ANTIOXIDANTS & REDOX SIGNALING
Volume 21, Number 13, 2014

DOI: 10.1089/ars.2013.5791

Discoveries

FORUM REVIEW ARTICLE

Heme Oxygenase in Neonatal Lung Injury and Repair

Phyllis A. Dennery

Abstract

Significance: Premature and sick neonates are often exposed to high concentrations of oxygen, which results in lung injury and long-term adverse consequences. Nevertheless, neonates are more tolerant to hyperoxia than are adults. This may be, in part, explained by the high lung content of heme oxygenase-1 (HO-1), the rate-limiting enzyme in the degradation of heme and an important stress protein. The abundance of HO-1 dictates its cytoprotective and deleterious effects. Interestingly, in response to hyperoxia, lung HO-1 mRNA is not further up-regulated in neonates, suggesting that lung HO-1 gene expression is tightly regulated so as to optimize cytoprotection when faced with an oxidative stress such as hyperoxia. ***Recent Advances:*** In addition to the lack of induction of HO-1 mRNA, neonatal lung HO-1 protein is observed in the nucleus in neonatal mice exposed to hyperoxia but not in adults, which is further evidence for the developmental regulation of HO-1. Nuclear HO-1 had unique properties independent of its enzymatic activity. In addition, there has been increasing evidence that nuclear HO-1 contributes to cellular proliferation and malignant transformation in several human cancers. ***Critical Issues:*** Since HO-1 has dual effects in cytoprotection and cellular proliferation, the titration of HO-1 effects is critical to ensure beneficial actions against oxidative stress. ***Future Directions:*** Much more has to be understood about the specific roles of HO-1 so as to manipulate its abundance and/or nuclear migration to maximize the therapeutic benefit of this pleiotropic protein in the neonatal lung. *Antioxid. Redox Signal.* 21, 1881–1892.

Introduction

SICK AND PREMATURE newborns are often exposed to high concentrations of oxygen, which leads to arrested alveolar and vascular development as seen in bronchopulmonary dysplasia (BPD) (6, 44, 45, 102), This has long-term implications for lung function in adolescence and adulthood, and it also impacts neurodevelopmental outcomes (26, 27, 34, 111, 112). The lung injury observed in BPD results, in part, from reactive oxygen species (ROS), which damage DNA and other molecules (7, 12, 14). Fortunately, antioxidant responses have evolved and protect against ROS, including heme oxygenase (HO), a stress protein that degrades heme to biliverdin. There are two forms of HO that have different roles: different regulation and different post-translational modifications (85). HO-1 is highly inducible in inflammation and hyperoxia among other stressors and has multiple transcriptional factor binding sites that regulate its induction with oxidative stresses (3, 56). Although HO is an integral protein of the smooth endoplasm reticulum, it can localize to other compartments, including caveolae (43, 47, 50), mitochondria (9, 19), and the nucleus, where it can mediate signaling functions (61).

This review will focus on how HO-1, through its pleiotropic effects, modulates lung injury and repair, and will describe how the regulation and expression of HO-1 is unique in the neonatal lung.

Oxidative Stress and Neonatal Lung Injury

The lung continues to develop in complexity and size throughout the postnatal period and into early childhood. At birth, the transition to air breathing and away from the low oxygen tension provided by the placental circulation represents an oxidative stress. In preterm human neonates, exposure to hyperoxia, inflammation, and ventilation at this critical time in development results in disruption of the normal developmental process in the vasculature, mesenchyme, and alveolar structure of the lung. This leads to a disruption of angiogenesis, increased fibroblast proliferation, and arrested

Department of Pediatrics, University of Pennsylvania, Philadelphia, Pennsylvania.

alveolar development, resulting in abnormal lung architecture with decreased ventilation/perfusion matching and impaired pulmonary compliance characteristic of BPD. In the mouse, high concentrations of oxygen alone can result in decreased cell proliferation, decreased pulmonary compliance, and altered architecture (109) as in BPD. ROS produced during hyperoxia cause DNA strand breaks and other chromosomal aberrations (7, 12, 14), which stimulate the expression of the genes involved in inhibiting cell cycle-progression (95). The majority of strand breaks occur in small airway epithelial (Type II) cells (84, 116). This can result in the simplification of alveoli as seen in BPD. In addition, the DNA strand breaks from hyperoxia lead to activation of the ataxia telangiectasia mutant (ATM)-related protein kinase-dependent p53 phosphorylation (53), which can result in either arrest or induction of transcription, induction of signal transduction pathways such as the serine threonine kinase AKT and phospho-Extracellular signal-regulated kinase (p-ERK), replication errors, and genomic instability, all of which are seen in carcinogenesis (1, 46) (Fig. 1).

Increased ROS also result in the activation of NF-E2-related factor 2 (Nrf2)-mediated pathways, which are a hallmark of the oxidative stress response, leading to the up-regulation of antioxidant defenses (16, 65, 83). This activation can also change metabolic signaling, resulting in the up-regulation of glucose-6-phosphate dehydrogenase (G6PDH) (103), the rate-limiting enzyme of the pentose phosphate shunt (PPS). With the latter, glucose is oxidized and nicotinamide adenine dinucleotide phosphate (NADPH) is produced, which provides reducing equivalents that detoxify ROS. In addition, the PPS facilitates the generation of ribose for the synthesis of macromolecules (Fig. 1). Although protective against oxidative stress, this response may enable the rapid proliferation of cancer cells even in adverse environments (10, 25).

