for hypoxia-inducible factor 1 (HIF-1). A role for HIF-1 in HO-1 gene regulation was established by the following observations: 1) HIF-1 specifically bound to an oligonucleotide spanning these sequences, 2) mutation of these sequences abolished HIF-1 binding and hypoxia-dependent gene activation in VSM cells, 3) hypoxia increased HIF-1α and HIF-1β protein levels in VSM cells, and 4) hypoxia-dependent HO-1 mRNA accumulation was not observed in mutant hepatoma cells lacking HIF-1 DNA-binding activity. Taken together, these data demonstrate that hypoxia induces HO-1 expression in animal tissues and cell cultures and implicate HIF-1 in this response.

Low cellular oxygen tension is a feature of both physiological conditions, such as adaptations to high altitude and physical endurance exercise (1), and pathophysiological conditions including ischemia, fibrosis (2), and neoplasia (3). Mammalian cells respond to hypoxia in part by increased expression of several genes that encode both tissue-specific and ubiquitous proteins (4). These proteins participate in diverse biological processes including erythropoiesis, which enhances the oxygen carrying capacity of the blood; angiogenesis, which permits delivery of oxygen carrying blood to hypoxia sites; glycolysis, as a means of energy production; xenobiotic detoxification; and cellular adaptation to stress. Hypoxia-inducible proteins within these respective categories include erythropoietin (EPO) (5), vascular endothelial growth factor (6), glycolytic enzymes (7–9), NAD(P)H:quinone oxidoreductase (10), and heat shock proteins (11, 12). Where examined, increased expression of specific proteins in response to hypoxia is regulated primarily at the level of gene transcription (although post-transcriptional mechanisms have also been characterized).

Another stress-associated protein whose expression is stimulated by hypoxia is heme oxygenase-1 (HO-1) (13, 14). HO-1, a microsomal membrane enzyme, catalyzes the first and rate-limiting reaction in heme catabolism, the oxidative cleavage of b-type heme molecules to yield equimolar quantities of biliverdin, carbon monoxide (CO), and iron. Biliverdin is subsequently converted to bilirubin by the action of biliverdin reductase. The expression of HO-1 is dramatically induced not only by the substrate, heme, but a variety of stress-associated agents, including heavy metals, hyperthermia, and UV irradiation (reviewed in Maines (15)). A common feature among these inducers, including heme, is that they generate reactive oxygen species and/or diminish glutathione levels. This correlation and the observation that bilirubin functions as an antioxidant (16) has led to the hypothesis that induction of HO-1 is part of a general response to oxidant stress and that this enzyme plays a protective role during such conditions (17–19).

Stimulation of HO-1 expression by most if not all inducers is controlled primarily at the level of gene transcription and in our studies on the regulation of the mouse HO-1 gene, we have identified two 5' distal enhancer regions, SX2 and AB1, that mediate gene activation by a variety of pro-oxidants including heme, heavy metals, TPA, hydrogen peroxide, and LPS (20–23). The mechanism of HO-1 induction by hypoxia has not been investigated and because this induction has been proposed to occur as a consequence of oxidative stress (13), we examined the role of the SX2 and AB1 enhancers in hypoxia-dependent gene activation. In this report we show that these enhancers do not mediate transcriptional activation of the HO-1 gene in response to hypoxia. Rather, this induction is mediated by a 163-bp fragment located directly downstream of the AB1 enhancer and approximately 9.5 kb upstream of the transcription initiation site. The active sequences within this fragment resemble the binding site for hypoxia-inducible factor-1 (HIF-1).

The abbreviations used are: EPO, erythropoietin; HO-1, heme oxygenase-1; AP-1, activator protein-1; CAT, chloramphenicol acetyltransferase; HIF-1, hypoxia-inducible factor-1; HypRE, hypoxia response element; VSM, vascular smooth muscle; CHO, Chinese hamster ovary; bp, base pair(s); kb, kilobase pair(s).
initially identified as a protein that binds to a site in the EPO gene enhancer required for transcription activation by hypoxia (24, 25). Additional data are provided to support a role for HIF-1 in HO-1 gene regulation.

