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Signaling Function of Heme Oxygenase Proteins

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Abstract

Significance: Many reports have underscored the importance of the heme degradation pathway that is regulated by heme oxygenase (HO). This reaction releases bile pigments and carbon monoxide (CO), which are important antioxidant and signaling molecules. Thus, the reaction of HO-1 would have significant cytoprotective effects. Nevertheless, the importance of this protein goes beyond its enzymatic action. New evidence outlines significant effects of inactive forms of the HO-1 protein. ***Recent Advances:*** In fact, the role of the HO protein in cellular signaling, including transcription factor activation, binding to proteins, phosphorylation, and modulation of protein function, among others, has started being elucidated. The mechanism by which the inducible form of HO-1, in particular, can migrate to various cellular compartments to mediate important signaling or how and why it binds to key transcription factors and other proteins that are important in DNA repair is also described in several physiologic systems. ***Critical Issues:*** The signaling functions of HO-1 may have particular relevance in clinical circumstances, including cancer, as redistribution of HO-1 into the nuclear compartment is observed with cancer progression and metastasis. In addition, along with oxidative stress, the pleiotropic functions of HO-1 modulate antioxidant defense. In organ transplantation, HO and its byproducts suppress rejection at multiple levels and in sepsis-induced pulmonary dysfunction, inhaled CO or modulation of HO activity can change the course of the disease in animals. ***Future Directions:*** It is hoped that a more detailed understanding of the various signaling functions of HO will guide therapeutic approaches for complex diseases. *Antioxid. Redox Signal.* 20, 1743–1753.

Introduction

Heme oxygenase (HO) is well known for its catalytic degradation of heme to biliverdin. This reaction generates unique byproducts such as carbon monoxide (CO), and it requires energy. In addition to its enzymatic function in the 1970s (15,63) described since the 1990s, HO has been described as an important antioxidant and anti-inflammatory defense (9,48), and emerging roles are reported every year, including a role in innate immunity, malaria, and diabetes (1,7,23,25,27), among others. Many of the functions ascribed to HO can be explained by its enzymatic function, which removes heme, a potent oxidant with known toxicities (12–14,18), and forms important antioxidant bile pigments (28,34,37) and signaling molecules such as CO (5,32,49,59). However, it is unlikely that the HO reaction can mediate cytoprotective effects *via* its byproducts if there is no available substrate as is the case in many tissues. Therefore, nonenzymatic roles of the HO protein have been reported. This review will focus on the signaling function of HO, its protein interactions, its subcellular localization, and the implications of these phenomena in biological and physiologic systems.

Structure of HO Proteins and Relevance to Signaling

Two isoenzymes of HO, the inducible form HO-1 and the constitutive form HO-2, have been well characterized. An additional form HO-3 was described, but this seems to result from differential splicing of HO-2 (41). The crystal structure of human (97), murine (50), and bacterial HO-1 (71) has been published, which enables a better understanding of the role of HO-1. If we compare the structure of HO-1 protein among species, many domains are highly conserved, including the alpha helical fold, which is responsible for substrate orientation within the heme pocket and the C-terminal, which is essential for binding to the smooth endoplasmic reticulum membrane. The distal helix always confers alpha-regioselectivity for the release of iron and biliverdin with the degradation of heme. The key heme iron ligand in the heme pocket is Histidine 25 (88). Mutant proteins with a histidine-to-alanine substitution

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are unable to degrade heme or to bind it in the heme pocket (43). The glycine 143 residue is also highly conserved among species, and it dictates the configuration of the heme pocket, thereby influencing the enzymatic function (58) of HO-1. Mutants with a deletion of the terminal 50 amino acids of the protein are able to bind heme but cannot degrade it to biliverdin (100). In addition, there is a highly conserved region in the rat HO-1 protein at amino acids 207–221 (LNIELSEELQALL) with >90% homology to a nuclear export sequence motif ($LX_{1-3}LX_{2-3}LXL$) found in the human immunodeficiency virus type 1 Rev protein (42) (Fig. 1). In humans, HO-1 protein has a novel helical fold with heme between the proximal and distal helices, but it still has histidine 25 as the heme iron ligand. The human HO-1 protein also has a flexible heme pocket that can open and close to perhaps regulate the catalytic activity (73). In addition, mutants at glycine 143 could result in an inactive HO-1 protein and a mutation at glycine 139 alters HO-1 function, converting it to a peroxidase (35). It is, therefore, reasonable to conceive that polymorphisms of the HO-1 distal helix could alter HO-1 function, thereby dramatically changing heme homeostasis, which would impact signaling (87).