Overall, oxidative stress in the lung affects the particularly vulnerable endothelial cells as well as alveolar type II cells, which are important in the recovery from lung injury. This leads to arrested alveolar development (6, 44, 45) as well as to the disruption of angiogenesis (102), which are characteristics of BPD. Protective responses against all aspects of this disease could mitigate this disease process. This article will explore the multiple roles of the antioxidant molecule HO in this process.

Important Aspects of HO-1 Regulation in Oxidative Stress

Induction of HO-1 is a generalized response to oxidative stress

There are two isoforms of HO. The constitutive form, HO-2 is found in abundance in the testes and brain and can be regulated by glucocorticoids *via* a glucocorticoid response element, but it is not readily inducible during oxidative stress (82). It plays a role in various signaling processes and neurotransmission. We have shown that HO-2 null mutant mice have increased evidence of oxidative stress after exposure to hyperoxia and that they accumulate reactive iron in the lung tissues, which exacerbates oxidative injury (22), proving that in neonatal animals, HO-2 also represents an important, albeit noninducible oxidative defense against hyperoxia.

The inducible isoform, HO-1, responds to the most oxidant stresses. This occurs *via* binding of the Nrf2/small Maf protein complex to the Maf recognition sites (multiple antioxidant response element [MARE]) (2, 3) (Fig. 2). Competitive binding between Nrf2 and heterodimer of BTB and CNC homology 1 (Bach1) at the MARE is important in down-regulating HO-1 expression (51, 98). Recently, transforming growth factor (TGF)-β was seen to suppress the transcriptional activation of HO-1 through Nrf2-independent mechanisms. In fact, TGF-β did not affect the stabilization or

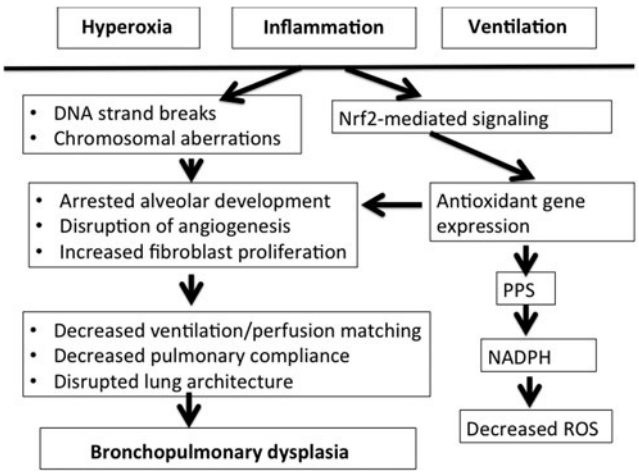

FIG. 1. Pathophysiology of bronchopulmonary dysplasia (BPD). In the neonatal lung, hyperoxia, ventilation and inflammation contribute to changes in cellular function, leading to blunted repair and persistent distortion in lung architecture. In addition, oxidative-mediated signaling *via* NF-E2-related factor 2 (Nrf2) results in activation of the pentose phosphate shunt (PPS) with resultant conversion of NADP to NADPH. This provides reducing equivalents to detoxify reactive oxygen species (ROS).

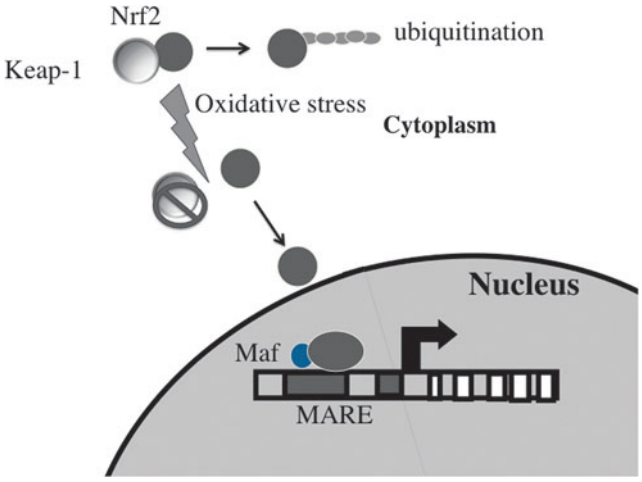

FIG. 2. Activation of the Nrf2 pathway with oxidative stress. Nrf2 is sequestered in the cytoplasm with Keap-1, facilitating its ubiquination and subsequent degradation. With oxidative stress, Nrf2 is released and migrates to the nucleus, where it binds to multiple antioxidant response elements (MARE) on heme oxygenase (HO)-1 and other genes to mediate gene transcription. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

nuclear accumulation of Nrf2 but induced the expression of Maf K and Bach1, which suppresses HO-1 transcription despite the accumulation of Nrf2 in the nucleus. Knockdown of Maf-1 and Bach1 abolished the TGF-β-dependent suppression of HO-1 through the substitution of Nrf2 for Bach1 on the MARE of HO-1 (72). Other binding sites found on the proximal and distal enhancer regions of the HO-1 promoter include STAT and NF-KB, which also regulate HO-1 gene transcription (58).

It is important to note that the Nrf2 signaling pathway not only results in HO-1 induction but also drives the expression of enzymes such as NAD(P)H:quinone reductase, an important electrophile-detoxifying enzyme, and G6PDH, the rate-limiting step in the PPS, thereby enhancing the generation of NADPH and reducing the generation of ROS (66). Nevertheless, the same genes downstream of Nrf2 may alter cellular metabolic fate and enable lung cancer cells to grow more rapidly (25). This will be discussed briefly in a later section.