MATERIALS AND METHODS

Animals—Pathogen-free Harlan Sprague Dawley rats (200–225 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN) and allowed to acclimate upon arrival for 7 d prior to experimentation. Animals were fed rodent chow and water ad libitum. Animals were exposed to hypoxia (7.0% O₂) in a 3.70-cubic feet plexiglass exposure chamber. Rats were supplied with rodent chow and water ad libitum during the exposures. At appropriate times, animal(s) were removed from the chamber and immediately sacrificed by decapitation. Tissues were removed and frozen in liquid nitrogen for subsequent RNA extraction. These experiments were carried out according to animal protocols approved by the Animal Care and Use Committee of The Johns Hopkins University School of Medicine.

Cell Culture—Primary cultures of rat aortic vascular smooth muscle (VSM) cells, passage 2–10, were generously provided by Dr. Michael Crow of the National Institute of Aging. Chinese hamster ovary epithelial cells (CHO cells) were obtained from American Tissue Cell Culture. CHO cells were maintained in Ham’s-F12 medium. Wild type Hepa1 c1c7 and mutant Hepa1 c4 cells (generously provided by Dr. O. Hankinson of the University of California at Los Angeles) were maintained in minimal essential medium—a supplemented with 10% fetal bovine serum. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂, 95% air. Cells were exposed to hypoxia (1% O₂, 5% CO₂, 95% air) in a tightly sealed modular incubator chamber (Billups-Rothenberg, Del Mar, CA) at 37°C. All experiments were performed with confluent cultures.

RNA Extraction and Northern Blot Analyses—Total RNA from cells and tissues was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method (26). Northern blot analyses were performed as described previously (23). Briefly, 10– or 15-μg (Hepa1 cells) aliquots of total RNA were fractionated on a denaturing agarose gel, transferred to nylon membrane by capillary action, and cross-linked to the membrane by UV irradiation. The nylon membranes were incubated in hybridization buffer (1% bovine serum albumin, 7% SDS, 0.5 mM phosphate buffer, pH 7.0, 1.0 mM EDTA) containing 32P-labeled rat HO-1 cDNA (27) (generously provided by Dr. S. Shibahara of Tohoku University, Japan) at 65°C for 24 h. The membranes were washed twice in buffer A (0.5% bovine serum albumin, 5% SDS, 40 mM phosphate buffer, pH 7.0, 1.0 mM EDTA) for 15 min at 65°C followed by four washes in buffer B (1% SDS, 40 mM phosphate buffer, pH 7.0, 1.0 mM EDTA) for 15 min at 65°C and exposed to x-ray film. To control for variation in either the amount of RNA in different samples or loading errors, blots were hybridized with a radiolabeled oligonucleotide probe (5′-ACGGTATCTGATCGTCTTCGAACC-3′) complementary to 18 S rRNA after stripping of the HO-1 probe. Autoradiographic signals were quantified by densitometric scanning (Molecular Dynamics, Sunnyvale, CA). All densitometric values obtained for the HO-1 mRNA transcript (1.8 kb) were normalized to values for 18 S rRNA obtained on the same blot. The HO-1 mRNA level in treated cells was expressed in densitometric absorbance units, normalized to control untreated samples, and expressed as fold induction compared to controls.

Western Blot Analyses—For HO-1 immunoblots, frozen tissues or cells were homogenized in lysis buffer (1% Nonidet P-40, 20 mM Tris, pH 8.0, 137.5 mM NaCl, 1 mM Na_2VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin). Protein concentrations of the lysates were determined by Coomassie Blue dye-binding assay (Bio-Rad). An equal volume of 2× SDS/sample buffer (0.125 mM Tris-HCl, pH 7.4, 4% SDS, and 20% glycerol) was added and the samples were boiled for 5 min. Samples (100 μg) were subjected to electrophoresis in a 12% SDS-polyacrylamide gel and then transferred electrothermally onto a polyvinylidene fluoride membrane. The membranes were incubated for 2 h with rabbit polyclonal antibody against rat HO-1 (1:1,000 dilution; StressGen Biotech Corp., Vancouver, Canada) followed by incubation with goat anti-rabbit IgG antibody for 2 h. Signal development was carried out using an ECL detection kit (Amersham Corp.).

