Regulation of endothelial heme oxygenase activity during hypoxia is dependent on chelatable iron

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Ryter, Stefan W., Minliang Si, Chen-Ching Lai, Ching-Yuan Su. Regulation of endothelial heme oxygenase activity during hypoxia is dependent on chelatable iron. Am J Physiol Heart Circ Physiol 279: H2889–H2897, 2000.—The regulation of heme oxygenase (HO) activity and its dependence on iron was studied in bovine aortic endothelial cells (BAEC) subjected to hypoxia-reoxygenation (H/R). HO activity was induced by hypoxia (10 h) and continued to increase during the reoxygenation phase. HO-1 protein levels were strongly induced by hypoxia from undetectable levels and remained elevated at least 8 h postreoxygenation. Addition of the Fe3+ chelator desferrioxamine mesylate (DFO) or the Fe2+ chelator o-phenanthroline had a partial inhibitory effect on HO activity and protein levels when added only during reoxygenation. Loading of BAEC with Fe3+ enhanced the activation of the HO-1 gene by H/R, whereas loading with l-aminolevulinic acid, which stimulates heme synthesis, had little effect. These results suggest that chelatable iron participates in regulating HO expression during hypoxia.

carbon monoxide; endothelium; stress response

THE FUNCTIONAL INTEGRITY of endothelial cells may influence both the risk of cardiovascular disease and the outcome of cardiac ischemia-reperfusion injury (40). Hypoxia, or low oxygen tension, occurs physiologically during ischemia or neoplasia (8, 13, 41). Both hypoxia and subsequent reoxygenation modulate the production of reactive oxygen species (ROS) (46, 52) and alter gene expression patterns in various cell culture systems including vascular endothelium (30, 51). Among the genes activated by hypoxia in vitro are erythropoietin, various cytokines (vascular epidermal growth factor, endothelin-1, platelet-derived growth factor-β, basic fibroblast growth factor, interleukin-1α, and interleukin-6), and the glucose transporter (13). Furthermore, hypoxia also modulates the expression of genes associated with small gas production: nitric oxide synthases (NOS, types II and III) (1, 24, 31, 32) and heme oxygenase-1 (HO-1) (22, 30).

Microsomal HO (EC 1.14.99.3), in association with NADPH-cytochrome P-450 reductase, catalyzes the rate-limiting step in the oxidative degradation of heme, forming carbon monoxide (CO), ferrous iron, and biliverdin IXα, which is subsequently converted to bilirubin IXα by NADPH-biliverdin reductase (BVR) (25, 44). The HO system consists of an inducible isozyme (HO-1) and two constitutive forms: heme oxygenase-2 (HO-2) and newly cloned heme oxygenase-3 (25, 28).

The activation of the HO-1 gene (32-kDa mammalian stress protein) occurs as a ubiquitous cellular response to chemical and physical stress, including heavy metals, nitric oxide (NO), and oxidants such as hydrogen peroxide (H2O2) or ultraviolet-A (UVA, 320–380 nm) radiation (15, 25, 37). The induction of HO-1 may also occur in vascular systems in response to hemodynamic stress and in the heart after a cardiac or renal ischemia-reperfusion episode (14, 35, 39).

Increasing evidence has accumulated in vitro and in vivo that HO-1 participates in cellular defense mechanisms against oxidative stress (33, 47). The activation of the HO-1 gene by oxidants may be enhanced by depletion of intracellular reduced glutathione (GSH) (21). The antioxidative effects of HO-1 may be related to: 1) the release of excess heme iron and its subsequent transfer to the ferritin pool, and/or 2) the physicochemical antioxidant properties of the bile pigments biliverdin and bilirubin (42, 47).

Similar to NO, the CO derived from HO activity functions as a second messenger in neural and vascular signaling (26). CO exerts its putative vasomodulatory effects by binding to and activating soluble guanylate cyclase in an autocrine or paracrine fashion, stimulating the production of cGMP (29). Although vascular CO production has typically been attributed to HO-2, the expression of endothelial HO-1 during vascular stress may serve an inducible vasoregulator function (10, 26). Furthermore, CO production in the heart may have a role in myocardial preservation after ischemia-reperfusion (27).