How the enzymatic functions of HO-1 influence oxidative stress

HO enables the cleavage of heme specifically at the alpha-methene bridge of the molecule, in a multistep manner. Iron is then reduced to its ferric state through the action of cytochrome cP450 reductase. Carbon monoxide (CO) is released by elimination of the alpha methylene bridge of the porphyrin ring (Fig. 3). Each byproduct of HO is considered as having a significant signaling or cytoprotective function. Heme is a pro-oxidant molecule that can participate in the formation of oxidative radicals, leading to oxidative injury. Therefore, the sequestration of heme and the subsequent degradation by HO has antioxidant benefits. CO has important biological roles, including neurotransmission, vasodilation, and signal transduction. This product is unique to the HO reaction. Currently, the use of CO-releasing molecules (CORM) is being investigated as a cytoprotective strategy in several clinical models (37, 41, 52, 55). Biliverdin is an important antioxidant that can prevent lipid peroxidation (110). This compound does not accumulate endogenously and is rapidly converted to bilirubin by biliverdin reductase. Bilirubin is documented to decrease lipid peroxidation even better than vitamin E (97). Overall, by sequestering heme and forming antioxidant and bioreactive molecules, HO and bilirubin can lead to cytoprotection against oxidative injury. Despite the antioxidant benefits of HO-1 byproducts, there are examples where even an inactive form of HO-1 is cytoprotective (38). The exact mechanisms for this effect are not yet well characterized.

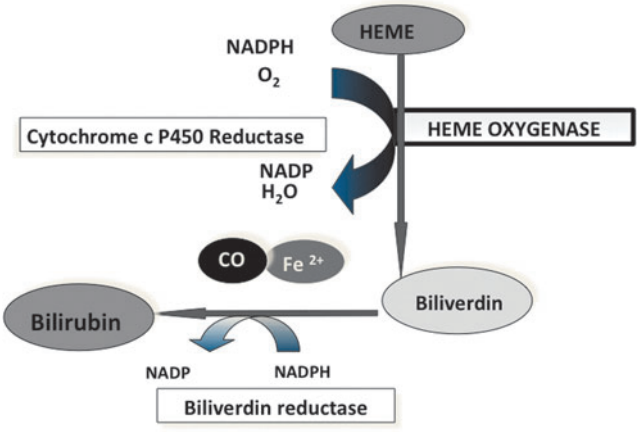

FIG. 3. Catalytic reaction of HO. Heme is degraded in an energy requiring process to biliverdin. This is then converted to bilirubin by the nonrate limiting biliverdin reductase. Iron (Fe) and carbon monoxide (CO) are released in equimolar amounts. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Subcellular localization of HO-1 and implications for oxidative stress

Although HO-1 is predominantly found in the smooth endoplasmic reticulum, where it is anchored at its c-terminus, it has been identified in the nuclear compartment and the nuclear envelope (33, 61, 73, 86) as well as in the mitochondria (19, 93) and caveolae (43, 47, 50).

In the mitochondria, a 27 kD HO-1 immunoreactive fragment was increased in a model of hepatotoxicity. The induction of mitochondrial HO-1 improved respiration and prevented a further drop in ATP levels (70). In cultured A549 cells as well as in primary small airway and epithelial cell cultures, mitochondrial localization was observed. In addition, *in vivo*, after exposure to cigarette smoke, this phenomenon occurred. The over-expression of HO-1 inhibited cigarette smoke-induced cell death and preserved cellular ATP levels (93). Therefore, the compartmentalization of HO-1 in the mitochondria may help protect against cigarette smoke-induced cell death. This form of HO-1 increases mitochondrial heme turnover, preserves liver ATP levels and energy metabolism (19). The mechanism by which HO-1 migrates to the mitochondria is not known, and no mitochondrial targeting sequence has been found on the HO-1 protein. The process may involve deletion of the C-terminus (93).

When HO-1 was over-expressed, it could be recovered in a detergent-resistant fraction containing caveolin-1 and was found in plasma membrane, cytosol, and isolated caveolae. Caveolin-1 physically interacted with HO-1, and HO activity increased in cells expressing caveolin-1 antisense transcripts, suggesting a negative regulatory role for caveolin-1 in the expression of HO-1 (50). Others also confirmed the binding of HO-1 protein to caveolin-1 using immuno-precipitation experiments (43, 47). In addition, HO-1 activity can be inhibited by caveolin-1. This binding occurred in the caveolin scaffolding domain, which plays an essential role in caveolin-related protein–protein interactions. The inhibition of HO activity by caveolin was correlated inversely to hemin concentration, suggesting that caveolin and hemin share a common binding site on the HO-1 protein (100). The proper distribution of HO-1 to caveolae appears to be required for normal toll-like receptor (TLR) signaling in response to inflammation (117).

With exposure to hypoxia and to hemin, we observed a faster migrating HO-1 immunoreactive band, which was enriched in nuclear extracts, suggesting that HO-1 could be cleaved to enables nuclear entry (61). The absence of HO-1 immunoreactive signal with an antibody against the C-terminus confirmed this as did the absence of a C-terminal sequence by gas chromatography/mass spectrometry. Furthermore, nuclear entry could be prevented by preincubation with a cysteine protease inhibitor, demonstrating the necessity for protease-mediated C-terminal cleavage for the nuclear transport of HO-1 (61). Nuclear localization was also