For HIF-1α and HIF-1β immunoblots, aliquots of crude nuclear extracts containing 15 μg of protein from cells exposed to 1% or 20% O₂...
were fractionated at 30 mA by SDS-PAGE using a 7% acrylamide, 0.23% bisacrylamide gel. The proteins were transferred to nitrocellulose membranes, incubated with affinity-purified HIF-1α or HIF-1β antibodies at 1:600 (v/v) dilution. The membrane was washed, incubated with horseradish peroxidase anti-immunoglobulin conjugate and detected with ECL reagents as described previously (28).

Plasmids, Transfections, and Enzyme Assays—Construction of the CAT reporter plasmids diagramed in Fig. 4 has been described previously (20–22). Subfragments (see Fig. 5) of fragment EH (formerly EH2) (22) were isolated after digestion with the appropriate endonucleases, blunt-ended, and cloned into the SpeI site, upstream of the minimal promoter and CAT gene in pMHO1CATΔ-33 (20). Mutation of the putative HIF-1 binding sites in the BT fragment was carried out by oligonucleotide-directed mutagenesis (29) using the single-stranded form of plasmid pMHO1CATΔ-33 + BT. The sequence of the mutagenic oligonucleotide was 5'-CGCTCTAGAACTAGCGAAGCTGGCGTGACGCTTTTCCTCTG-3'. The first 14 residues are derived from the multiple cloning sites of pBluescript II SK+, the parent plasmid of pMHO1CATΔ-33. Residue 15 corresponds to nucleotide 324 in the sequence presented in Fig. 6A.

Plasmids (10 μg) were transiently transfected into cells using Lipofectin Reagent (Life Technologies, Inc.) according to the manufacturer's protocol. The cells were transfected overnight after which time the plates were washed twice with serum-free media and then incubated in complete media at 1 or 20% O2 for 24 h. Preparation of cellular extracts and measurement of CAT activity were carried out as described previously (30). CAT enzyme standard was purchased from Sigma and used for calculation of cellular enzyme activities.

Electrophoretic Mobility Shift Assay—Rat VSM cells were exposed to 20% or 1% O2 for 6 h and crude nuclear extracts were prepared as described previously (24). Electrophoretic mobility shift assay using 7 μg of nuclear extracts was performed in binding buffer containing 25 mM Tris-HCl (pH 7.6), 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 5 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride, and 1.2 mM sodium vanadate, with 104 cpm of oligonucleotide probe (24, 31). Oligonucleotide competition experiments were performed with 1 or 3 ng (10- or 30-fold excess) of unlabeled wild-type or mutant HO-1, or 3 ng of unlabeled wild-type or mutant EPO double-stranded oligonucleotides. After preincubation for 5 min at room temperature, labeled wild-type HO-1 probe was added and incubated on ice for 15 min. In the supershift assays, 1 μl of preimmune serum or antiserum (28) specific for HIF-1α at 1:3 dilution or HIF-1β at 1:6 dilution was added to the reaction mixture and incubated for 30 min on ice prior to electrophoresis.

RESULTS

Hypoxia Induces HO-1 Expression in Rat Tissues—Rats were exposed to 7% O2 for 0, 0.5, 1, or 2 h and HO-1 expression in various tissues was monitored by RNA blot analysis. Results from a representative experiment are shown in Fig. 1. A marked increase in the steady-state level of HO-1 mRNA was observed in the lung, liver, heart, and aorta, and in all cases this induction was evident within 30–60 min of exposure to hypoxia. Within the time period examined, the highest levels of induction were observed in the liver (32-fold) and lung (16-fold). Reexposure of rats to 21% O2 for 15 or 30 min after 2 h of hypoxia resulted in a rapid decline in the amount of HO-1 mRNA in all tissues examined. HO-1 mRNA accumulation was also observed in the kidney and adrenal gland but was less pronounced. No induction was observed in the spleen (data not shown).