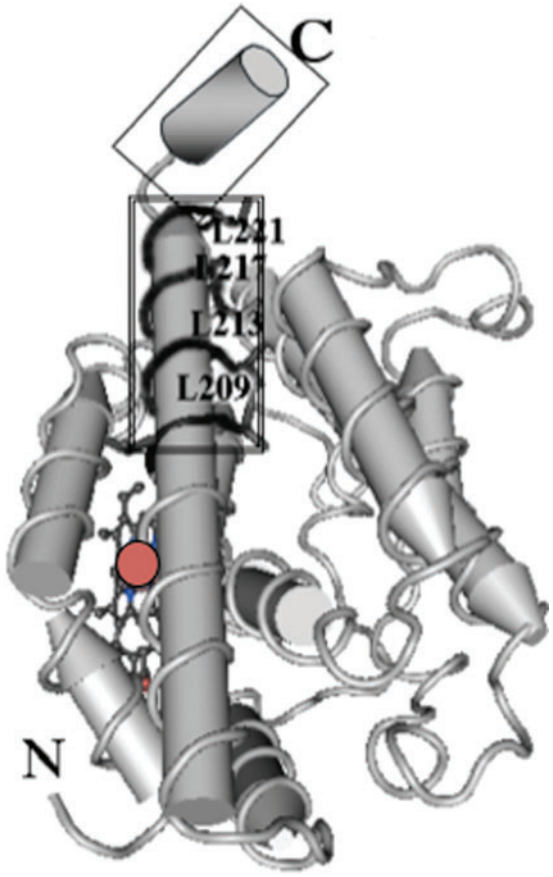

FIG. 1. Structure of rat HO-1 protein. The C-terminal region, which is key to anchoring the protein in the smooth endoplasmic reticulum, and a leucine-rich region with homology to a nuclear export sequence motif are highlighted. The heme binding site is shown in red. Adapted from (86). HO, heme oxygenase. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

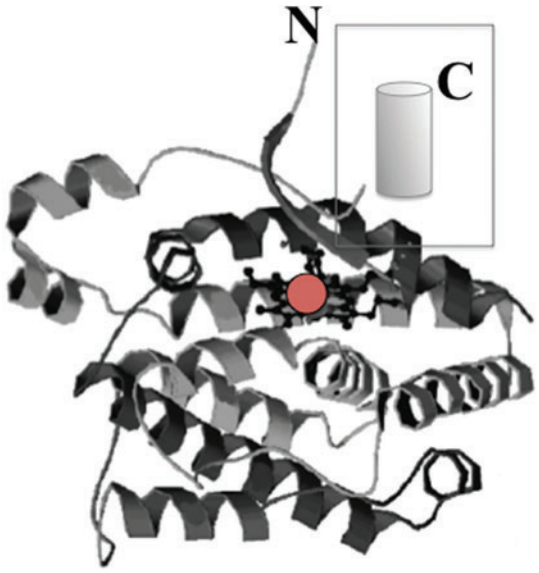

FIG. 2. Structure of algal HO-2 protein. The C-terminal region is highlighted. The heme binding site is shown in red. Adapted from (84). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

The HO-2 protein has similar properties to HO-1 in its ability to degrade heme (91). Its crystal structure has been elucidated in algae in complex with heme (84). The algal HO-2 shares 20% homology to the human protein (www.ncbi.nlm.nih.gov/genbank/). The protein contains an additional N-terminal segment not found in HO-1. Nevertheless, the heme-binding site is conserved across the species (84) (Fig. 2). In solution, the ferric heme complex exists as a dimer form, whereas apo-HO-2 exists as a monomer. When it is a dimer, HO-2 partially covers the surface of the heme-binding site and may decrease heme accessibility, although this needs to be further refined. On HO-2, the heme-binding histidine residue is at amino acid 16 (84). Protein sequencing reveals that HO-2 differs from HO-1 in that it has cysteine residues (84). These are present near polar residues and are involved in forming the heme regulatory motif. The cysteine residues can form a ligand with heme iron and can be oxidatively modified (66), which may have importance in the signaling role of the protein during oxidative stress. HO-2 can also be phosphorylated at key serine residues by protein kinase C, which enables a change in the activity of HO-2 enzyme (95).

