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# Forum Review

# Hypoxia and Carbon Monoxide in the Vasculature

STELLA KOUREMBANAS

## ABSTRACT

**Hypoxia is sensed by all mammalian cells and elicits a variety of adaptive and pathophysiological responses at the molecular and cellular level. For the pulmonary vasculature, hypoxia causes increased vasoconstriction and vessel-wall remodeling. These responses are mediated by complex intracellular cascades leading to altered gene expression and cell–cell interaction. Hypoxia transiently increases the transcriptional rate of the heme oxygenase-1 (HO-1) gene, resulting in increased production of carbon monoxide (CO) and bilirubin. CO has vasodilatory and antiinflammatory properties in the vasculature, whereas bilirubin is an antioxidant. Both enzymatic products could thus modulate the hypoxic cellular response. Accumulating data suggest that CO inhibits the hypoxic induction of genes encoding vasoconstrictors and smooth muscle cell mitogens in the early hypoxic phase. During chronic hypoxia, low CO levels tilt the balance toward increased production of growth factors and vasoconstrictors that promote vessel-wall remodeling. Mice null in the HO-1 gene manifest decreased tolerance to hypoxia with right ventricular dilatation and infarction, whereas targeted lung overexpression of HO-1 prevents hypoxia-induced inflammatory responses and protects against the development of pulmonary hypertension. Such observations point to CO as a critical modulator of the body's adaptive responses to hypoxia.** Antioxid. Redox Signal. 4, 291–299.

## INTRODUCTION

HYPOXIA is a physiologic regulator of important biological processes, including erythropoiesis, angiogenesis, glycolysis, and tissue remodeling. It appears that all cell types in the body are capable of "sensing" oxygen and can respond acutely with modification of protein(s) and alterations in redox state. Chronically, hypoxia induces changes in gene expression eliciting a variety of adaptive responses at the cellular and tissue level. In the pulmonary vasculature, acute hypoxia causes vasoconstriction with a rapid rise in pulmonary vascular resistance, whereas chronic hypoxia induces vascular remodeling characterized by smooth muscle proliferation and deposition of extracellular matrix. These structural changes are the hallmark of pulmonary hypertension. Other pathological conditions linked to hypoxia include blood stasis associated with venous insufficiency, tumor vascularization leading to tumor growth and metastasis, cerebral and myocardial ischemia, as well as chronic heart and lung diseases. These hypoxia-mediated pathologic conditions are the result of complex cell–cell and cell–matrix interactions regulated by growth factors, basement membrane components, metalloproteases, cytokines, and gas molecules that function as chemical or biological messengers. This review discusses the molecular mechanisms underlying these cellular interactions with an emphasis on the gas molecule, carbon monoxide (CO), as a key modulator of the hypoxic response.

## OXYGEN SENSING MECHANISMS

Recently, significant progress has been made in identifying the cellular responses to hypoxia and unraveling the precise mechanisms by which cells sense changes in oxygen ($O_2$) tension. One long-held view has been the heme-protein sensor hypothesis whereby hypoxia is detected by an al-

Department of Medicine, Division of Newborn Medicine, Children's Hospital, Department of Newborn Medicine, Brigham & Women's Hospital, and Department of Pediatrics, Harvard Medical School, Boston, MA 02115, U.S.A.

losteric shift in a heme-containing protein. In the presence of $O_2$, this protein can bind $O_2$ at a heme site attaining a "relaxed" configuration, whereas the absence of $O_2$ confers a "tense" configuration to the heme protein (13). This idea is supported by evidence that cobalt chloride induces the erythropoietin gene presumably by maintaining a tense configuration of the heme-protein, thus mimicking hypoxia. In contrast, CO, a molecule known to interact with heme groups, can inhibit the hypoxic induction of erythropoietin by behaving similar to $O_2$ and shifting the heme-protein to the relaxed configuration.

