Heme Oxygenase and Acute Lung Injury: The Functional Significance of Heme Oxygenase Induction

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Introduction

In spite of major advances in intensive care, the morbidity and mortality of patients with acute respiratory distress syndrome (ARDS) remains high, and therapy is at best supportive [1, 2]. ARDS is characterized by refractory hypoxemia secondary to non-hydrostatic pulmonary edema [3] and is precipitated by a range of common predisposing factors not necessarily related to primary lung injury, such as polytrauma, severe burns, sepsis, gastric aspiration, surgery necessitating cardiopulmonary bypass (CPB), lung resection, hemorrhagic shock, and oxygen toxicity [4].

Early in the course of the syndrome, activated inflammatory cells move into the air spaces releasing reactive oxygen species (ROS) capable of initiating or amplifying injury to the alveolar capillary membrane. Accumulating evidence suggests that ROS such as superoxide, hydrogen peroxide and the hydroxyl radical, and reactive nitrogen species (RNS) such as peroxynitrite, are central to the pathogenesis of ARDS. Thus elevated levels of oxidatively damaged biomolecules are frequently found in blood and bronchoalveolar lavage (BAL) fluid taken from patients with established ARDS [5–8]. Moreover, upregulation of the enzymatic antioxidant system (superoxide dismutase [SOD], glutathione peroxidase, and catalase) has also been described in these circumstances [9]. Such enzymes work in concert to detoxify ROS and limit molecular damage and cytotoxicity.

Although discovered some thirty years ago [10], the importance of the heme oxygenase system in modulating the pulmonary stress response has only recently been appreciated. Current research suggests that heme oxygenase induction is an adaptive mechanism designed to prevent cellular damage during conditions of oxidant stress. Here, we summarize the current knowledge on the functional aspects of heme oxygenase and suggest areas for future research which may prove beneficial to the critically ill patient with ARDS.

Heat Shock Proteins

While the chemical reactions involved in the generation and removal of ROS and related toxic metabolites have been studied in great detail, less is known about the cellular and molecular changes involved in stress-mediated cytoprotection. The stress response is a highly conserved cellular defense mechanism defined by the rapid and specific expression of stress proteins, with concomitant transient inhibition of non-

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stress protein gene expression. The heat shock response represents a distinct aspect of the stress response and leads to the production of a diverse group of inducible and constitutive proteins termed heat shock proteins (HSP). Synthesis of HSP is induced ubiquitously after exposure of cells and tissues to elevated temperatures, or to a variety of other types of stress, including pro-oxidants. In general, the constitutively expressed proteins act as chaperones for other cellular proteins by binding to nascent polypeptides to prevent premature folding and to translocate proteins into organelles [11]. The inducible proteins confer thermotolerance to cells [12] and can protect them from stress-induced damage by preventing protein denaturation or by repairing such damage [13].

Heat Shock Protein (HSP) 32

Among the many classes of stress proteins, HSP 70 and heme oxygenase-1 (HSP 32) are the best characterized with respect to lung biology. Here, we focus on the properties of the highly-conserved, heme oxygenase-1 (HO-1). Like many heme proteins, heme oxygenase exists in a number of isoforms. Three have been identified to date (Table 1), which are the products of three separate, distinct genes. Each isoform displays a different primary structure, regulation, antigenicity, molecular weight, and tissue distribution. The heme oxygenase-3 (HO-3) isoform was identified in 1997 [14], long after characterization of HO-1 [15] and heme oxygenase-2 (HO-2) [16]. HO-1 and HO-2 are homolgous for only 40% of their amino acid sequence, but both isoforms retain similar enzyme activities and require the same co-factors/co-enzymes [16, 17]. HO-3 is related to HO-2, sharing 90% of the amino acid sequence. Both contain two heme regulatory motifs, which may confer heme regulatory functions [18]. HO-3, however, is a poor heme catalyst [14]. Heme oxygenase activity is highest in the spleen, where senescent erythrocytes are sequestered and destroyed, but this activity is observed to a certain extent in all systemic organs. All heme oxygenase isoforms are cellular in origin and are associated with the smooth endoplasmic reticulum to which they are anchored by a C-terminal lipophilic domain [19].