Despite the similarity in the reaction catalyzed by the two isoforms of HO, different roles, regulation, and post-translational modifications have been described for the two proteins (92). HO-1 is highly inducible in oxidative stress and has multiple transcriptional factor binding sites that regulate its induction in oxidative stress (4). Most important is the binding of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and small Maf proteins to the antioxidant response elements (AREs) in the distal enhancers of the HO-1 promoter, leading to its rapid induction in oxidative stress (76). Heme levels regulate the inhibition of HO-1 induction through Bach1, an antagonist of Nrf2 (69) (Fig. 3). In the regulatory region of the human HO-1 gene, other binding sites have been identified that can modulate the induction of HO-1 in oxidative stress (4). The HO-2 protein may function as an oxygen sensor for

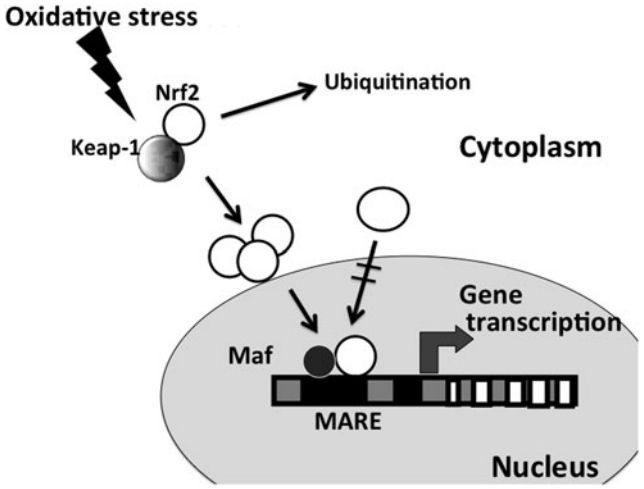

FIG. 3. Transcriptional regulation of HO-1 in oxidative stress. Under basal conditions, nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is bound to kelch-like ECH-associated protein-1 (Keap-1), which leads to its ubiquination and degradation. With oxidative stress, Nrf2 dissociates from Keap-1 and translocates to the nucleus, where it mediates HO-1 gene transcription. If Bach1 binds to the antioxidant response element on the HO-1, distal enhancers lead to the inhibition of gene transcription.

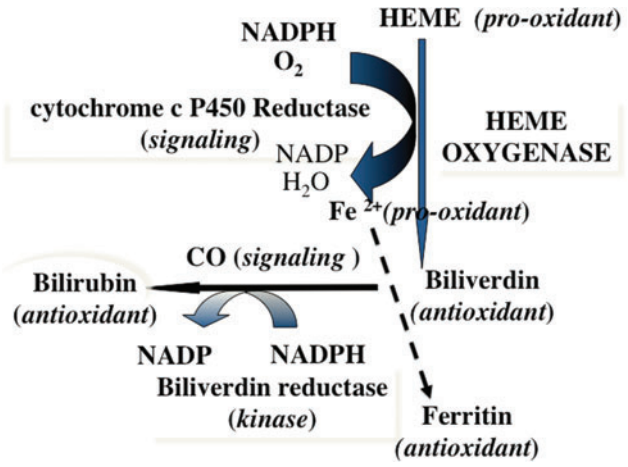

FIG. 4. Antioxidant potential of the heme degradation pathway. Heme is degraded by the rate-limiting enzyme HO in an energy-dependent pathway. Each substrate and by-product has a specific antioxidant, pro-oxidant, or signaling function (italics). In addition, the pro-oxidant Fe^{2+} is sequestered by ferritin, which is co-induced with HO-1. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

calcium-sensitive potassium channels in various cells (101). In contrast to HO-1, the HO-2 gene is not inducible by oxidative stress nor is the protein regulated by oxidants despite having cysteine residues, which are amino acids that are typically modulated by redox changes (66). Nevertheless, the gene has sites that enable induction *via* glucocorticoids. In fact, in the 14-day-old rat brain, exposure to corticosterone in the post-partum period resulted in a marked enhancement of HO-2 immunoreactivity in many neuronal populations, and this was mediated *via* a glucocorticoid element found on the HO-2 promoter (62,74).