In recent years, reports have identified different $O_2$-sensitive ion channels that could function as $O_2$ sensors (34). However, it is unclear whether the channels themselves are responsive to low $O_2$ or whether nearby sensors transmit the hypoxic signal. Alternatively, NADPH oxidase has also been implicated in $O_2$ sensing. In the presence of $O_2$, this enzyme alters the redox state of the cell by the generation of superoxide, whereas under hypoxic conditions decreased superoxide production results in a more reduced cellular state. Thus, decreased levels of reactive oxygen species (ROS) would signal hypoxia. However, although transgenic mice lacking the $gp91^{phox}$ signaling subunit of NADPH oxidase demonstrated a decrease in ROS generation, the $K^+$ current and vasoconstrictor responses to hypoxia were not inhibited (2).

A more recent hypothesis proposes the mitochondrial electron transport chain complex IV (cytochrome *c* oxidase) to be the $O_2$ sensor via mitochondrial ROS production. Hypoxia inhibits the activity of cytochrome oxidase resulting in upstream electron release and generation of superoxide. In support of this theory, $\rho^\circ$ cells, which lack mitochondrial DNA and electron transport chain activity, do not generate ROS during hypoxia and do not up-regulate the expression of hypoxia-inducible genes (6). By genetic approaches utilizing *Drosophila melanogaster* as a model organism, Wingrove and O'Farrell (79) recently reported that acute and chronic responses to hypoxia are impaired in flies with a mutation in the cyclic GMP (cGMP)-dependent protein kinase gene and amplified in transgenic flies overexpressing nitric oxide synthase (NOS). These studies couple nitric oxide (NO) signaling and ROS generation in the cellular responses to hypoxia.

The most exciting discoveries about $O_2$ sensing were recently reported in *Science* by two groups (21, 22) who simultaneously identified a hypoxia-inducible factor (HIF)-specific prolyl hydroxylase enzyme to be responsible for the $O_2$-dependent regulation of the HIF transcription factor (discussed in the next section on hypoxia-regulated transcription factors). In the presence of $O_2$ and iron, this enzyme targets a highly conserved proline residue in human HIF-1α and attaches an OH group. Hydroxylation of this proline is both necessary and sufficient for the binding of the tumor suppressor protein, von Hippel–Lindau (VHL), which leads to HIF-1α ubiquitination and degradation under normoxia (18, 35, 50). The requirement for proline hydroxylase activity and subsequent binding to VHL would potentially explain the stabilization of HIF-1αunder hypoxia, as well as the reported findings that heavy metals that compete with iron and iron chelators mimic the hypoxic response in multiple systems. Regardless of the specific sensor(s), it is very clear that $O_2$ sensing is not restricted to specific cell types, but is a universal biological response necessary for survival and adaptation to hypoxia.

## HYPOXIA-REGULATED TRANSCRIPTION FACTORS

Hypoxia is well known to regulate the expression of several target genes. The key transcription factor mediating most of these hypoxic responses is HIF-1 (14, 72, 73). HIF-1 is a ubiquitous basic helix–loop–helix PAS heterodimer that is activated by hypoxia and is composed of two subunits, HIF-1α and HIF-1β or ARNT (74). The biological activity of HIF-1 is determined by the regulation of the HIF-1α subunit, which occurs primarily at the protein level. Under nonhypoxic conditions, HIF-1α is ubiquitinated and subject to proteosomal degradation (18, 25, 60). In renal carcinoma cells, this has been shown to occur via the function of the VHL tumor suppressor protein that binds HIF-1α and targets it for ubiquitination (50). As discussed above, recent reports describe a HIF-specific proline hydroxylase that modulate(s) HIF-1 by posttranslational modification in an oxygen-dependent manner. HIF-1 binding sites have been identified in the 5′ or 3′ flanking regions of genes encoding erythropoietin (63, 64), vascular endothelial growth factor (VEGF) (32), glucose transporter (9), endothelin-1 (ET-1) (17, 83), and several glycolytic enzymes (62, 73). This transcription factor, therefore, is central to hypoxic signaling and the control of vital processes, including erythropoiesis, angiogenesis, glycolysis, and the regulation of vascular tone.