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Chelatable iron levels have been shown to influence HO-1 gene expression after cellular exposure to oxidizing agents. In addition to the well-known prooxidant properties of iron, including the production of the hydroxyl radical (·OH) via the Fenton reaction, the formation of hypervalent oxidizing complexes, the promotion of lipid peroxidation, and cellular sensitization to ROS (2,3), iron has direct regulatory roles in gene expression (17). In this study, we examined the regulation of HO-1 expression in bovine aortic endothelial cells (BAEC) subjected to simulated hypoxia or hypoxia-reoxygenation and its dependence on intracellular iron. We show that the endothelial HO-1 response to hypoxia can be modulated by agents that alter intra-cellular iron status.

MATERIALS AND METHODS

Chemicals. L-Aminolevulinic acid (ALA), biliverdin, bilirubin, β-NADPH, (reduced form), desferrioxamine mesylate (DF), DMSO, (−)-epigallocatechin gallate (EGCG), ferric ammonium citrate, glucose-6-phosphate, glucose-6-phosphate dehydrogenase (G-6-PDH, type XV from baker’s yeast), hemin (bovine), 8-hydroxyquinoline hemisulfate, N-acetyl-L-cysteine (NAC), o-phenanthroline, 3,3′,4,5,7-pentahydroxyflavone dihydrate (quercetin), sucrose, and Tris-HCl, were from Sigma (St. Louis, MO). Pefabloc SC, leupeptin, and pepstatin A were from Boehringer Mannheim (Indianapolis, IN). Chloroform was from Aldrich (Milwaukee, WI).

Primary culture. BAEC were isolated from fresh bovine thoracic aorta (male) (Y-T Packing, Springfield, IL) according to the procedure described by Fenselau and Mello (11). Aorta were washed in PBS containing antibiotics (100 μg/ml penicillin and 200 μg/ml streptomycin). Isolated aorta were tied with surgical suture, filled with a 0.1 mg/ml solution of collagenase type IA (Sigma) in PBS, and incubated for 30 min at 37°C with gentle agitation. The collagenase suspension was recovered, centrifuged for 5 min at 500 g, and resuspended with complete growth media. Cells were maintained in DMEM containing 4.5 g/l glucose, supplemented with penicillin-streptomycin, L-glutamine solution, and 10% fetal bovine serum (FBS). The cells were grown in humidified incubators containing an atmosphere of 95% air-5% CO2 at 37°C for various intervals (10–24 h). Upon opening the tank, cell monolayers were rinsed with 95% N2-5% CO2 saturated PBS. Cells were either harvested immediately on ice or replaced with complete oxygenated growth media and restored to an atmosphere of 95% air-5% CO2 at 37°C for various reoxygenation time intervals.

The sham-treated cells were subjected to 10 h in a minimal volume of normoxic growth media (3 ml). The sham H/R-treated cells were subjected to 10 h in a minimal volume of normoxic growth media (3 ml) followed by replacement with 8 ml normoxic growth media for 2 h. Normoxic control cells were treated with a normal volume of growth media (8 ml) for 10 h only. Other variations of control conditions specific to each experiment are stated in the figure legends.

Chemical treatments. For chemical treatments, DFO, ALA (dissolved in PBS), or o-phenanthroline (dissolved in DMSO) was added to culture media at a final concentration of 100 μM immediately before the hypoxia treatment and/or added to the reoxygenation media after hypoxia. In the latter case, the final concentration of DMSO did not exceed 0.1%, a concentration that did not affect basal HO activity. Antioxidant compounds NAC and EGCG (dissolved in PBS) or quercetin (dissolved in DMSO) were added to the deoxygenated complete media immediately before hypoxia treatment, at final concentrations of 100 μM, or up to 10 mM for NAC. DFO-Fe3+ (1:1 molar ratio) was prepared by vortex mixing stoichiometric amounts of DFO and ferric ammonium citrate. Iron loading of endothelial cells was performed as described (3). An equimolar amount (5 μM) of 8-hydroxyquinoline hemisulfate and Fe3+ (as ferric ammonium citrate) was added to 10 ml of PBS and applied to the cell monolayers for 1 h before hypoxia treatment. As a positive control for HO induction, BAEC were seeded at 0.5 × 106 cells per 100-mm plate and cultured for 3 days, then treated with sodium m-arsenite (100 μM) for 30 min in PBS, rinsed, and allowed to recover for 14 h in complete conditioned media (36).