associated with a reduction of HO activity (61). We also demonstrated that nuclear HO-1 regulates the activation of various transcription factors, including AP-1, an important mediator of the antioxidant response (61). In preliminary work, we document the activation of Nrf2 by HO-1 protein and the lack of ARE activation in HO-1 null mutant mouse embryonic fibroblast (MEF) cells transfected with an ARE-driven luciferase reporter, further indicating that not only does Nrf2 regulate HO-1 but also HO-1, in turn, regulates Nrf2 (Biswas, unpublished observations, 2013). Interestingly, despite reduced activity, nuclear HO-1 protected cells against hydrogen peroxide-mediated toxicity and prevented oxidative DNA damage in HO-1 null mutant MEF cells stably infected with nuclear HO-1 that were exposed to hyperoxia (61). The effects of nuclear HO-1 appear to be cell specific. After exposure to hyperoxia, cultured tracheal smooth muscle cells that were recovered from aborted human fetuses showed nuclear distribution of HO-1 only when they were not proliferating (73) Our studies reveal that neonatal mice do not induce HO-1 but have increased nuclear HO-1 in response to hyperoxia in the acute phase of hyperoxic exposure, but this nuclear expression does not persist during room air recovery (Yang, unpublished observations). This pattern of over-expression may be beneficial, because adults that induce lung HO-1 and do not demonstrate nuclear localization are more susceptible to oxidative stress. Corroborating this, mouse HO-1 null mutant MEFs with stable over-expression of nuclear HO-1 show decreased cellular proliferation and are relatively tolerant to 24 h of hyperoxia compared with MEFs expressing cytoplasmic HO-1 or empty vector controls (Fernando, unpublished observations). Intriguingly, transgenic mice over-expressing nuclear HO-1 in type II cells initially showed improved alveolarization with (3 days) hyperoxic exposure but had increased oxidative DNA damage and abnormal lung histology and pulmonary function tests as adults (69), raising the possibility that the duration of nuclear HO-1 protein signaling is key to cytoprotective responses to injury and repair.

Overall, these observations suggest that the level, localization, and duration of expression of HO-1 may be extremely important in determining its cytoprotective and proliferative effects.

Physiologic Effects of HO-1 That Influence Oxidative Lung Injury

To mitigate neonatal lung injury and enhance repair, HO would need to prevent the key pathologic aspects of BPD, namely increased oxidative stress, increased inflammation, disrupted vascular development, and disrupted alveolarization. All of these roles have been documented for HO-1, in particular. In addition, maladaptive consequences of the proliferative actions of HO-1 have been observed

HO-1 abundance and localization alters cellular differentiation and proliferation

Although cell proliferation is disrupted by hyperoxia, others have suggested that decreased cell proliferation may be beneficial in acute hyperoxic injury (57, 71). Nevertheless, long-term suppression of cell proliferation could lead to arrested lung development. *In vivo*, disruption of HO-1 in the neonatal mice had little effect at 3 days or exposure (23), but when the animals were allowed to recover in air for 11 days, they had significant dysregulation of cell-cycle gene expression compared with similarly exposed wild-type (WT) (115). *In vitro,* tracheal smooth muscle cells from human fetuses exposed to hyperoxia showed nuclear distribution of HO-1 only when they were in a nonproliferative state (73). These results are in agreement with several other publications showing that HO-1 is anti-proliferative in smooth muscle cells (59, 75). In contrast, in epithelial cells, HO activity is associated with pro-proliferative effects (18) and, in endothelial cells, knockdown of HO-1 suppressed proliferation (118). HO-1 also influences naive T-cell homeostatic proliferation (13) and in the differentiation of induced pluripotent stem cells in response to oxidative stress (60), as well as Wnt signaling-mediated differentiation of preadipocytes to adipocytes. (106).

To maximize the cytoprotective effects HO-1, one should account for the specific effects of its subcellular localization and expression levels. To this effect, lung (Type II cell)-specific transgenic mice expressing high or low levels of full-length HO-1 (cytoplasmic) or C-terminally truncated HO-1 (nuclear) were generated (69). Mice were exposed to hyperoxia for 3 days as neonates and then allowed to recover in room air for approximately 8 weeks. During recovery from hyperoxia, the mice expressing low levels of full-length HO-1 had normal alveoli and minimal oxidative damage, whereas those expressing high levels of HO-1 had increased alveolar wall thickness with type II cell hyperproliferation, worsened pulmonary function, and evidence of abnormal lung cell hyperproliferation at 8 weeks of age. In the mice expressing C-terminally truncated HO-1 in the nucleus, increased lung DNA oxidative damage, increased poly (ADP-ribose) polymerase protein expression, and reduced poly (ADP-ribose) hydrolysis as well as reduced pulmonary function were observed during recovery from hyperoxia. This demonstrates that low cytoplasmic levels of HO-1 protect against hyperoxia-induced lung injury by attenuating oxidative stress, whereas high cytoplasmic levels worsen lung injury by increasing type II cell proliferation, alveolar wall thickness, thereby impeding gas exchange. Enhanced lung nuclear HO-1 impairs recovery by disabling poly (ADP-ribose)-dependent regulation of DNA repair (69). Interestingly, when HO-1 null mutant mice exposed to hyperoxia as neonates were evaluated after 11 days of recovery in room air, these mice exhibited significant changes in lung alveolarization and altered expression of genes which were important in cell proliferation and DNA damage (115). With regard to fibroblasts' myofibroblast proliferation, myocardial infarct was reduced with both *in vivo* and *in vitro* HO-1 over-expression (62) Overall, as a regulator of cell proliferation and differentiation, moderate levels of HO-1 could have a significant impact on lung injury and repair processes in hyperoxia. Perhaps in the neonatal lung exposed to hyperoxia, basal moderate over-expression of HO-1 in epithelial and endothelial cells promotes their proliferation; whereas it suppresses the over-proliferation of smooth muscle cells and fibroblasts, thereby maintaining lung vascularization and alveolarization while suppressing pulmonary hypertension and fibrosis during the repair phase. This would mitigate the phenotypic changes of BPD (Fig. 4).