Several other transcription factors have also been reported to regulate the expression of specific genes under hypoxia. These include activator protein-1 [tyrosine hydroxylase (49)], early growth response-1 (Egr-1) [tissue Factor (86)], high-mobility group I (Y) [cyclooxygenase-2 (23)], nuclear factor interleukin-6 [interleukin-6 (84, 85)], and nuclear factor-κB [cyclooxygenase-2 (61)]. Of these, Egr-1 is a zinc-finger transcription factor that is induced by hypoxia (87, 89) and up-regulates the expression of chemokines, procoagulant, and permeability-related genes in response to ischemic stress (88). Unlike HIF-1, which is primarily regulated by hypoxia at the posttranslational level, hypoxia induces Egr-1 gene transcription via the activation of an ets binding site-serum response element potentially involving the activity of the transcription factor, Elk-1 (87). Egr-1 plays a crucial role in tissue factor expression and fibrin deposition, thus promoting pulmonary vascular thrombosis under severe hypoxic conditions (6% $O_2$ exposure). Like Egr-1, HIF-1 may also play an important role in lung homeostasis. HIF-1 regulates the expression of growth factors such as ET-1 and VEGF under hypoxia and is itself regulated by the growth factors angiotensin II and platelet-derived growth factor (PDGF) under normoxic conditions (58). The growth-promoting actions of these factors have been implicated in pulmonary vascular cell proliferation and altered vessel-wall remodeling. Mice heterozygous null in HIF-1α have less severe pulmonary hypertension when exposed to chronic hypoxia (10% oxygen), suggesting a role for this transcription factor in pulmonary vascular disease under milder degrees of hypoxia (91).

## ENDOTHELIAL CELL RESPONSES TO HYPOXIA

One of the earliest effects of hypoxia on endothelial cells is to increase their adhesiveness for neutrophils (3, 40). Hypoxia induces a calcium-dependent exocytosis of the Weibel– Palade bodies leading to the release of von Willebrand factor and to the overexpression of P-selectin that sustains neutrophil binding to endothelium (54). This hypoxia-inducible effect and the changes in expression of several cell-surface molecules lead to the recruitment, rolling, adhesion, and activation of neutrophils in ischemic tissues [reviewed by Michiels *et al.* (39)].

With more prolonged hypoxia (hours to days), there is an increase in the transcriptional rate of PDGF-B (26) and ET-1 genes (27), both encoding potent vasoconstrictors. Hypoxia also increases the expression of the VEGF gene in endothelial cells (32, 48) and its receptor Flt-1 (11). The production of the extracellular matrix protein thrombospondin-1 is increased in human endothelial cells exposed to hypoxia (53), and basic fibroblast growth factor (bFGF) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) are released from intracellular stores and stimulate smooth muscle cell (SMC) proliferation (38). The production of the vasodilator NO is suppressed under hypoxic conditions through multiple mechanisms, including a decrease in the transcriptional rate of the endothelial NOS gene and destabilization of its mRNA (31, 37). Unlike endothelial NOS, inducible NOS expression is enhanced by hypoxia in pulmonary artery endothelial cells (52). Regardless of the inhibitory or stimulating effects of hypoxia on NOS gene expression, NO production has been shown to be reduced under hypoxic conditions (67). In conclusion, the increased production of mitogens combined with the suppression of endothelial NO production would be expected to enhance SMC growth and vascular remodeling. In the pulmonary vasculature, chronic hypoxic exposure induces medial hypertrophy of the pulmonary vessels (16, 29, 57, 71). Therefore, the imbalance of growth factors and vasodilators produced by the endothelial cells may promote pulmonary vascular remodeling under hypoxic conditions.