HO activity. Crude endothelial protein extracts were prepared as follows: cell monolayers were rinsed with PBS and then scraped directly in 300 μl sonication buffer A (containing 0.25 M sucrose, 20 mM Tris-HCl, 50 μg/ml Pefabloc SC, 4 μg/ml pepstatin, and 4 μg/ml leupeptin; pH 7.4) sonicated on ice twice for 15 s, and centrifuged for 20 min at 18,000 g. The protein concentration of the resulting supernatant was determined by using the commercial Bio-Rad assay (Bio-Rad, Hercules, CA) with BSA as the standard (5). The HO activity was measured by the spectrophotometric determination of bilirubin production as previously described (18). Final reaction concentrations were 25 μM heme, 2 mM glucose-6-phosphate, 2 U G-6-PDH, 1 mM β-NADPH, 1 mg/ml crude endothelial cell extract, and 2 mg/ml partially purified bovine liver BVR preparation. Reaction mixtures were incubated for 60 min in a 37°C water bath in the dark. The reactions were terminated by addition of 2 volumes chloroform. Bilirubin concentration in the chloroform extracts was determined using a Hitachi U-2000 scanning spectrophotometer (Hitachi Instruments, St. Louis, MO) by measuring optical density (OD) at 464 and 530 nm. HO activity was reported as picomoles bilirubin per milligram endothelial cell protein per hour, assuming an extinction coefficient of 40 mM−1cm−1 for bilirubin in chloroform. Partially purified BVR was prepared from fresh bovine liver (Y-T Packing) according to the protocol of Tenhunen and colleagues, stopping at step III (45).

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The resulting fraction was assayed for the rate of biliverdin conversion by NADPH difference spectroscopy.

**Western immunoblot analysis.** Protein extracts were prepared as described for enzymatic activity determination and were then diluted with 1 volume of 2× loading buffer (20). Proteins were separated on an SDS-polyacrylamide gel (10% for HO-1 analysis) and transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) using a Hoeffer apparatus. Membranes were blocked with 5% BSA in Tris-buffered saline with Tween 20 (pH 7.4) for 30 min. Membranes were incubated for 18 h with polyclonal rabbit anti-rat HO-1 antisera at a 1:1,000 dilution (PA-3–019, Affinity Bioreagents, Golden, CO), which reacts with bovine HO-1 (19). The membranes were washed and then incubated with goat anti-rabbit IgG horseradish peroxidase conjugate (New England Biolabs, Beverly, MA) at a 1:2,000 dilution. Immune complexes were visualized using the horseradish peroxidase substrate tablet set (Sigma).

**Viability assays.** Total cellular lactate dehydrogenase (LDH) activity was measured as described previously (4) by monitoring the consumption of NADH during the conversion of pyruvic acid to lactate. LDH content in samples was calculated from ΔOD₄₅₀ at 30°C normalized against milligrams of cell protein and expressed as the percentage LDH content of control samples.

**Data analysis.** Enzymatic activity experiments were performed with triplicate determinations from separate protein extracts. Data are expressed as means from 2–3 experiments (n = 6–9 total determinations, ±SD) and analyzed for statistical significance by one-way ANOVA with Fisher’s protected least-significant difference test. Data were considered significantly different from controls at P < 0.05 and reported as P < 0.01.