How the Enzymatic Function of HO Alters Signaling

The HO reaction enables the cleavage of heme in a specific manner using a multistep reaction. Iron is reduced to its ferric state through the action of cytochrome C p450 reductase, and CO is released by elimination of the a-methylene bridge of the porphyrin ring. Biliverdin is formed in an energy-requiring step and is then further converted to bilirubin by biliverdin reductase (BVR), which is non-rate limiting (Fig. 4). Many have proposed that the released iron is sequestered by ferritin (11). Each byproduct of HO has a significant cellular function.

Heme

The substrate heme is a pro-oxidant molecule that can participate in the formation of oxidative radicals, leading to oxidative injury (11). In a recent study, the addition of methemoglobin with a heme polymer enhanced the production of reactive oxygen species from macrophages (29). In a model of heme overload in hemopexin-null mice, excess heme in plasma promoted the production of reactive oxygen species and reduced nitric oxide (NO) availability (96). These observations suggest an important role for heme as a pro-oxidant and pro-inflammatory molecule. Therefore, the sequestration of heme and its degradation by HO may serve an important antioxidant function. In addition, the binding of heme to ferritin, which is co-induced with HO-1 (11), may prevent oxidative stress. In cultured alveolar epithelial cells, HO-1 overexpression resulted in a nearly threefold induction of ferritin. This was accompanied by compensatory increases in transferrin receptor expression, resulting in altered intracellular iron distribution and protection against iron toxicity from heme degradation (33).

Carbon monoxide

The production of CO has systemic effects, including regulation of odor response adaptation, long-term potentiation, memory, vision, and hearing, among others (21,65,72). The mechanisms by which CO mediates its effect are complex. It may have both deleterious and beneficial effects on mitochondrial biogenesis and function (6,53) as well as calcium availability (53), thereby influencing apoptosis. In addition, through its activation of p38 mitogen-activated protein (MAP) kinase, CO alters various signaling kinases and prevents apoptosis in some models (54) but may enhance it in others, as suggested by an increased eryptosis or red cell death, in the presence of a CO-releasing molecule, CORM-2 (51). In addition, CO can repress protein synthesis and elongation (79). Interestingly, CO can also inhibit toll-like receptor (TLR)-4 signaling by regulating the interaction of TLR4 with caveolin (24). Overall, depending on the mechanism by which CO mediates its signaling function, it can have differential effects, which help explain the role of HO-1 as an antioxidant and anti-inflammatory mediator in some cases.

Bile pigments

Biliverdin and bile pigments such as bilirubin, which result from the degradation of biliverdin, are important antioxidants that can prevent lipid peroxidation more efficiently than

vitamin E (28,67,81,82). When lung grafts were exposed to biliverdin during cold storage, these were protected against cold ischemia/reperfusion injury and had significantly improved function. This was explained by the scavenging properties of the bile pigment (83). Both bilirubin and biliverdin were effective at suppressing the oxidative burst, resulting in phorbolester-stimulated neutrophils (45). Nevertheless, biliverdin was thrice less effective than bilirubin at inhibiting peroxynitrite-mediated protein tyrosine nitration (46). Since enzymes modify their activity (94), this suggests that biliverdin plays an important role in modulating the activity of a variety of enzymes, including kinases. In fact, incubation of macrophages with biliverdin resulted in a rapid increase in phosphorylation of Akt and upregulation of interleukin-10 expression (98). Further, incubation of cultured macrophages with biliverdin induced phosphorylation of endothelial NO synthase at Ser-1177 in a time- and dose-dependent fashion, (70) and this effect was mediated by BVR, which is also a serine/threonine/tyrosine kinase, which inhibits apoptosis and is a kinase-independent activator of protein kinase Cβ (64). Through this kinase, biliverdin also regulates one of the three major MAPK signal transduction pathways, including MEK/ERK/Elk and protein kinase Cδ (38). This is an important mechanism that controls mitogenic and stress stimuli for gene activation (52).