## SMOOTH MUSCLE CELL RESPONSES TO HYPOXIA

As mentioned above, all mammalian cells are capable of sensing oxygen. Indeed, SMC also respond to hypoxia, evident by hypoxia regulation of gene expression, as well as cell contractility. Acute hypoxia inhibits potassium ($K_v$) channel activity on SMC, resulting in cell depolarization and contraction (76). Chronic hypoxia also inhibits the mRNA expression of the voltage-gated $K^+$ channel $\alpha$ subunits in pulmonary artery SMC (75). Under hypoxic conditions, vascular SMC demonstrated a more pronounced proliferative response to mitogens such as ET-1 and PDGF-BB (45). Hypoxia has also been shown to prolong the lifespan of vascular SMC by inducing the activity of telomerase, resulting in telomere stabilization (42).

Hypoxia induced the phosphorylation of the telomerase catalytic component (TERT), resulting in nuclear translocation of this protein, increased telomerase activity, and significantly extended the replicative lifespan of vascular SMC with longer population doublings (42). Increased responsiveness to endothelial-derived mitogens and prolongation of vascular SMC lifespan under hypoxia are crucial processes underlying the pathogenesis of hypoxia-induced vascular remodeling and pulmonary hypertension. However, critical checks and balances are required to maintain vascular homeostasis and adaptation to hypoxia. To this end, vascular SMC also up-regulate the expression of the heme oxygenase-1 (HO-1) gene, which has multiple biological effects that are reviewed in this issue of *Antioxidants & Redox Signaling*. The role of HO-1 and its enzymatic products in maintaining vascular homeostasis with emphasis on its effects on the pulmonary vasculature under hypoxia will be reviewed in the next section.

## HEME OXYGENASE ENZYMATIC ACTIVITY AND GENE EXPRESSION

Heme oxygenase (HO) is the enzyme responsible for physiological heme degradation into equimolar amounts of biliverdin, CO, and iron. Biliverdin is converted to bilirubin by the enzyme biliverdin reductase, and both biliverdin and bilirubin have been shown to function as antioxidants (66). CO, similar to NO, is a gas molecule that can activate guanylyl cyclase, resulting in increased production of the second biological messenger, cGMP. More recently, CO has been shown to have cGMP-independent effects by directly activating 105pS $K_{ca}$ channels (24). It has been suggested that CO functions as a neurotransmitter in the brain (70) and as a coneurotransmitter with NO in the enteric nervous system (81). There are three known isoforms of HO (HO-1, HO-2, and HO-3) that are the products of unique genes. HO-1 is inducible by many diverse agents in virtually all cells (7), whereas HO-2 is constitutively expressed in most tissues and highly concentrated in the brain and testes (59, 68). HO-3 is also constitutively expressed and has no significant enzymatic activity, but instead functions as a heme-binding protein (36).

Exposure of aortic or pulmonary SMC to hypoxia significantly increases the transcriptional rate of the HO-1 gene (44). Of interest, depending on the vascular bed or the degree of hypoxia, endothelial cell expression of HO-1 is differentially regulated by hypoxia. For example, HO-1 mRNA levels are suppressed in hypoxic human umbilical vein endothelial cells (43, 47) and induced in aortic and pulmonary artery endothelial cells (15, 46). The increase in HO-1 in SMC was subsequently shown to be mediated by HIF-1 binding to the hypoxia response element at approximately −9 kb of the HO-1 promoter (30). However, this may be cell-specific or additional mechanisms may be operative in that HIF-1 does not transactivate the HO-1 gene in pulmonary artery endothelial cells (15), and a mutant Chinese hamster ovary-derived cell line defective in HIF-1$\alpha$ still maintained hypoxia inducibility of the HO-1 gene (80).