**RESULTS**

We have established a system for studying the hypoxic regulation of the 32-kDa HO-1 protein using primary cultures of BAEC as a vascular cell model. In BAEC, a sustained hypoxia treatment induced steady-state HO-1 protein levels to a high degree compared with the virtually undetectable level in control (normoxic) BAEC cultures. The apparent induction of HO protein after hypoxia treatment was maintained during the reoxygenation phase and remained elevated at least 8 h postreoxygenation (Fig. 1).

Total heme oxygenase enzyme activity in cultured BAEC was induced five- to sixfold (922.50 ± 218.5 pmol·mg protein⁻¹·h⁻¹) over basal values (206.50 ± 57.50 pmol·mg⁻¹·protein⁻¹·h⁻¹) by a bolus of sodium m-arsenite (50 μM). A hypoxia treatment of 10 h increased total HO activity two- to threefold (541 ± 129 pmol·mg⁻¹·protein⁻¹·h⁻¹) over normoxic controls (Fig. 2). Varying the initial hypoxic interval produced comparable increases in HO activity in the range of 10–24 h (Fig. 2). After a 10-h hypoxic interval, total activity appeared to increase to an apparent maximum 2 h postreoxygenation (1,086 ± 383 pmol·mg⁻¹·protein⁻¹·h⁻¹) and remained elevated at least 3 h postreoxygenation (Fig. 3).

Addition of the ferric iron chelator DFO (100 μM) during the hypoxic phase or during the entire H/R treatment abolished the induction of HO protein (Fig. 4A) and activity (Fig. 4B). Inclusion of DFO during the reoxygenation phase only, however, did not interfere with the manifestation of HO activity by H/R treat-
Those observed after hypoxia, H/R, or iron treatment in elevations of HO activity severalfold higher than activity after the hypoxia or H/R treatments, resulting versus the PBS-pretreated normoxic control (199.8 ± 6 SD (n = 6–9); **P < 0.01.

Fig. 3. Effect of reoxygenation interval on HO activity in BAEC. BAEC were seeded, cultured, and refed as described for Fig. 2. Cells were subjected to sustained hypoxia in a minimal volume (3 ml) of deoxygenated growth media for 10 h followed by varying reoxygenation intervals (0–3 h). The 10-h sham-treated cells were subjected to 10 h incubation in 3 ml normoxic growth media. The 10-h to 2-h sham-treated cells were subjected to 10 h incubation in 3 ml normoxic growth media followed by replacement with 8 ml normoxic growth media for 2 h. Normoxic control cells were treated with 8 ml growth media for 10 h only. Cells were harvested for enzymatic activity determination. HO-activity values were expressed as means ± SD (n = 6–9); **P < 0.01.

Under normoxic conditions, the DFO (100 μM) neither stimulated HO activity nor affected basal HO activity (Fig. 4B). The DFO treatment (100 μM for 10 h) did not significantly affect cell viability under normoxic conditions as determined by the cellular content of LDH (data not shown).

Similar results were obtained using the ferrous iron chelator o-phenanthroline, which abolished the induction of HO-1 protein (Fig. 5A) and activity (Fig. 5B) when included during hypoxia. The o-phenanthroline (100 μM) did not affect HO activity under normoxic conditions. The DMSO vehicle also did not affect basal HO activity at the concentration used to deliver the chelator (0.1%). The o-phenanthroline (100 μM) did not significantly affect cell integrity under normoxic conditions (data not shown).

Because chelation of intracellular iron diminished the hypoxic response with respect to HO-1 activation, we examined the effect of iron supplementation on the response. The loading of endothelial cultures with Fe³⁺ (as ferric ammonium citrate-8-hydroxyquinoline) stimulated the expression of the HO-1 gene under normoxic conditions and amplified the induction of HO-1 in combination with the hypoxia (10 h) and H/R (10 h-2 h) protocols (Fig. 6A). The iron loading (5 μM) had a minimal effect on HO activity after a normoxic recovery period (10 h) (322 ± 38 pmol-mg⁻¹-protein⁻¹-h⁻¹) versus the PBS-pretreated normoxic control (199.8 ± 33 pmol-mg⁻¹-protein⁻¹-h⁻¹). Iron preloading had a dramatic synergistic effect on the manifestation of HO activity after the hypoxia or H/R treatments, resulting in elevations of HO activity severalfold higher than those observed after hypoxia, H/R, or iron treatment alone (Fig. 6B) under similar cell culture conditions (i.e., PBS pretreatment). The PBS pretreatment used for the sham and iron-loading conditions had the effect of diminishing the absolute HO induction response attainable by the hypoxia and H/R treatments. Nevertheless, the superinduction attainable by iron loading and H/R in combination is at least as great as that attainable by H/R under the conditions used in Fig. 3.