Induction of HO-1 Expression in VSM Cells Subjected to Hypoxia—Previous studies have demonstrated hypoxic-dependent induction of HO-1 expression in CHO cells (13) and VSM cells (14). The latter were selected for investigation of the regulatory mechanism of HO-1 induction because of the physiological relevance of VSM cells (see below) and because they exhibited more consistent and higher levels of induction than CHO cells (data not shown). Exposure of VSM cells to 1% O2...
produced an initial rise in the steady-state amount of HO-1 mRNA at 4 h (4.0-fold), peak levels at 8 h (6.0-fold), prior to a drop in the levels by 24 h (2.4-fold) and 48 h (1.6-fold) of continuous hypoxia (Fig. 2A). The level of HO-1 mRNA accumulation and the temporal profile are similar to those observed after exposure of VSM cells to 0% O₂ (14). Induction of HO-1 expression in cultured VSM cells appears to be delayed relative to that observed in the aorta. This variation, however, is not surprising given the differences in the treatment conditions and the cellular environments, particularly the presence of multiple cell types (i.e. endothelial cells) in the in vivo studies. The accumulation of HO-1 mRNA in cultured VSM cells correlated with increased levels of HO-1 protein (Fig. 2B).

To further delineate the molecular basis for increased expression of HO-1 in response to hypoxia, we examined whether HO-1 mRNA induction was dependent on gene transcription. Cells were pretreated for 1 h with actinomycin D, a potent inhibitor of RNA synthesis, prior to exposure to hypoxia. As shown in Fig. 3, actinomycin D (1 μg/ml) completely inhibited hypoxia-induced HO-1 mRNA accumulation.

Identification of the Hypoxia-responsive Elements in the Mouse HO-1 Gene—To identify cis-acting DNA sequences that mediate HO-1 gene activation in response to hypoxia, VSM cells were transiently transfected with plasmids containing various segments of the 5′-flanking region of the mouse HO-1 gene linked to the Escherichia coli CAT gene. Transfected cells were exposed to 20% O₂ or 1% O₂ for 24 h and hypoxia-dependent fusion gene regulation was assessed by measuring CAT activity in cellular extracts. Analysis of approximately 12.5 kb of the HO-1 5′-flanking region indicated that the hypoxia-responsive element (HypRE) resides within a 900-bp fragment, EH, located 9 kb upstream of the transcription initiation site (Fig. 4). By analyzing subfragments of EH in a manner analogous to that described above, the HypRE was localized to a 163-bp BsrBI/TaqI fragment, BT (Fig. 5). Interestingly, this fragment is immediately downstream of enhancer sequences, AB1, previously shown to mediate transcriptional activation of the HO-1 gene in response to various agents including hemoglobin.

| CONSTRUCT | CAT ACTIVITY (x 10⁻³ UNITS) |
|-----------|-----------------------------|
| Control   | 81.1                        |
| Hypoxia   | 724.0                       |
| Fold Ind  | 8.9                         |

Fig. 5. Functional analysis of the EH subfragment in VSM cells after hypoxia. The diagrammed fragments were isolated after digestion with the indicated restriction endonucleases, blunt-ended and cloned into the vector pMHO1CAT-33 which contains a minimal HO-1 promoter (−33 to −73). VSM cells transiently transfected with the appropriate CAT construct were exposed to 20% O₂ or 1% O₂ for 24 h and then analyzed for CAT activity. The data represent the average value from three independent experiments and the mean fold induction of CAT activity in extracts of cells exposed to 1% O₂ compared to that in extracts of cells exposed to 20% O₂. Standard error of the mean was <20% for all values shown. E, EcoRI; A, AflII; B, BsrBI; T, TaqI; R, RsaI; H, HindIII.