Table 1. Principle distribution and function of the three isoforms of heme oxygenase

Isoform	Expression	MW	Main location	Function
HO-1 (HSP32)	inducible	32-kDa	spleen and liver	stress protein, protects against oxidative stress
HO-2	constitutive*	36-kDa	brain and testes	signal transduction in neural tissues, role in germ cell development and epidermal cells
HO-3	constitutive	33-kDa	spleen, liver, thymus, prostate, heart, kidney, liver, brain, testes	heme-dependent regulatory processes

* HO-2 is induced only by adrenal glucocorticoids

Reaction Mechanisms

Heme oxygenase catalyzes the first and rate-limiting step in the oxidative degradation of heme (iron protoporphyrin [Fe-PP] IX) to biliverdin, releasing equimolar amounts of carbon monoxide, and iron (Fe^{2+}) [2] (Fig. 1). In mammals, biliverdin is reduced to bilirubin by biliverdin reductase [20] and following conjugation with glucuronic acid, excreted. The active site on heme oxygenase does not recognize the metal moiety of metalloporphyrins, rather having specificity only toward the side chains of the porphyrin ring. Hence, zinc-protoporphyrin (Zn-PP), tin protoporphyrin (Sn-PP) and cobalt protoporphyrin (Co-PP) can inhibit enzyme activity by competing against Fe-PP. The reaction mechanism has been well characterized [21–23].

Carbon monoxide is produced by heme oxygenase predominantly in the liver and spleen, bound to hemoglobin, and excreted by the lungs. Carbon monoxide can act as a heme ligand, and may possibly have a physiological role similar to nitric oxide (NO). Thus, carbon monoxide activates soluble guanylate cyclase, leading to increased cyclic 3,5-guanosine monophosphate (cGMP) production, and may be an important regulator of vascular tone [24] and a modulator of neurotransmission in the lung [25].

Inducers of HO-1

Apart from its major substrate heme, HO-1 is strongly, and rapidly, induced by a long list of seemingly unrelated agents, including heat, ultraviolet (UV)-A irradiation, endotoxins, hyperoxia, ozone, paraquat, sulfhydryl reagents, peroxynitrite, superoxide/hydrogen peroxide, glutathione depletion, metal ions, human prion protein, the

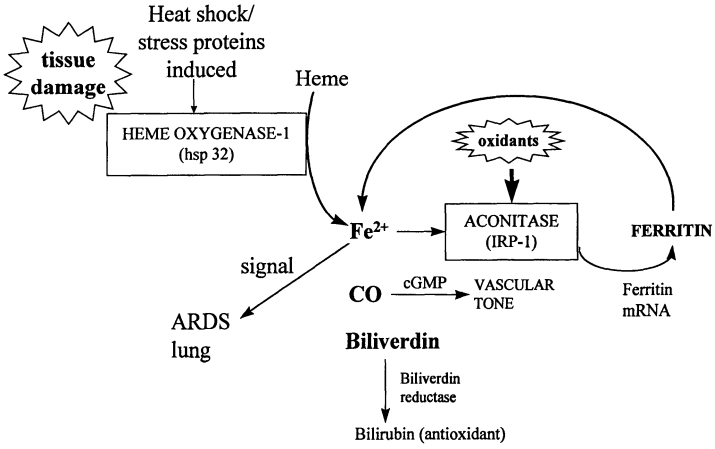

Fig. 1. Heme oxygenase (HO) and intracellular iron control. Heme oxygenase is induced during the stress response, releasing iron (Fe^{2+}), carbon monoxide (CO) and biliverdin. All heme oxygenase products are biologically active: CO modulates vascular tone via cGMP, biliverdin is reduced to bilirubin, which may have antioxidant properties, and iron may act as a gene regulator, participate in Fenton reactions or induce ferritin synthesis via iron regulatory protein (IRP-1)

NO donors sodium nitroprusside/S-nitroso-N-acetylpenicillamine (SNP/SNAP), prostaglandins, inflammatory cytokines, tumor-promoting phorbol esters, hormones, cyclic adenosine monophosphate (cAMP), and the specific lipid peroxidation product 4-hydroxy-2-nonenol (HNE) [26]. Some authors [27–29] have suggested oxidative stress linking these agents, either directly, or via glutathione depletion, which would imply HO-1 induction represents a response to oxidative stress rather than a response to a direct effect on heme metabolism.

The molecular regulation of HO-1 expression has been well characterized *in vitro* and *in vivo*. Induction of heme oxygenase is regulated primarily at the mRNA level. Increases in HO-1 levels can be brought about by increased transcription rates, as in the case of induction by metals, or by message stabilization for the induction of HO-1 by heat. Indeed, HO-1 is the most transcriptionally-sensitive protein discovered to date. The HO-1 gene promoter region contains several transcriptionally-active regulatory sites, such as heat shock factor, nuclear factor-κB (NF-κB), activator protein-1 (AP-1), antioxidant response elements, and metal regulatory binding sequences; which are known to be sensitive to oxidative stress [30–33].