The inclusion of ALA (100 μM), the primary precursor for heme synthesis, during the hypoxia had a slight additive effect on the induction of HO activity after H/R. ALA, however, also weakly stimulated HO activity after a 10-h treatment under normoxic conditions (Fig. 7.)

The possibility that the inhibition of HO activity by DFO could be due to direct antioxidant effects of the chelator was examined. The inclusion of Fe³⁺ + DFO (100 μM) in the hypoxic phase did not decrease H/R-inducible HO activity (Fig. 8). Significant inhibition of
H/R-inducible HO activity was observed with the inclusion of NAC (100 μM and 10 mM) or quercetin (100 μM) in the hypoxic phase, but not with green tea polyphenolic EGCG at 100 μM (Fig. 8).

**DISCUSSION**

Hypoxia causes distinct alterations in the pattern of gene expression in cell culture systems, which may overlap with heat shock and glucose-regulated responses, depending on the cell culture system used (30, 50). However, induction of the 32-kDa protein HO-1 appears to be a ubiquitous hypoxic response in mammalian cells (22, 29, 30, 51). In this study we observed activation of HO-1 protein and activity by H/R in BAEC. The response began during the hypoxic phase and remained elevated during the reoxygenation phase.

HO-1, which participates in heme detoxification and iron redistribution (33) and potentially influences vasoregulatory processes (26), may play an important role in endothelial adaptation to hypoxia (10, 29). CO, which is released from heme by HO activity, has been shown to directly affect vascular tone (23, 43, 49). Previously Morita and colleagues (29) demonstrated that hypoxia induced HO-1 protein levels and activity in vascular smooth muscle. The accompanying increase in CO elevated cGMP in these cells and in separate cells subjected to conditioned media from the hypoxia-treated cells (29). An elevation in cGMP production regulates vascular processes, including the stimulation of vasodilation or the inhibition of platelet aggregation and smooth-muscle proliferation (10). An increase in the capacity for CO production relative to the capacity for NO production from endothelial cells during hypoxia may have importance in vascular signaling events leading to adaptive processes. In this

Fig. 5. Effect of o-phenanthroline (OP) on hypoxia-inducible HO activity in BAEC. BAEC were seeded, cultured, and refed as described for Fig. 2. Cells were subjected to sustained hypoxia in 3 ml deoxygenated growth media for 10 h followed by a 2-h reoxygenation interval. Sham-treated cells were subjected to 10 h in 3 ml normoxic growth media followed by replacement with 8 ml normoxic growth media for 2 h. Normoxic control cells were treated with 8 ml growth media for 10 h only. Cells were also treated with the ferrous iron chelator OP (100 μM), which was included either: 1) during hypoxia treatment, 2) during the entire H/R treatment, 3) during reoxygenation only, or 4) in normoxic controls for 10 h. Cells were harvested for Western immunoblot analysis (A) and enzymatic activity determination (B). HO activity values were expressed as means ± SD (n = 6); **P < 0.01 vs. normoxic control, *P < 0.01 vs. H/R.

H/R-inducible HO activity was observed with the inclusion of NAC (100 μM and 10 mM) or quercetin (100 μM) in the hypoxic phase, but not with green tea polyphenolic EGCG at 100 μM (Fig. 8).