HO-1 reduces inflammation

Through the degradation of heme, HO may have cytoprotective effects against systemic infections (Fig. 5). The

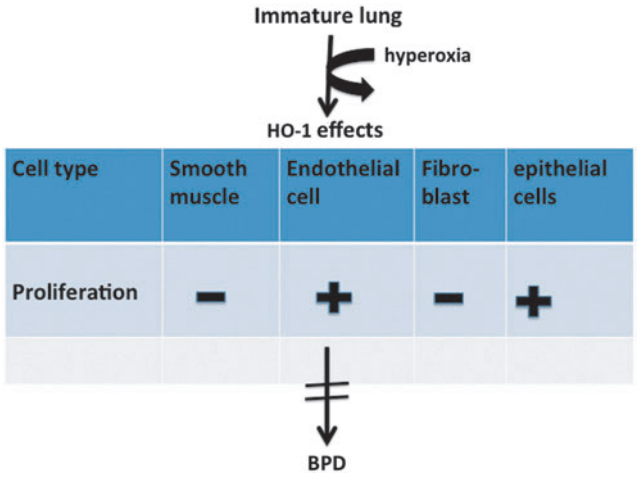

FIG. 4. Effects of HO-1 on cell proliferation. Increased proliferation is shown by a +, decreased proliferation by a −, based on existing literature. In the developing lung exposed to hyperoxia, the net effect of HO-1 on the different cell lineages prevents the phenotypic changes of BPD. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

regulation of HO-1 expression in macrophages was strictly required for protection against mycobacterial infection in mice, and HO-1-deficient mice were more susceptible to intravenous mycobacterium avium infections, and failed to mount a protective granulomatous response in mice lacking mature B cells (91). In a mouse model of noneosinophilic asthma, HO-1 provided anti-inflammatory effects by inhibiting the p-STAT3-RORγt pathway (120). HO can also work along with the scaffold protein caveolin-1 and negatively regulate TLR-4 signaling. In LPS-challenged cystic fibrosis macrophages, HO-1 accumulated intracellularly. The over-expression of HO-1 or the stimulation of CO release with a CORM enhanced caveolin-1 expression in the macrophages and re-established HO-1 cell surface localization,

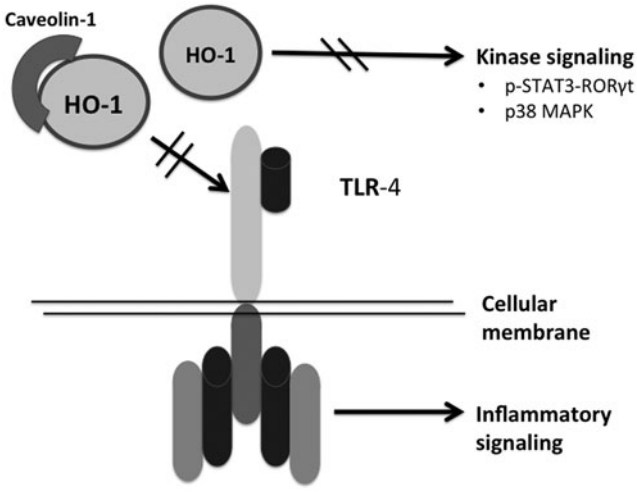

FIG. 5. Mechanisms by which HO-1 influences inflammation. HO-1 can bind to caveolin to prevent toll-like receptor (TLR)-4 signaling. In addition, the activation of p-STAT3-RORγ and p38 MAP kinase signaling is reduced by HO-1, which also dampens inflammatory responses.

which restored the normal TLR signaling pattern (119). Mice deficient in Irak-M, an important regulator of TLR-4, that were exposed to 95% oxygen had reduced mortality compared with WT mice, and this was associated with increased expression of HO-1 and Nrf2 (5). Treatment of the mice *in vivo* and incubation of cells *in vitro* with metalloporphyrins that suppress HO activity decreased survival and reduced the number of live cells after hypoxic exposure; this attenuated anti-inflammatrory cytokines, including interleukin-10, and up-regulated pro-inflammatory cytokines (4). In a premature lamb model, intra-amniotic endotoxin decreased lung caveolin-1 expression. This was associated with increased expression of HO-1 (54). It was not obvious whether this induction of HO-1 later resulted in improved lung histology or function. The HO-1 null mice developed by Poss and colleagues (77, 78) show evidence of oxidative damage and chronic inflammation. Surprisingly, the exposure of neonatal (< 12 h old) HO-1 null mice to hyperoxia did not result in increased lung inflammation compared with WT littermates, suggesting developmental differences in the effects of HO-1 (23).

Since inflammation is an important component of BPD, abundance and caveolar localization of HO-1 may serve to mitigate this disease process.

Multiple roles of HO-1 in angiogenesis and vascular proliferation

There is significant evidence in both humans and animals that the disruption of lung vascular development disrupts alveolarization. *In vitro*, transfection with the human HO-1 gene increased blood vessel formation (24). In endothelial cells, hemodynamically relevant cyclic strain stimulated HO-1 gene expression and inhibited cell death (63). *In vivo*, lentiviral vectors with microRNA sequences controlled by vascular endothelium cadherin were used to study the role of lung endothelial HO-1 in mice exposed to hyperoxia (119). When HO-1 was knocked down by 55% in the lung endothelium, there was a twofold increase in apoptosis and ROS generation, and this had the same effect on lung injury and survival as silencing HO-1 in multiple lung cell types. Furthermore, HO-1 regulated caspase 3 activation and autophagy in the endothelium during hyperoxia (119). Pulmonary inflammation arterial remodeling and right ventricular hypertrophy were attenuated in transgenic mice over-expressing HO-1 in Type II cells exposed to hyperoxia. Type II cell-specific over-expression of HO-1 also reduced hyperoxia-mediated pulmonary edema, hemosiderosis and prevented the loss of blood vessels observed in similarly exposed WT animals (28). Interestingly, lung-specific HO-1 over-expression neither prevented alveolar simplification nor altered ferritin and lactoferrin levels in this model, suggesting that HO-1 over-expression primarily protects the vascular system through iron-independent antioxidant and anti-inflammatory pathways (28). In summary, HO-1 plays an important vasculoprotective role in the lung, and this could be beneficial for preventing BPD.