HO-1 and Oxidative Stress

Antioxidant Properties: Ferritin and Bilirubin: Initially, researchers focused on the role of HO-1 in maintaining cellular heme protein levels. Over the past decade, however, it has become increasingly clear that HO-1 functions as a defense against oxidative stress, though the precise mechanism has yet to be defined clearly as the products of heme degradation are all biologically active [34–36], and heme itself is a pro-oxidant compound [37, 38]. Heme oxygenase-mediated cytoprotection is proposed to occur via the removal of pro-oxidant heme, production of bilirubin, and the induction of ferritin synthesis which sequesters redox active iron [39–42]. Bilirubin scavenges lipid peroxyl radicals and terminates radical chain reactions *in vitro* at low oxygen concentrations and is thought by some to be a potent antioxidant [35].

Pro-Oxidant Properties: Low Molecular Mass Iron: The role of iron in the heme oxygenase system is considered by the authors to be largely overlooked. Formation of ferrous ions can lead to hydroxyl radical production and biomolecular damage via the Fenton reaction [43]. We are exploring the pro-oxidant capacity of heme oxygenase in lipid and cell preparations. Preliminary data [44] indicate lipid peroxidation in a microsomal-lipid system, which is blocked by iron chelators and Sn-PP, suggesting an HO-mediated reaction driven by catalytic free iron (Fig. 2). Similarly, we have shown an increase in bleomycin-detectable iron in rat pulmonary artery smooth muscle cells on induction of HO-1 [45]. Free iron has many other functions in the cell, including gene regulation. Released iron can inhibit further production of nitric oxide synthase (NOS) by inhibiting its nuclear transcription [36]. Iron increases heme oxygenase levels, but decreases 5-aminolaevulinate synthase (ALAS) transcription [46].

NO inhibits and activates heme oxygenase activity in different settings. SNAP induces activation of heme oxygenase and decreases heme iron in pulmonary artery endothelial cells. It also increases iron-responsive (enzymes requiring iron for their activation) gene products, ferritin and mitochondrial aconitase, secondary to the re-

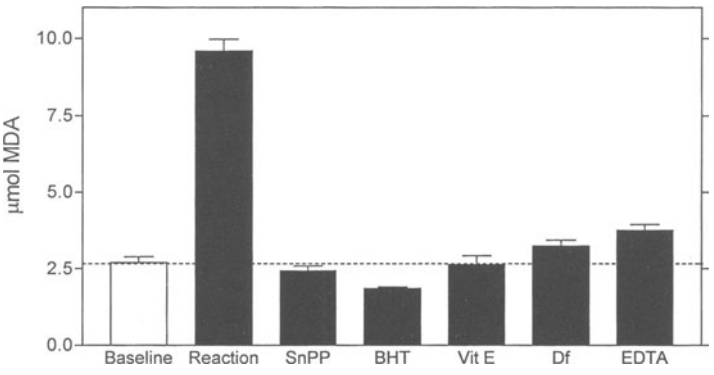

Fig. 2. Heme oxygenase-mediated lipid peroxidation (LPO), where baseline is microsomes (source of heme oxygenase) and liposomes. Addition of hemoglobin, and NADPH (reaction) causes LPO, which is inhibited by Sn-PP (tin protoporphyrin, heme oxygenase inhibitor); the chain-breaking antioxidants butylated hydroxytoluene (BHT), vitamin E; and the iron chelators desferrioxamine (Df) and EDTA

lease of iron from heme stores [47]. Recent evidence has shown similar localization of NOS and HO-2 in blood vessels and the autonomic nervous system, suggesting a coordinated physiological role [24].

ARDS and Oxidative Stress

Animal Models

If critical defenses such as haptoglobin, which can inhibit hemoglobin-stimulated lipid peroxidation [37] and hemopexin, which inhibits hemin-stimulated peroxidation [38] are overwhelmed, heme may catalyze oxidant mediated cellular damage. Heme oxygenase may offer antioxidant protection against this type of injury. Thus, both *in vivo* and cell culture experiments demonstrate that pre-treatment with hemoglobin protects against heme-mediated oxidant injury probably due to the induction of HO-1. In this regard transfection with HO-1 cyclic desoxyribonucleic acid (cDNA) has been shown to protect coronary vessel endothelial cells from heme-induced injury [48]. Further, HO-1 protects against rhabdodmyolysis in rats, where high levels of heme are released from myoglobin and cause renal injury [40]. Moreover, methemoglobin increases both HO-1 and the intracellular iron-storage and antioxidant protein ferritin in cultured endothelial cells [49]. Additionally, it has been shown that pateints with septic shock complicated by disseminated intravascular coagulation (DIC) display neutrophil activation and margination which oxidizes hemoglobin to heme-releasing methemoglobin, and subsequently induces both heme oxygenase and ferritin [50].