Fig. 6. Effect of iron loading on hypoxia-inducible HO expression in BAEC. BAEC were seeded at 0.5 × 10^6 cells per 100-mm plate and cultured 3 days before assay. Cells were treated for 1 h in PBS containing 5 μM 8-hydroxyquinoline and 5 μM Fe^{3+} as ferric ammonium citrate. Cells were rinsed twice in PBS and subjected to either: 1) sustained hypoxia in 3 ml deoxygenated growth media for 10 h, 2) sustained hypoxia in 3 ml deoxygenated growth media for 10 h followed by a reoxygenation interval of 2 h, or 3) restoration to 3 ml complete growth media for 10 h under normoxic conditions. Sham-treated cells were incubated for 1 h in PBS, followed by 10 h in 3 ml normoxic growth media. Cells were harvested for Western immunoblot analysis (A) and enzymatic activity determination (B). HO activity values were expressed as means ± SD (n = 6); **P < 0.01, ***P < 0.001 vs. normoxic control, *P < 0.01 vs. corresponding treatments in absence of iron preload.
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regard, HO-1 may serve as an inducible vasoregulator response to ischemia or hypoxia. A potential role for HO-1 in vascular protection is illustrated by the observation that HO-1 null mice (HO-1−/−) develop right ventricular dilation and infarction relative to wild-type mice after a chronic hypoxia challenge (48). This study indicated that HO-1 may confer protection on cardiomyocytes against injury during chronic hypoxia and associated pulmonary hypertension, although the contributory role of CO remains unclear.

In addition to its putative vasoactive roles, HO may function to remove catalytic free heme, which may build up during stress conditions (15). It is not clear whether hypoxia increases endothelial intracellular free-heme levels or causes intravascular hemolysis. Furthermore, although bilirubin, an HO reaction product, has demonstrated serum antioxidant properties (42), it remains unclear if intracellular bilirubin production leads to a significant increase in cellular membrane antioxidant capacity.

Our study was designed to examine the role of intracellular iron in the regulation of the HO gene by hypoxia. The preloading of BAEC with ferric iron dramatically increased the induction of HO-1 protein levels by hypoxia or H/R. These results suggest that iron either enhances oxidative damage produced during hypoxia or increases a “regulatory” iron pool that is necessary for the induction of the gene. We have observed that the incorporation of PBS (instead of fresh growth media) during the prehypoxic period or the use of serum-free buffers (i.e., Krebs-Henseleit buffer, pH 7.4) during the hypoxic phase had the general effect of decreasing the magnitude of HO induction after H/R, which is likely due to a modulation of the cellular iron status. Despite the diminished HO-1 induction effect after H/R in cells pretreated with PBS, preincubation with iron restores the HO-1 effect to a value equal to or greater than that achieved by the H/R response under the standard conditions. In the comparison of Fig. 3 (H/R 10 h−2 h; 1,086 ± 383 pmol·mg−1·protein−1·h−1) with Fig. 6 (iron loading + H/R 10 h−2 h; 1,650 ± 694 pmol·mg−1·protein−1·h−1), the restoration of a half-maximal response to a maximal response by preaddition of Fe3+ supports the hypothesis that iron is involved in the induction mechanism.

At least three general mechanisms have been proposed for the role of iron in gene activation: 1) modulation of cellular redox state by promoting the generation of ROS, which in turn triggers redox-sensitive component(s) of signal transduction pathways including kinases, phosphatases, or specific transcription factors, leading to transcriptional regulation of the target gene; 2) posttranscriptional regulation of genes by direct interaction of iron with iron regulatory proteins, as exemplified by the regulation of ferritin and transferrin receptor synthesis; and 3) regulation of signal transduction by heme iron-containing proteins that serve as oxygen sensors (2, 7, 17, 50).