Overall, each of the products of the HO reaction play a role in important cellular signaling processes in different ways (see Fig. 4). This results in cytoprotective effects.

Nonenzymatic Signaling Roles of HO

Despite the many signaling effects of HO-1 byproducts, even the inactive form of HO can mediate various signaling pathways. Hori *et al.* (43) evaluated the role of the enzymatically inactive HO-1 protein and showed that the mutated form of HO-1 was still able to protect against oxidative injury despite its lack of activity. In addition, overexpression of the native HO-1 was associated with increased cell susceptibility to hydrogen peroxide (H_2O_2). This was explained by an over-abundance of iron with absent heme metabolism. Overexpression of the mutant protein was considered as interfering with the binding of naturally occurring HO-1 to nicotinamide adenine dinucleotide phosphate-cytochrome c P450 reductase, thereby suppressing the intrinsic enzyme activities and resulting in dominant-negative effects. In addition, the mutant protein was associated with increased catalase and glutathione levels that would confer antioxidant benefits (43). How inactive HO-1 regulated the activity of other antioxidant enzymes was not made entirely clear. We verified this observation in NIH-3T3 cells and further demonstrated that the inactive form of HO-1 protein enhanced resistance to H_2O_2 toxicity but did not protect against heme-mediated cell death (56). This is likely due to the fact that sequestration and/or degradation of heme by the active form of HO-1 is important to prevent heme toxicity, whereas an inactive form which is unable to degrade heme would not have such a function.

Interactions Between HO and Other Proteins That Affect Signaling

Although there are significant hydrophobic forces around the heme pocket that could inhibit interactions between HO-1 and other proteins, the hydrophilic portions of the protein favor binding (85). We have previously shown that HO-1 binds to HO-2 at the largely hydrophilic residues of the HO-2 protein (99) (Fig. 5). The significance of this binding is not yet clear. Others have shown that HO-1 binds to a variety of other protein substrates, which may alter the activity of these proteins, thereby modifying cellular function. In one such example, HO-1 protein was seen to bind to cytochrome c p450 reductase in cytochrome c p450 reductase cDNA-transfected HEK293 cells that were exposed to hypoxia (Fig. 5). This then modified the ability of HO-1 to translocate to the nucleus and promoted its oligomerization into higher-ordered complexes, thereby stabilizing the protein (57). This may have relevance in the regulation of cytochrome c p450 reductase function and expression by HO-1.

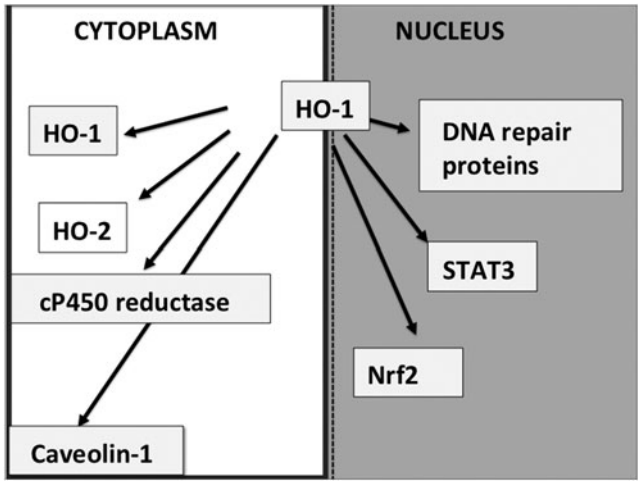

FIG. 5. Binding of HO-1 with various proteins. HO-1 can bind to itself, HO-2, and cytochrome c P450 reductase in the cytoplasm; whereas in the nucleus, it binds to various DNA repair proteins and also binds to transcription factors such as STAT3 and perhaps Nrf2. It can also bind to caveolin-1. STAT3, signal transducer and activator of transcription-3.