**Fig. 6. A**, nucleotide sequence of the hypoxia-responsive region of the mouse HO-1 gene. A partial sequence of the EH fragment is presented. The sequence of the AB1 enhancer, between the AflII and BsrBI recognition sites, has been previously reported (18). Binding sites for the transcription factors CCAAT/enhancer binding protein (C/EBP) and AP-1 (both in the AB1 fragment) and for HIF-1 are bracketed. **B**, mutational analysis of the BsrBI/TaqI (BT) subfragment in hypoxic VSM cells. The sequence of the 5′ end of the BT fragment (starting at residue 324 in the sequence shown in A) is presented along with the mutations (underlined) within the putative HIF-1 binding sites (upper case letters). VSM cells transiently transfected with the corresponding wild-type and mutant BT/CAT fusion constructs were treated and analyzed as described in the legend to Fig. 4. The data represents the average of three independent experiments. Standard error of the mean was <15% for all values shown.
HIF-1 Mediates HO-1 Gene Activation by Hypoxia

**FIG. 7.** Binding of nuclear factors from VSM cells to the HO-1 enhancer. A, oligonucleotides. The sense strand of double-stranded oligonucleotides is shown. All oligonucleotides also contained the single-stranded sequence 5'-GATC-3' at their 5' ends. Potential and proven HIF-1 binding sites in the wild-type HO1 (Hw) and EPO (Em) oligonucleotides, respectively, are highlighted in **boldface**. Nucleotides substitutions in the mutant HO1 (Hm) and EPO (Em) oligonucleotides are underlined. B, electrophoretic mobility shift assay. 7-μg aliquots of nuclear extract, prepared from VSM cells exposed to 20% O2 (lanes 1 and 1) or 1% O2 (lanes 2–8) for 6 h, were incubated with 32P-labeled Hw probe in the absence (lanes 1 and 2) or presence of the indicated amount (in ng) of unlabeled Hm (lanes 3 and 4), Hw (lanes 5 and 6), Em (lanes 7), or Em (lane 8) oligonucleotide and DNA binding activities were analyzed by gel electrophoresis and autoradiography. Position of free probe (FP) and complexes containing HIF-1 or constitutive binding activity (C) is indicated. C, supershift assay. Mobility shift assays were performed as described above in the absence (lanes 5 and 6) or presence of polyclonal antiserum raised in rabbits against recombinant human HIF-1α (lane 2) or HIF-1β (lane 4), or in the presence of the respective preimmune serum (lanes 1 and 3). Position of supershift complexes (S) is indicated.

and cadmium (22). Neither the AB1 enhancer nor the SX2 enhancer (Fig. 4) were responsive to hypoxia in VSM cells.

A partial sequence of the EH fragment, is shown in Fig. 6A. The sequence of the AB1 segment (residues 166–326) has been published previously (22). Computer analysis of the EH sequence indicated that it contains two motifs similar to the consensus binding site, 5′-BACGTGCK-3′, for HIF-1 (9). Both of the putative HIF-1 binding sites 5′-GACGTGCT-3′ (sense strand) and 5′-GACGTGCC-3′ (antisense strand) are located within the hypoxia-responsive BT fragment (residues 324–486). To assess the role of these putative HIF-1 binding sites in hypoxia-dependent gene activation, the CTCG trinucleotide of both elements was mutated to AAA in the context of the BT fragment (Fig. 6B). Previous studies have demonstrated that analogous mutations within the HIF-1 binding site of the hypoxia-responsive EPO enhancer, 5′-TACGTGCT-3′, abolish both protein binding and enhancer function (24, 32) (see below). The mutant BT fragment was unresponsive to hypoxia (Fig. 6B), demonstrating that these sequences are necessary for HO-1 gene activation in hypoxic VSM cells.

**FIG. 8.** Expression of HIF-1α and HIF-1β protein in VSM cells. Nuclear extracts were prepared from VSM cells exposed to 20% O2 or 1% O2 for 6 h. Fifteen-μg aliquots were fractionated by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with affinity-purified antibodies raised against recombinant human HIF-1α (top) or HIF-1β (bottom). Position of molecular mass markers of 86 and 121 kDa is indicated.