In our study, we show that inclusion of a chelator DFO during the hypoxic phase attenuates the HO-1 induction response; it is clear that the signal for HO-1 activation has occurred primarily during the hypoxic phase. The response is downregulated by both ferric...
and ferrous iron chelators present during hypoxia, suggesting that the presence of intracellular iron, rather than its steady-state valence, is the critical factor. Curiously, when incubated in the reoxygenation phase alone, o-phenanthroline apparently had a slight inhibitory effect on HO activity, suggesting that Fe$^{2+}$ could be important for any HO-1 synthesis occurring postreoxygenation. DFO is a water-soluble metal chelator that is thought to remain in the extracellular media, whereas o-phenanthroline is a lipophilic membrane-permeable iron chelator. Both chelators, whether intracellular or extracellular, restrict the potential of the bound iron from catalyzing Fenton-type reactions (16).

Cellular iron status has been shown previously to influence the regulation of several genes after exposure to oxidants under normoxic conditions, in particular, HO-1 after UVA radiation and H$_2$O$_2$ treatment (16) or matrix metalloproteinases after UVB radiation (6). In our system the activation of HO-1 during hypoxia bears an important resemblance to the activation of the gene by oxidants, because both phenomena can be downregulated by pretreatment or concurrent treatment with iron-chelating agents. HO-1 differs from most other hypoxia-inducible genes in that its induction responds to both high and low oxygen tensions.

Recent work using fluorescence probes has suggested that in cultured cardioocytes a substantial ROS production may occur during the hypoxic phase before reoxygenation (52). Murphy and co-workers (30) argued that hypoxia represents a prooxidant state and suggested that in cultured cardiocytes a substantial ROS production may occur during the hypoxic phase before reoxygenation. On the other hand, Semenza (38) has proposed a model that diminished rather than augmented production of ROS during hypoxia, which represents an antioxidant state and regulates the hypoxia-inducible factor (HIF-1) and associated gene-expression phenomena under hypoxia. Metal chelators such as DFO have been shown to activate HIF-1 under normoxic conditions, leading to the activation of several hypoxia-regulated genes, including erythropoietin, phosphoenolpyruvate carboxykinase I, and vascular epidermal growth factor (reviewed in ref. 7). In our studies, however, metal chelators attenuate HO-1 expression under hypoxia, but do not stimulate the expression of the gene under normoxic conditions, suggesting that DFO acts by removing “catalytic” or “regulatory” iron required for the activation of HO-1.

DFO has been suggested to directly scavenge reactive oxygen species including ·OH and O$_2^-$. (12). The inclusion of DFO (100 μM) saturated with equimolar Fe$^{3+}$ in the hypoxic phase did not decrease the hypoxic induction of HO expression. Furthermore, the inclusion of antioxidants such as EGCG and NAC at comparable concentrations had no inhibition or partial inhibition, respectively, on the hypoxic induction of HO activity. The significant inhibition by quercetin at 100 μM may be due in part to the metal-binding properties of this flavonoid. We conclude that the inhibitory effects of DFO are unlikely to be related to its direct antioxidant effects, but rather to the scavenging of reactive iron. The generation of ROS by chelatable iron during hypoxia cannot be excluded, however, from the induction mechanism, because NAC nearly completely inhibited HO expression during hypoxia at high concentrations (10 mM).

Other secondary effects of DFO such as inhibition of cell growth and DNA synthesis, although unlikely to be relevant to the current study, cannot be excluded from potential mechanisms of action (15). Because DFO cannot remove iron from heme groups, it is unlikely that DFO would directly interfere with a hemeprotein-based oxygen-sensing mechanism. However, DFO inhibits heme synthesis, which would have an indirect inhibitory effect on a hemoprotein-based mechanism. The stimulation of heme synthesis by ALA treatment in our studies produced a slight additive effect when included during the hypoxia treatment. However, the magnitude of the additive effect was not greater than that produced by the same concentration of ALA under normoxic conditions. It is likely that the ALA treatment stimulates HO-1 slightly by production of heme, which acts through a separate mechanism than that which regulates the hypoxic activation of the gene.

In summary, we have shown that prototypical metal-chelating agents can serve as pharmacological modulators of the vascular HO response to hypoxia. Natural plant flavonoids and polyphenolic compounds that exhibit metal-chelating properties could be used as a starting point in the development of strategies for nontoxic pharmacological manipulation of HO activity in vascular systems.

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