In other experiments, the nuclear fractions of cells that were transfected with a FLAG tagged HO-1 were isolated and purified with a FLAG column. The bands recovered were isolated and subjected to matrix-assisted laser desorption/ionization–time of flight mass spectrometry (55). Using this model, we observed that the nuclear HO-1 protein can also bind to various nuclear proteins, including poly (ADP-ribose) polymerase-1 (PARP1), nucleolin, 14-3-3 protein major isoform, and adenosine tri-phosphate (ATP)–dependent DNA helicase II, among others (104) (Fig. 5). The proteins PARP1 and PARP2 play an important role in the epigenetic control of cell death and autophagy pathways, and PARP1 is key to repairing DNA strand breaks (102). In cultured cells, overexpression of nucleolin decreased, whereas reduction of nucleolin increased ultraviolet-damaged DNA repair efficiency. Interestingly, cotransfection with proliferating cell nuclear antigen rescued this effect, suggesting that the binding of proteins to nucleolin could be a protective strategy to ensure DNA repair (103). As for 14-3-3 proteins, these play critical roles in the regulation of cell fate through binding to proteins targeted by protein kinases. They are key to co-ordinating progression through the cell cycle and regulating DNA damage after injury (68). The ATP-dependent DNA helicase II enzyme is important for double-strand break repair and has a high affinity to nicks in double-stranded DNA (61). It binds to

chromosomal ends and recruits telomerase for telomere elongation and repair telomeric of ends even in the absence of telomerase (31). Although the precise mechanisms of binding are not yet described, these interactions would likely result in changes in DNA repair and synthesis.

In other studies involving prostate cells in culture, HO-1 was seen to directly interact with the transcription factor signal transducer and activator of transcription-3 (STAT3), forcing its cytoplasmic retention (30).

In preliminary reports, we also observed that HO-1 protein binds to a 40 kDa Nrf2 immunoreactive fragment. This interaction was associated with nuclear migration of this Nrf2 fragment (Biswas, C., unpublished). This could have significant implications on oxidative gene regulation.

Others have shown that oligomerization of HO-1 protein is essential for its stability in the endoplasmic reticulum. Although HO-1 has enzymatic function as a monomer, it can also form dimers and oligomers when it is anchored to the endoplasmic reticulum. This oligomerization cannot occur when HO-1 is truncated and lacks the c-terminal amino acids. Therefore, this c-terminal sequence may be essential for the self-assembly of HO-1 in the endoplasmic reticulum (44).

Role of Post-translational Modifications of HO Protein in Signaling

Based on computational analysis, phosphorylation of HO-1 could occur at various residues on the protein (77). In fact, Salinas *et al.* (77) demonstrated that protein kinase Akt/PKB can phosphorylate recombinant human HO-1 *in vitro* and in HEK293T cells at Ser188 (78). Interestingly, this phosphorylation only slightly changed the activity and binding affinity of the HO-1 protein. Perhaps another function of HO-1 was enabled by this phosphorylation, but this was not tested.

Subcellular Localization of HO Protein and Signaling

More recently, the localization of HO-1 has been intensively studied in the context of oxidative stress and cancer. Although HO-1 is an integral protein of the smooth endoplasm reticulum, it can localize to other compartments, including caveoli, mitochondria, and the nucleus, where it can mediate signaling functions (Fig. 6). When HO is anchored to the smooth endoplasmic reticulum, it faces the cytosol, and, therefore, heme breakdown products are generated in the cytosol. This facilitates iron recycling to ferroportin for iron export and to ferritin for iron storage (39).

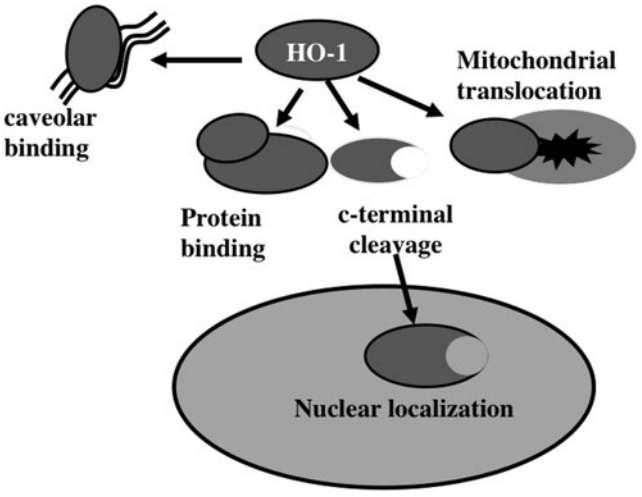

FIG. 6. Subcellular localization of HO-1 protein. The HO-1 protein can translocate to multiple subcellular compartments, including mitochondria and caveoli, and it can migrate to the nucleus after c-terminal cleavage.