The HypREs Bind HIF-1 and HIF-1 Expression Is Increased in Hypoxic VSM Cells—Double-stranded oligonucleotides were synthesized corresponding to wild-type and mutant HO-1 and EPO sequences (Fig. 7A). The wild-type HO1 oligonucleotide (Hw) was radiolabeled and incubated with nuclear extracts prepared from VSM cells that had been incubated in 1% or 20% O2 for 6 h. The Hw probe detected a constitutively-expressed DNA-binding activity (C) that was present in both nonhypoxic (Fig. 7B, lane 1) and hypoxic (lane 2) cells as well as a DNA binding activity present only in hypoxic cells (lane 2). Inclusion of 1 or 3 ng (representing a 10- or 30-fold molar excess) of unlabeled mutant HO1 oligonucleotide (Hm) had no effect on the binding of the inducible factor(s) to the Hw probe (lanes 3 and 4), whereas binding was partially and completely inhibited by 1 and 3 ng, respectively, of unlabeled Hw oligonucleotide (lanes 5 and 6). Binding of the inducible factor(s) to the Hw probe was also blocked by excess wild-type (Ew), but not mutant (Em), EPO oligonucleotides (lanes 7 and 8). Inclusion of antiserum raised against HIF-1α (Fig. 7C, lane 2) or HIF-1β (lane 4) resulted in the loss of the inducible complex and formation of a supershifted complex, suggesting that HIF-1α and HIF-1β are components of the hypoxia-inducible HypRE binding activity. The respective preimmune sera (lanes 1 and 3) had no effect on the HIF-1/DNA complex that formed in the absence of antiserum (lane 5).

HIF-1α and HIF-1β protein levels in nuclear extracts from non-hypoxic and hypoxic VSM cells were also quantified by immunoblot assay (Fig. 8). Levels of HIF-1α were extremely low in nonhypoxic cells and increased dramatically in response to hypoxia. Multiple HIF-1α isoforms were detected in hypoxic VSM cells. Compared to HIF-1α, HIF-1β protein levels were higher in nonhypoxic VSM cells and showed a more modest induction in response to hypoxia. These features of HIF-1 ex-
expression are similar to those previously described for Hep3B cells (28). Taken together, these results demonstrate that (i) exposure of VSM cells to 1% O₂ increases nuclear HIF-1 DNA-binding activity as well as HIF-1α and HIF-1β protein, (ii) HIF-1 specifically binds to HO-1 sequences necessary for gene activation in response to hypoxia, and (iii) mutations in the HO-1 enhancer that result in a loss of transcriptional activity in hypoxic cells also result in a loss of HIF-1 binding.

**HO-1 mRNA Is Not Induced by Hypoxia in Cells Lacking HIF-1 DNA Binding Activity**—To further investigate the role of HIF-1 in HO-1 gene regulation, two clones of mouse hepatoma cells, wild-type Hepa1 c1c7 and its mutant derivative Hepa1 c4, were analyzed for HO-1 expression under hypoxic conditions. Hepa1 c4 cells are deficient in HIF-1 (33) and lack HIF-1 DNA-binding activity. These cells were incubated for 0, 4, 8, and 16 h at 1% O₂, and HO-1 expression was subsequently assessed by Northern blot analyses. In wild-type Hepa1 cells, HO-1 mRNA levels increased 14.0-fold at 4 h, 7.8-fold at 8 h, and 4.3-fold at 16 h of hypoxia (Fig. 9). In mutant Hepa1 c4 cells, the basal level of HO-1 mRNA was significantly higher but no appreciable increase was observed under hypoxic conditions. These data provide further evidence that HIF-1 plays a critical role in HO-1 gene activation in response to hypoxia in cultured cells.

**DISCUSSION**

Much interest in HO-1 has been generated recently by reports demonstrating the induction of HO-1 expression by a variety of pro-oxidants including UV irradiation, hypoxia, and endotoxin, and the protection this induction affords against heme- and non-heme-mediated oxidant injury (reviewed in Choi and Alam (34)). The present study demonstrates that acute hypoxic stress also stimulates HO-1 expression both in animal tissues and in cell cultures. In VSM cells, induction of HO-1 expression is regulated at the level of gene transcription and results from several lines of investigation demonstrate that this activation is mediated by HIF-1.