Clues of the role of HO-1 in lung cytoprotective defenses in humans

So far, there have been only two reported cases of HO-1 deficiency in humans. A 6-year-old boy with severe growth

restriction was evaluated for persistent hemolytic anemia with a paradoxical absence of hyperbilirubinemia. He also suffered from severe endothelial damage as well as from iron deposition in his kidneys and liver. Sequence analysis revealed complete loss of exon 2 of the maternal allele and a two-nucleotide deletion within exon 3 of the paternal allele for HO-1 (113). Another case of human HO-1 deficiency was recently reported in a 12 year-old girl with congenital asplenia, who presented with severe hemolysis, inflammation, and nephritis, refractory to therapy. Mutation analysis showed homozygous missense mutations in exon 2 on chromosome 22q12, which would result in the absence of the functional HO-1 protein. Furthermore, the patient's kidneys were devoid of HO-1 immunostain (81). These two cases show common phenotypes involving inflammation, hemosiderosis, and oxidative stress. There are no reports of abnormal lung function in these patients. Perhaps a second insult would be needed to unmask the lung phenotype.

Several HO-1 promoter polymorphisms have been documented in lung diseases (30, 36, 90). Since GT dinucleotide repeats in the 5″ flanking region of the human HO-1 gene can modulate its transcription, differences in GT repeat length could alter HO activity (Fig. 6). The frequency of longer repeat alleles was significantly higher in the smokers with chronic pulmonary emphysema than in smokers without it, suggesting that diminished HO-1 promoter activation was associated with increased susceptibility to emphysema (114). *In vitro* studies showed that hydrogen peroxide induced HO-1 only in the short and medium promoter repeats, suggesting that longer GT repeats prevent HO-1 induction. Other functional polymorphisms were tested as well. No association between the various single nucleotide polymorphisms of HO-1 and lung function decline could be found, nor was there any evidence that three promoter polymorphisms affected the regulation of HO-1 gene (101). In 44 asbestos-exposed subjects without mesothelioma and 78 asbestos-exposed subjects with mesothelioma, long GT repeats were significantly higher in the asbestos-exposed subjects with mesothelioma, suggesting that decreased induction of HO-1 is associated with a higher risk of malignant mesothelioma (68). In 749 French subjects aged 20–44, lung function was assessed and compared with the length of HO-1 promoter polymorphisms. The long allele carriers showed lower forced expiratory volume in 1 second/forced vital capacity (FEV1/FVC) than other noncarriers with steeper decline in FEV1 over time than noncarriers. In addition, HO-1 in the serum was lower in rapid decliners than in normal decliners, suggesting that HO-1 may be a predictor of lung function decline in these patients (87). Overall, lower inducibility of HO-1 is associated with worsened lung disease in humans. As evidence in children and neonates, a boy with marked elevation of serum bilirubin during autoimmune hemolytic anemia was seen to be a homozygous carrier of short GT dinucleotide-repeat promoter polymorphism (42), and short GT repeat alleles have been associated with prolonged neonatal jaundice (11). No information exists about neonatal lung disease and HO-1 promoter polymorphisms.

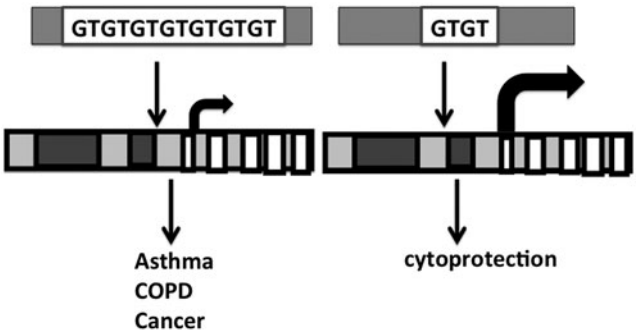

FIG. 6. Proposed effects of promoter polymorphisms on HO-1 in lung disease. Long GT repeats on the HO-1 promoter (*left*) are associated with the development of several lung pathologic states. It remains to be determined whether short GT repeats are protective against lung disease.

Maturation alters the role and regulation of HO-1 in oxidative stress

Overall, HO-1 can exert pleiotropic effects in the lung depending on localization and abundance (Fig. 7). This is a very important consideration when devising therapeutic strategies using HO-1 to prevent BPD.