It is now clear that HO-1 provides cellular protection against many agents other than heme. Accumulation of heme oxygenase mRNA and protein is strongly stimulated by treatment of cultured human skin fibroblasts with near-UV radiation, hy-

drogen peroxide, or the sulfhydryl reagent sodium arsenite [27], as well as infective agents such as the influenza virus [51]. The increased expression is thought to occur via transcriptional activation rather than increased mRNA stability [52]. Antisense transfection studies have shown that lowering HO-1 increases *in vitro* damage by UV-A irradiation [29]. In further work, it was shown that this was a generalized response, occurring in several different human and animal cell types [28].

Gram-negative sepsis is a common predisposing condition leading to lung injury and ARDS. Endotoxin is frequently used to induce lung injury comparable to ARDS in animal models. Recently endotoxin challenge has also been shown to lead to the expression of HO-1 in bronchoalveolar epithelial tissue and cultured macrophage cells [31]. Additionally, the transcription factor AP-1-dependent induction of HO-1 by hemoglobin, has been shown to protect against subsequent lethal endotoxemia [53]. These studies taken together support a protective role for HO-1 induction in animal models of sepsis and ARDS.

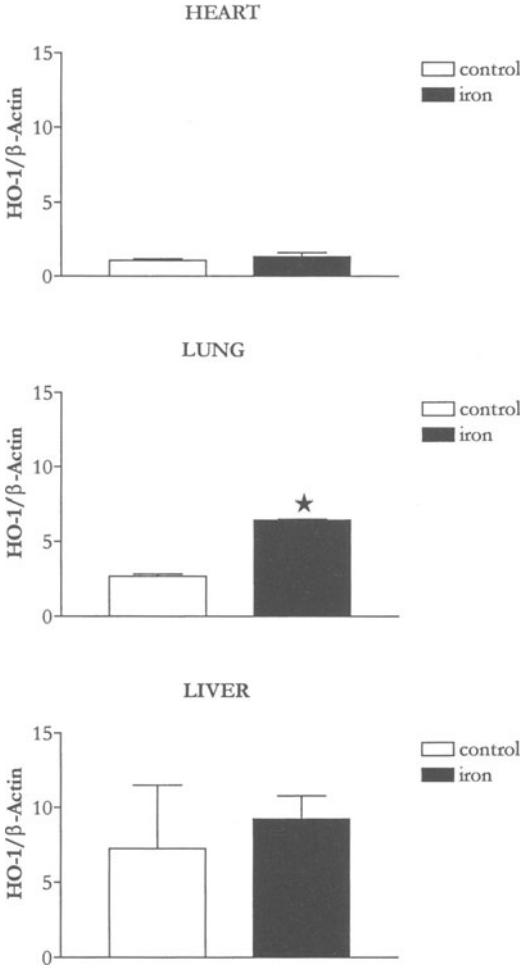

Fig. 3. HO-1 protein levels in the heart, lung and liver of iron-overloaded (filled bars) and normal rats (clear bars). A significant increase of HO-1 is seen in the lung of iron-overloaded rats

Hyperoxia causes pulmonary microvasculature damage and is often used as a model of oxidant-induced lung injury. Furthermore, hyperoxia also leads to increased HO-1 expression in rat lung in a variety of cell types such as the bronchial and alveolar epithelium, and interstitial and inflammatory cells [31]. A protective effect associated with HO-1 induction has been demonstrated under these conditions. Thus, HO-1 over-expression arrests cell growth and protects against hyperoxic oxidant injury in pulmonary epithelial cells [54]. A lag in the cell cycle, possibly mediated by cGMP, is thought to allow the cell time to repair damaged DNA [55]. Conversely hypoxia can also lead to HO-1 gene expression as shown by *in vitro* studies in various cultured cells including epithelial cells, fibroblasts, macrophages, and smooth muscle cells, this increase being associated with AP-1 activation and not mRNA stability [56].

We have demonstrated a rapid rise in HO-1 protein in lung tissue of plasma iron-overloaded rats when compared with other tissues [57] (Fig. 3). The functional significance of this finding is unclear, but may have implications for ARDS, as we have previously demonstrated increased levels of iron accumulation in BAL fluid from patients with ARDS [58].