Exposure of vascular SMC to hypoxia results in significantly increased HO-1 activity as measured by bilirubin production in the cell microsomal fraction, by release of CO in

the media, and by increases in SMC cGMP content (44). However, this increase is transient, peaking at 15 h of hypoxia and returning to almost baseline levels by 48 h. This response may have important biological implications for vascular cell–cell interactions given the known effects of CO on hypoxic gene expression. Specifically, exogenous CO has been reported to inhibit the hypoxic induction of the erythropoietin gene (13) and formed the basis for the heme-protein sensor hypothesis described above. In addition, exogenous CO has been shown to inhibit the hypoxic induction of the VEGF (12), ET-1, and PDGF-B genes (28). Moreover, the accumulation of CO secondary to increased HO-1 activity at 24–48 h of hypoxia may be responsible for a negative feedback inhibition on HO-1 gene expression (44). CO may inhibit hypoxic gene expression by attenuating the DNA-binding activity of HIF-1 (19, 33), although CO has not been shown to alter HIF-1 production at low concentrations (5% CO) (33) as it does at high exposure (>80% CO) (19). Therefore, the SMC-derived CO may have inhibitory effects on endothelial cell gene expression by suppressing the induction of hypoxia-inducible genes through alteration of HIF-1 function.

## CO ALTERS ENDOTHELIAL CELL–SMOOTH MUSCLE CELL INTERACTIONS UNDER HYPOXIA

CO produced by vascular SMC can diffuse across cell membranes and lead to paracrine effects on adjacent endothelial cells. Indeed, in endothelial–SMC coculture experiments, SMC-derived CO inhibited the hypoxic induction of the endothelial-derived PDGF-B and ET-1 genes (43) and resulted in decreased production of these SMC mitogens with a secondary decrease in SMC proliferation in response to hypoxia (43, 45). An increase in ET-1 and PDGF-B production occurred only after 48 h of hypoxia when the HO-1 gene and its enzymatic products were no longer induced (43). Vascular SMC-derived CO also has direct autocrine effects on vascular SMC by inhibiting the expression of E2F-1, the cell cycle-specific transcription factor that regulates the transition of cells from the $G_1$ to S phase of the cell cycle (45). Therefore, although vascular SMC proliferate faster and have a longer lifespan under hypoxic conditions, their growth is suppressed by CO in the early phase (24 h) of hypoxia, but this effect is limited due to the transient induction of CO under hypoxia, summarized schematically in Fig. 1. These findings suggest that if CO production could be sustained at elevated levels under hypoxic conditions, the excess SMC proliferation would be inhibited, resulting in amelioration or prevention of hypoxia-induced vascular remodeling. This was shown in cell culture studies (43, 45) and can be tested *in vivo* using the rat and mouse models of hypoxic pulmonary hypertension.

## HO-1 MODULATES THE PULMONARY RESPONSES TO HYPOXIA

Exposure of adult rats to hypoxia (10% $O_2$ environment) resulted in a transient, but significant, threefold increase in lung HO-1 mRNA and protein levels that peaked at 9–15 h, but returned to baseline at 48 h (8), similar to the expression pattern in cultured hypoxic vascular SMC (44). Exposure of adult rats to an altitude chamber to achieve hypoxia resulted in increased HO-1 mRNA and its enzymatic activity (determined by bilirubin levels) at 1 day with a decrease by 3–14 days (4). Although HO-1 mRNA expression had a second peak at 21 days of exposure to high-altitude hypoxia, this was not associated with increased activity. Therefore, lung HO-1 activity is up-regulated in the *in vivo* lung during early chronic hypoxia. The lack of HO-1 activity with prolonged hypoxia may be permissive for pulmonary vascular proliferation.

Sustained induction of HO-1 expression in adult rat lungs achieved with serial injection of hemin or $NiCl_2$ inhibited the development of structural remodeling and pulmonary hypertension by chronic hypoxia (8). Interestingly, HO-1 null mice did not exhibit an exaggerated hypertensive state after 5–7 weeks of hypoxia, nor did they show signs of more severe vascular remodeling on morphometric studies when compared with the wild-type controls (90). However, these mice manifested a maladaptive response to hypoxia in response to elevated pulmonary vascular resistance with severe right ventricular dilatation and infarction (90). Whereas the left ventricle did not sustain any damage, the right ventricular injury was due to lipid peroxidation and oxidative injury of the right ventricular cardiomyocytes in HO $-/-$, but not in wild-type mice. As hypoxia has been shown to increase ROS production as discussed above and reviewed by Chandel and Schumacker (5), HO-1 activity may be protective under hypoxic conditions not only due to the vasodilating properties of CO, but also due to the antioxidant effects of bilirubin (66). HO-1 has been reported to be induced by oxidant stress (7) and to impart protection in this setting (51). Indeed, mice null in HO-1 manifest decreased tolerance to oxidant stress (55, 56), similar to the reported case of human HO-1 deficiency (82). The hypoxic studies using HO-1 null mice did not predict the protection imparted by targeted lung-specific HO-1 overexpression against the development of hypoxia-induced vascular remodeling. Lung-specific HO-1 overexpression ameliorated hypoxia-induced vasoconstriction and prevented right ventricular hypertrophy and pulmonary vascular remodeling up to 8 weeks of hypoxic exposure (41).