Most studies on HO-1 have been done in adult animals. Neonatal rodents are more tolerant to hyperoxia and have a high expression of lung HO-1 than do adults (29, 96). This is not attributable to less inflammation (8, 32, 89). Theoretically, antioxidant defenses up-regulated at birth could protect the lung against oxidative injury (29). In fact, HO-1 is found at highest levels in the first postnatal days and then decreases to adult values by the second week of life (21). There may be a benefit to having high constitutive levels of this cytoprotective molecule at the time of delivery, where the neonatal animal transitions to the relatively hyperoxic *ex-utero* environment. At birth, with the transition from the placenta to air breathing, pulmonary vessels are exposed to oxidative stress and undergo remodeling. Although some argue that the lungs from HO-1-deficient mice develop normally after birth,

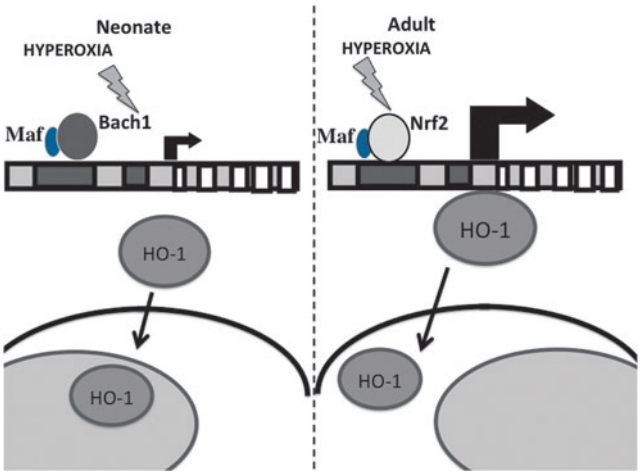

FIG. 7. Maturational differences in HO-1 gene regulation and protein localization. In response to hyperoxia, neonates (*left*) do not up-regulate HO-1 mRNA but translocate HO-1 to the nucleus. In contrast, adults (*right*) induce HO-1 mRNA but do not translocate HO-1 to the nucleus. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

which suggests that HO-1 induction plays no role in the development of the vasodilator response and remodeling which occurs at birth (94), we show evidence to the contrary. In fact, HO-1 null mutant mice showed mild alveolar simplification, disorganization, and reduced secondary crest formation. These defects were more pronounced when these mice were challenged with dexamethasone treatment. The latter further decreased levels of both endothelial and alveolar epithelial markers (121).

Despite the cytoprotective effects of moderate HO-1 overexpression, we have shown that at high levels, HO-1 may be deleterious (108), resulting in enhanced oxidative stress and apoptosis and decreased cell proliferation *in vitro* (99) and, when targeted to type II cells *in vivo*, this leads to a maladaptive over-proliferation of type II cells and perhaps a failure to differentiate to type I cells which are important for recapitulation of the normal alveolar epithelium. This then manifests as increased alveolar thickness and decreased lung function (69). In neonatal rats exposed to hyperoxia, no significant increase in lung HO-1 mRNA was seen in contrast to similarly exposed adults (21). In addition, with prolonged hyperoxic exposure, lung HO-1 mRNA only increased after 10 days in neonatal mice (107); whereas this occurred within 24 h in adult mice (74). Furthermore, neonatal lungs have enhanced expression of Bach1, suggesting a developmental cue to prevent further up-regulation of HO-1 in hyperoxia (48). These differences may explain the lack of up-regulation of neonatal lung HO-1 in hyperoxia, but other mechanisms could also be involved. The role of microRNAs in regulating HO-1 abundance is being explored (39, 40, 79, 80, 92, 119), but it is not known whether there are developmental differences in microRNAs that explain both the increased abundance and the relative lack of hyperoxic induction of lung HO-1 in neonatal mice.

Using a pig and mouse model, HO-1 expression was investigated during adaptation to extrauterine life. HO-2 expression was constitutive, whereas HO-1 protein was induced after birth in the blood vessels and airways, peaking at 14 days in the pig and at 4 days in the mouse. Inhibitors of HO-1 did not alter vasodilatory responses in the pigs (94), suggesting that these effects may not be related to the enzymatic activity of HO-1.

In the acute phase of hyperoxic exposure (3 days), neonatal mice have increased nuclear HO-1 compared with similarly exposed adults (115). This pattern of overexpression may be beneficial, because adults that induce lung HO-1 and do not demonstrate nuclear localization are more susceptible to oxidative stress. Corroborating this, HO-1 null mutant MEFs with stable over-expression of nuclear HO-1 show decreased cellular proliferation and are relatively tolerant to 24 h of hyperoxia compared with MEFs expressing cytoplasmic HO-1 or empty vector controls (Fernando, unpublished observations). Intriguingly, transgenic mice over-expressing nuclear HO-1 in type II cells initially showed improved alveolarization with (3 days) hyperoxic exposure but had increased oxidative DNA damage and abnormal lung histology and pulmonary function tests as adults (69), raising the possibility that the duration of nuclear HO-1 protein signaling is key to cytoprotective responses to injury and repair.

Overall, these observations suggest that the level, localization, and duration of expression of HO-1 may be extremely important in determining its cytoprotective and proliferative effects in the neonatal lung.

Maladaptive Consequences of HO-1 Overexpression and Cellular Localization in the Lung

Obviously, HO-1 plays a significant role in cellular proliferation. Important features of tumorigenesis are excessive proliferation and invasiveness (67, 105). Therefore, by enhancing cellular proliferation and mitigating oxidative stress, HO-1 could also promote tumor cell growth (Fig. 8). Many examples suggest that HO-1 abundance and localization are associated with tumor formation (20, 31). In nonsmall lung cells, cancer patients with a high HO-1 expression ratio in tumor tissue compared with normal tissue had a significantly poorer prognosis and a higher metastatic rate compared with those with a low HO-1 expression ratio (20). *In vitro*, the invasive and migratory abilities of A549 and H441 lung cancer cells significantly increased after high (20-fold increase) exogenous HO-1 over-expression and significantly decreased after HO-1 silencing. Furthermore, HO-1 overexpression positively correlated with the expression of metastasis-associated proteins (104). Adenocarcinoma is the most prevalent subtype of lung cancer, and it is often associated with mutations in the Kras oncogene (49). MAP kinase signal amplification characteristic of Kras lung adenocarcinomas drives toward the progression of malignancy (64). These result in constitutive signaling, which regulates proliferation, differentiation, and cell survival (15). Oncogenic ras alone results in a permanent G1 arrest, as in senescence, but can transform most immortal rodent cells to a