Nuclear localization

We have shown that HO-1, under hypoxic and hyperoxic stress, can migrate to the nucleus, where it can alter transcription factor activation and regulate its own expression. In cultured 3T3 cells exposed to hyperoxia, nuclear localization of HO-1 protein was observed (56). In the nuclear extracts of cells transfected with a FLAG-HO-1 cDNA exposed to hypoxia and eluted on a FLAG column, a peak with a molecular mass of 34 kDa was recovered, which was identified as HO-1 protein lacking the C terminus by liquid chromatography/tandem mass-spectrometry (LC/MS/MS). After deduction of the FLAG molecular weight, the nuclear HO-1 fragment had a mass corresponding to the first 237 amino acids of rat HO-1 protein (56). Using an antibody directed toward the C terminus of HO-1, loss of all HO-1 immunoreactivity was noted in the nuclear extracts, suggesting that the nuclear form of HO-1 does not contain the C terminus (55). The mechanism by which HO-1 was released from its C-terminal anchor in the smooth endoplasmic reticulum was considered cleavage at a PEST-like domain in the protein, thereby enabling its transfer to the nucleus (55).

In HEK cells, coexpression of cytochrome P450 reductase prevents the translocation of HO-1 under hypoxia, but has no effect on HO-2 cellular distribution. This coexpression resulted in inactivation of HO-1 enzymatic activity, whereas the activity of HO-2 was not altered. This suggests that mutations of cytochrome P450 reductase could promote nuclear HO-1 accumulation and could lead to changes in signaling (57).

Others investigated whether HO-2 can also migrate to the nucleus. Cells transfected with amino terminal green fluorescent protein (GFP) fusion proteins of HO-2 demonstrate a perinuclear distribution of HO-2-GFP signal and homogeneous cytosolic and nuclear fluorescence with mutation of the C-terminus of HO-2.

One protein that binds to nuclear HO-1, heterogeneous nuclear ribonucleoprotein K (HnRNPK), is a modulator of DNA transcription and of protein translation, depending on its distribution in the nucleus or the cytoplasm (20). Under nonstimulated conditions, hnRNPK is found predominantly in the cytoplasm of lung cells. When HO-1 cDNA was overexpressed in the nucleus of alveolar type II cells in newborn mice, HNRPK migrated with HO-1 to the nucleus, whereas when HO-1 was disrupted, HNRPK was found predominantly in the cytoplasm of lung cells (104). The function of HnRNPK in the cytoplasm is to inhibit protein translation, whereas its function in the nucleus is to modulate DNA transcription in association with b-catenin (20). Therefore, subcellular distribution of HO-1 could alter transcription and translation, not as a transcription factor or as an RNA binding protein, but through its interactions with other proteins, including HNRMPK. In the HO-1 null mutant mice, there was significant upregulation of various lung genes without an accompanying change in the corresponding protein,

suggesting dysregulation and dysynchrony of transcription and translation (104).

Our group has also shown that HO-1 can bind to nuclear DNA repair enzymes, which could result in altered DNA damage (104) during oxidative stress and tumorigenesis. In fact, other preliminary studies show that in HO-1 null MEF cells stably transfected with nuclear HO-1 cDNA, exposure to hyperoxia results in protection against oxidative DNA damage (Fernando, P.A., unpublished observations).

Overall, the physiological role of nuclear HO-1 in oxidative stress may be to modulate and enhance the transcription of important antioxidant proteins and to improve DNA repair protein function.

In HO-2, a perinuclear distribution of the GFP fusion protein is seen. However, when the carboxy terminal deleted form of HO-2-GFP was transfected, the signal was distributed between the cytoplasm and the nucleus (57), suggesting that HO-2 also can translocate to the nucleus.