The above summarized studies point to a critical cytoprotective role of HO-1 activity in hypoxic and oxidant-induced injury, probably through overlapping pathways. HO-1 has also been shown to have antiinflammatory effects (reviewed by Dr. L.E. Otterbein in this issue). In studies using HO-1 null mice, expression of HO-1 was critical in determining cardiac xenograft survival (65) and survival from endotoxin-induced oxidative stress and end-organ dysfunction (78). Up-regulation of HO-1 also protected livers from genetically obese Zucker rats against *ex vivo* cold ischemia/reperfusion injury (1). More recently, HO $-/-$ mice exhibited lethal pulmonary ischemic injury compared with wild-type controls, but were rescued by inhaled CO (10). In this model, activation of guanylyl cyclase by CO with a direct increase in cGMP levels inhibited the hypoxic induction of plasminogen activator inhibitor-1, resulting in decreased fibrin deposition and lung inflammation. In the mouse model of hypoxic pulmonary hypertension, hypoxic wild-type mice manifested a

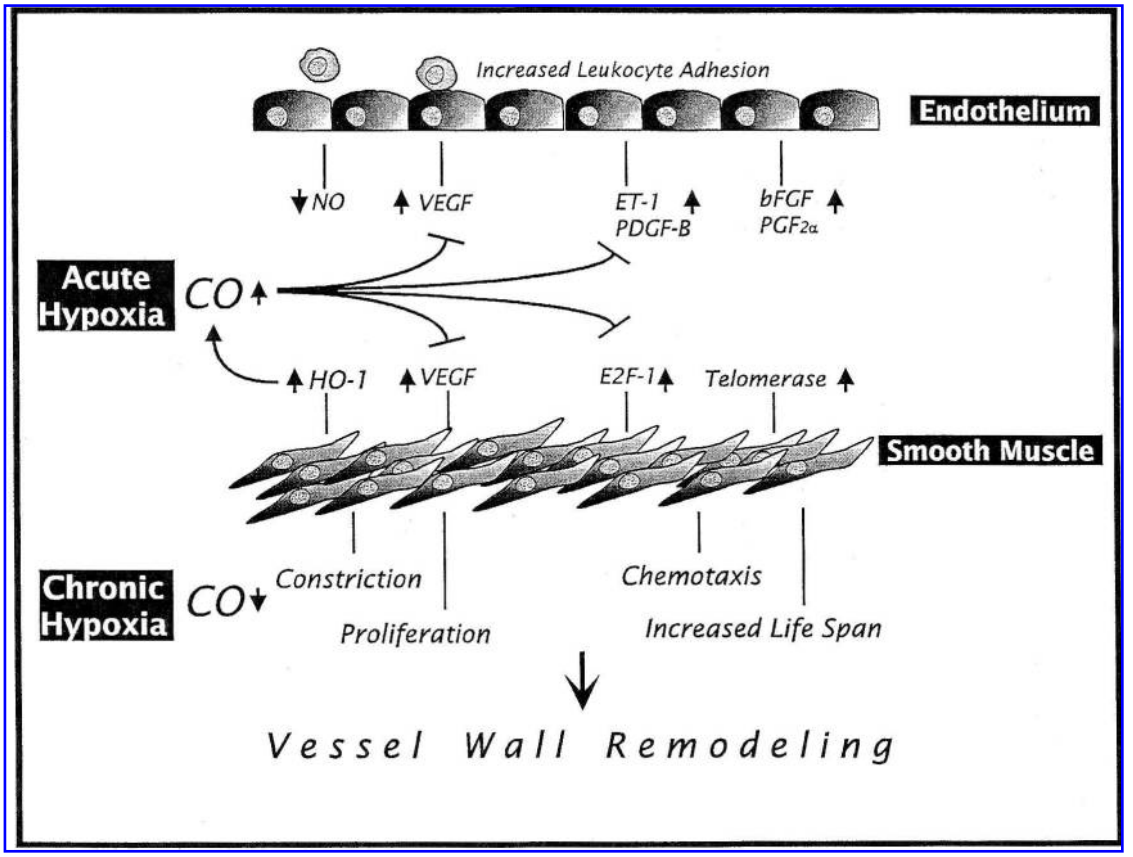


**FIG. 1. CO modulates cell–cell interactions in the vessel wall.** Transient increases in SMC-derived CO protect the vessel wall against hypoxia-induced SMC proliferation via autocrine and paracrine mechanisms. During chronic hypoxia, CO levels are low, shifting the balance toward increased production of SMC-mitogens, SMC-cycle progression, and telomere stabilization resulting in cellular proliferation and vessel-wall remodeling.

striking inflammatory response in the lung parenchyma prior to the development of vascular remodeling. In contrast, HO-1 overexpressing mice had no inflammatory cell infiltration and revealed significantly reduced expression of cytokines and chemokines in response to hypoxia (41). Therefore, HO-1 is a multifaceted enzyme whose activity modulates hyperoxic, hypoxic, and proinflammatory responses via pathways that have not yet been fully characterized.