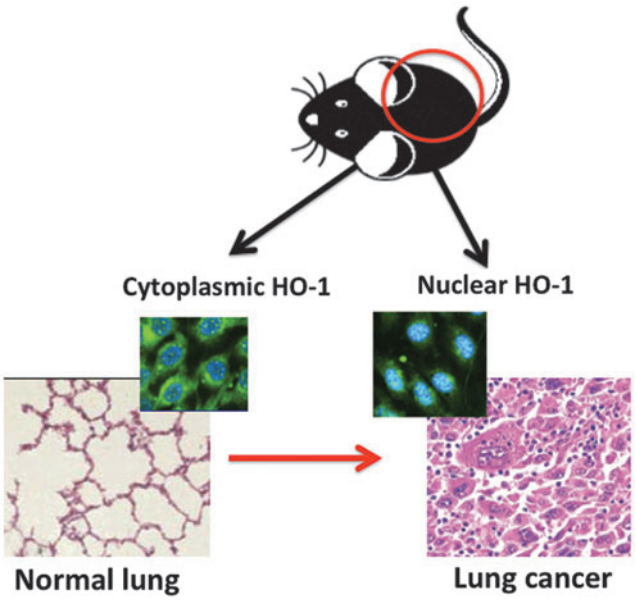

FIG. 8. Association of nuclear HO-1 with lung cancer. In both rodents and humans, increased nuclear distribution of HO-1 (*inset* on the right where DAPI nuclear stain and HO-1 are co-localized as shown by the cyan color) is associated with abnormal lung histology (as shown in the hematoxylin and eosin-stained tissue on the right). With cytoplasmic localization of HO-1 (*left inset*), lung tissue histology is more likely to be normal (*left*). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

tumorigenic state with the inactivation of tumor suppressors such as p53 (88). Growing tumors also preferentially utilize glycolysis over mitochondrial oxidative phosphorylation for glucose-dependent ATP production even in the presence of oxygen (35, 76). In addition to energy, glycolysis generates intermediates that are important to cell growth such as ribose-5-phosphate, a key intermediate in nucleotide biosynthesis (17) which supports the proliferation of tumor cells. In neonatal HO-1 null mutant mice and WT littermates exposed to hyperoxia for 3 days and allowed to recover in room air for 11 days, six DNA damage-response genes were down-regulated in the WT; whereas these were up-regulated many-fold in the knockout, suggesting that HO-1 disruption modifies DNA repair pathways which are important in tumorigenesis (115). We also show that transgenic mice with cytoplasmic over-expression of HO-1 in type II cells exposed to 3 days of hyperoxia as neonates had increased numbers of multinucleated hyper-proliferating type II cells and foamy macrophages but no evidence of fibrosis or inflammation (69). This correlated with lung lesions on MRI with enhanced p-ERK (which is seen in early tumorigenesis) and PCNA staining (69). Interestingly, adult nuclear HO-1 transgenics exposed to hyperoxia as newborns also had increased p-ERK (69). Furthermore, HO-1 null mutant MEF cells stably transfected with nuclear HO-1 showed the most migration toward EGF in an agarose assay, suggesting that nuclear HO-1 is a stronger stimulus for abnormal cell migration than cytoplasmic HO-1 (69). Lastly, G6PDH synthesis and activity was facilitated in nuclear HO-1-infected MEFs more so than in cytoplasmic HO-1-infected MEFs or empty vector controls (Biswas, unpublished observations). This suggests that nuclear HO-1 in conjunction with hyperoxia results in a metabolic switch that favors cancer cell survival. The precise mechanisms by which abundance and localization of HO-1 influence lung tumorigenesis in hyperoxia remain to be determined.

Conclusions

HO is a complex and pleiotropic protein that has multiple roles depending on its abundance, intracellular localization, and duration of action. In the lung, many examples demonstrate its beneficial cytoprotective effects. Nevertheless, equal evidence exists which shows that abnormally high levels are detrimental. In the neonatal lung, HO-1 is found at high abundance but is not further inducible in response to hyperoxia, suggesting the importance of tight regulation of this protein. Furthermore, excessive induction of HO-1 may have abnormal consequences, including favoring a tumorigenic phenotype. In order to take advantage of this important protein, caution should be taken to enhance its cytoprotective properties while preventing adverse effects due to excessive expression, prolonged action, or subcellular localization.

Acknowledgment

This work is funded by grants HL-58752 and HL-111907 from the National Institutes of Health.

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Address correspondence to:
Dr. Phyllis A. Dennery
Department of Pediatrics
University of Pennsylvania
3451 Walnut St.
Philadelphia, PA 19104

E-mail: dennery@email.chop.edu

Date of first submission to ARS Central, December 10, 2013; date of acceptance, January 1, 2014.

Abbreviations Used

Bach1 = heterodimer of BTB and CNC homology 1
BPD = bronchopulmonary dysplasia
CO = carbon monoxide
CORM = CO-releasing molecules
G6PDH = glucose-6-phosphate dehydrogenase
HO = heme oxygenase
MARE = multiple antioxidant response element
MEF = mouse embryonic fibroblast
NADPH = nicotinamide adenine dinucleotide phosphate
Nrf2 = NF-E2-related factor 2
p-ERK = phospho-Extracellular signal-regulated kinase
PPS = pentose phosphate shunt
ROS = reactive oxygen species
TGF = transforming growth factor
TLR = toll-like receptor