HIF-1 was initially identified as a hypoxia-inducible nuclear factor in Hep3B hepatoma cells that bound to sequences necessary for hypoxic activation of the EPO gene, whose product is expressed primarily in fetal liver and the kidney. Subsequent studies demonstrated that HIF-1 is present and/or functions in cell lines of various tissue origin, including HeLa cervical carcinoma cells, Ltk⁻ fibroblast, C₆C₁₂ skeletal myoblasts and CHO cells, suggesting that this transcription factor plays a more general role in the hypoxic response of mammalian cells and may regulate the expression of other hypoxia-inducible genes (32). Indeed, HIF-1 binding sites have been identified in other functional classes of hypoxia-inducible genes including those coding for inducible nitric oxide synthase (35), vascular endothelial growth factor (36, 37), and the glycolytic enzymes aldolase A, enolase 1, lactate dehydrogenase A, and phosphoglycerate kinase 1 (7, 9). Given the potential significance of increased HO-1 activity in hypoxic cells (see below), the identification of the HO-1 gene as a target for HIF-1 regulation lends further support to the hypothesis that HIF-1 coordinates homeostatic transcriptional responses to cellular hypoxia.

In addition to HIF-1, the AP-1 transcription factors, comprised of homo- and heterodimers of the Fos and Jun family of proteins, have been implicated in hypoxia-dependent gene activation. For instance, in several cell types, both the mRNA levels of individual Fos and Jun family members and AP-1-specific DNA binding activity are increased after exposure to hypoxia (10, 38, 39). Furthermore, an AP-1 binding site within the promoter of the tyrosine hydroxylase gene is required for hypoxia-dependent transcription activation (40). Both SX2 and AB1 enhancers contain binding sites for various transcription factors, including CCAAT/enhancer binding protein and Sp1, but the dominant element within both fragments is the consensus sequence, T(C/G)C(T/G)A, which is present in five copies (20, 22). Mutation of these elements, which are targets for various AP-1 member proteins (20) (data not shown), dramatically diminishes or completely abolishes gene activation by all SX2- and AB1-dependent inducers thus far tested including heme, heavy metals, hydrogen peroxide, 12-O-tetradecanoyl-phorbol-13-acetate, and lipopolysaccharide (22, 23, 30, 41). That a single type of element regulates mouse HO-1 induction by diverse agents argues for a common mechanism in the mode of action of these agents and is consistent with the oxidant stress hypothesis. Clearly, neither the SX2 nor the AB1 enhancer is responsive to hypoxia in VSM cells. These results attest to the uniqueness of hypoxia as an inducer of the mouse HO-1 gene and argue against the previous suggestion that hypoxic induction of HO-1 in CHO cells occurs as a consequence of oxidative stress (13).

The significance of increased HO-1 expression under hypoxic conditions is not clearly understood. The utilization of distinct transcription factor pathways for HO-1 gene activation by hypoxia and various pro-oxidants, however, suggests that this significance may be less related to the antioxidant activity of CO, like NO, can activate guanylate cyclase resulting in elevated levels of cGMP, which among other activities, causes relaxation of VSM cells and inhibition of platelet aggregation. Recent studies have in fact established that, under physiological conditions, maintenance of normal vascular tone and blood flow can be attributed in part to enzymatically derived CO (42, 43). Therefore, changes in vascular tone, endothelial permeability, and/or coagulant function observed under hypoxic conditions (44, 45) may in part be a consequence of HO-1 induction and localized production of CO. Consistent with this role of

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HO-1, hypoxic VSM cells exhibit increased production of CO, derived primarily if not exclusively from induced HO-1 activity, and increased levels of cGMP (14). Furthermore, the HO-1-derived CO from VSM cells may have paracrine effects as it modulates cGMP levels and gene expression in endothelial derived CO from VSM cells may have paracrine effects as it modulates cGMP levels and gene expression in endothelial cells in an in vitro co-culture system (46). Finally, with respect to VSM cells, HO-1 induction and the regulatory effects of CO may be of particular significance in pathophysiological conditions such as atherosclerosis where smooth muscle cells are exposed to hypoxic environments prior to and during development of lesions (47, 48).

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