As a product of HO-1 activity, CO can also have toxic effects that may be tissue- and dose-dependent. For example, in neonatal mouse lungs, HO-1 gene transfer exacerbated lung injury as measured by protein carbonyls and 8-isoprotanes (77). CO exposure at 10–110 ppm induced NO-mediated endothelial cell apoptosis (69), and high levels of CO resulted in pronounced perivascular injury in the brain of rats due to increased peroxynitrite and NO levels (20). These findings point to potential injurious effects of CO, as well as a cross-talk between NO and CO that may regulate tissue responses to enhanced HO-1 activity (reviewed by Dr. L. Hartsfield in this issue).

## PERSPECTIVES

An abundance of data has accumulated in the past decade to support the notion that, as one of the enzymatic products of HO-1 activity, CO is a novel signaling molecule critical to the maintenance of cellular homeostasis: CO regulates cGMP production, modulates gene expression under hypoxia, and may have potent antiinflammatory properties that are just beginning to be unraveled. Hypoxia has been shown to play a more global role in human diseases, including ischemic cardiovascular disorders, pulmonary hypertension, chronic lung diseases of infancy and adulthood, angiogenesis, and cancer. The rapidly growing evidence that CO and bilirubin (the other enzymatic product of HO-1) are modulator(s) of hypoxic signaling pathways renders HO-1 a key therapeutic target of these biological processes.

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## ABBREVIATIONS

bFGF, basic fibroblast growth factor; cGMP, cyclic GMP; CO, carbon monoxide; Egr-1, early growth response-1; ET-1, endothelin-1; HIF, hypoxia-inducible factor; HO, heme

oxygenase; NO, nitric oxide; NOS, nitric oxide synthase; PDGF, platelet-derived growth factor; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; ROS, reactive oxygen species; SMC, smooth muscle cell(s); VEGF, vascular endothelial growth factor; VHL, von Hippel–Lindau.

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Address reprint requests:
*Stella Kourembanas, M.D.*
*Division of Newborn Medicine*
*Children's Hospital, Enders 9*
*300 Longwood Avenue*
*Boston, MA 02115*

*E-mail:* stella.kourembanas@tch.harvard.edu

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