Lung Injury and Induction of HO-1 in Humans

Understandably, most of our knowledge on the functional consequences of heme oxygenase induction comes from animal models and cell culture work. However, a growing body of evidence indicates widespread upregulation of HO-1 in various disease states in humans. It has been reported that heme oxygenase is induced in alveolar macrophages during phagocytosis of red blood cells, and is found in BAL macrophages from patients with interstitial lung disease [59].

Generation of carbon monoxide through the HO-1 pathway contributes to the hemodynamic compromise of endotoxic shock. Increased carbon monoxide generation in critical illness, and pulmonary carbon monoxide production in healthy and critically ill humans has been shown [60, 61]. Further, we have shown increased HO-1 mRNA levels in white blood cells and lung tissue taken from patients with ARDS compared to controls (Y. Chen, unpublished data).

Beneficial vs Adverse Effects

Evidence confirming the hypothesis that induction of heme oxygenase confers cytoprotection during the stress response is emerging but remains inconclusive. Many early studies evaluating the effect of oxidative agents on cells, measured HO-1 mRNA and protein levels, but not enzyme activity. Furthermore, it has been demonstrated that heme oxygenase does not protect MCF-7 cells against menadione-induced oxidant stress [62]. Indeed, the protective effect of heme oxygenase may actually reside in the induction of ferritin, rather than HO-1 itself, as seen in a rodent rhabdomyolysis model [40]. This was confirmed in endothelial cells, where it was the induction of ferritin and not heme oxygenase that protected cells from hydrogen peroxide [39]. And more recently, intratracheal lipopolysaccharide (LPS) was found

to rapidly induce ferritin protein in the rat lung independently of its mRNA synthesis or heme oxygenase enzyme activity [63]. Additionally, intratrachael administration of hemoglobin was found to induce lung HO-1 in the rat and protect against hyperoxia; although inhibitor studies showed that the protection was not mediated by increased heme oxygenase enzyme activity [64]. Finally, Takahashi et al. [65] showed increases in HO-1 mRNA only after prolonged exposure of rat lungs to ozone, suggesting that HO-1 is involved in the recovery process from lung damage by ozone, rather than the acute respiratory response.

Perhaps the most interesting work has come from the studies in a genetically manipulated murine heme oxygenase deletion. Compared to wild type animals HO-1, but not HO-2, knock-outs had serum iron deficiency and pathological iron-loading in the kidneys and liver, suggesting that HO-1 is crucial for iron homeostasis in the body [66]. In further work, the HO-1 knock-outs were more susceptible to oxidative stress in both *in vitro* and *in vivo* models [67]. In an HO-2 knock-out model, lung hemoproteins and iron content were found to be significantly increased without increased ferritin during hyperoxia, suggesting accumulation of available redox-active iron [68]. The absence of HO-2 was associated with induction of HO-1 and increased oxygen toxicity *in vivo*, apparently due to accumulation of lung iron. In fact, the discovery of two heme regulatory motifs on HO-2 independent of the active site [18], suggests HO-2 can limit oxidant stress by binding heme, an antioxidant function which HO-1 does not appear to possess. In HO-1 knock-out mice the same group reported decreased markers of oxidative injury after hyperoxic exposure [69]. These studies appear to give conflicting results regarding the possible beneficial or adverse effects of these enzymes. However, it has been suggested that heme oxygenase mediates cytoprotection against hyperoxia within a narrow range of gene expression, so at lower levels it may be protective whereas at high levels protection is obviated by increased redox active iron release [70].

Conclusion

Heme oxygenase plays a potentially crucial role in modulating the effects of oxidant stress in critically ill patients with ARDS. Tools for manipulation are available, in that Sn-PP, a selective heme oxygenase inhibitor, has been used for treatment in a rat model of hyperbilirubinemia [71]. However, whether blocking heme oxygenase expression and/or activity, or even upregulating the enzyme would be clinically desirable, remains unclear. Indeed, inhibitors, given their porpyhrin structure, may even influence other vital protein functions. Second, HO-1 up-regulation not only provides the cell with resistance to oxidative stress, but also releases catalytically-active free iron. Suppressing iron release may prevent formation of toxic oxygen free radicals, but we may inadvertently affect useful signaling functions within the cell. As with all antioxidant interventions the circumstances of individual patients will probably determine whether HO-1 up-regulation or inhibition is desirable. Determining the functional significance of the pro-oxidant/antioxidant redox balance thus remains as important as ever.

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