Mitochondrial localization

In lung epithelial cells exposed to cigarette smoke, subcellular compartmentalization of HO-1 was demonstrated. With exposure to cigarette smoke, lipopolysaccharide, or hemin, HO-1 protein increased in cytosolic and mitochondrial fractions of human alveolar or bronchial epithelial cells (80). The authors concluded that this may serve to protect against mitochondrial-mediated cell death, because HO activity increased with mitochondrial localization, in contrast to nuclear localization of HO-1 where HO activity decreases (80). Nevertheless, the precise mechanism of action of metabolically active mitochondrial HO-1 has not been elucidated.

In rats, indomethacin treatment triggered HO-1 translocation to the mitochondria of the gastric mucosa (19). This was associated with a decreased level of mitochondrial protein and lipid peroxidation. The enhanced mitochondrial translocation of HO-1 was also correlated with the amelioration of gastric mucosal injury.

The precise mechanism by which HO-1 localizes to the mitochondria was recently clarified. The distribution of mouse HO-1 from the endoplasmic reticulum to the mitochondrial-associated membrane (MAM) depends on efficient lipid modification through palmitoylation. To corroborate this, a palmitoylation inhibitor, 2-bromopalmitate reduced MAM enrichment of HO-1 protein (60).

Caveolar localization of HO-1

Caveolae-rich fractions from mesenchymal cells that were treated with various stimuli contained both HO-1 and caveolin-1 or caveolin-2 (47). In the rat, an interaction between caveolin-1 and HO-1 involving the caveolin scaffolding domain at amino acids 82–101 has been demonstrated. This plays an essential role in caveolin function and alters the enzymatic kinetics of the HO reaction. When hemin binds to HO-1, this interferes with the HO-1/caveolin binding (89). In fact, HO activity dramatically increased in cells expressing caveolin-1 antisense transcripts, suggesting a negative regulatory role of caveolin-1 in the regulation of HO activity. Interestingly, colocalization experiments in endothelial cells from the control and rats exposed to chronic hypoxia demonstrated an association between HO-2, caveolin-1, and large-conductance calcium-activated potassium channels (75), suggesting that both isoforms of HO can localize to caveolae.

Autoregulatory Signaling Function of HO-1

In our studies evaluating the function of the nuclear form of HO-1 protein, it was noted that nuclear localization of HO-1 decreased HO activity in cells exposed to hypoxia (55). In addition, an enzymatically inactive mutant form of HO-1 protein that was delivered into the cells was able to regulate a variety of transcription factors, including activator protein-1 (AP-1), AP-2, and Brn3, to a similar extent as the active form of the protein (56). However, only the native active HO-1 protein was able to modulate other transcription factors, including STAT1, STAT3, and STAT4. In contrast, the DNA binding activity of the transcription factor nuclear factor kappa B (NFκB) was downregulated after the delivery of both active and inactive forms of the HO-1 protein, as was that of stimulatory protein-1 (56). This may be the mechanism by which HO-1, despite having no characteristics of a transcription factor, alters gene expression (Fig. 7). These data also indicate that the activity of HO-1 is not required for its signaling function such as transcription factor activation.

Interestingly, since HO-1 protein enhanced the transcriptional activity of the transcription factor AP-1, it served to regulate the transcription of its own gene (56). In fact, the AP-1 sequence is an essential part of the ARE, which is found in abundance on the distal enhancer region of the HO-1 promoter (3). Therefore, the transfection of either active or inactive HO-1 protein altered HO-1 promoter activation. This suggests that transcription factor activation provides a mechanism for autoregulatory modulation of HO-1.

Another interesting characteristic of HO-1 is its ability to alter phosphorylation. After focal cerebral ischemia, treatment with Ad-HO-1 significantly inhibited cerebral inducible nitric oxide synthetase protein expression without affecting endothelial nitric oxide synthetase (eNOS). In fact, Ad-HO-1 preserved NO bioavailability by increasing eNOS phosphorylation, thereby preserving NO bioavailability (22). In addition, Ad-HO-1 administration was associated with decreased superoxide production and peroxynitrite generation. The

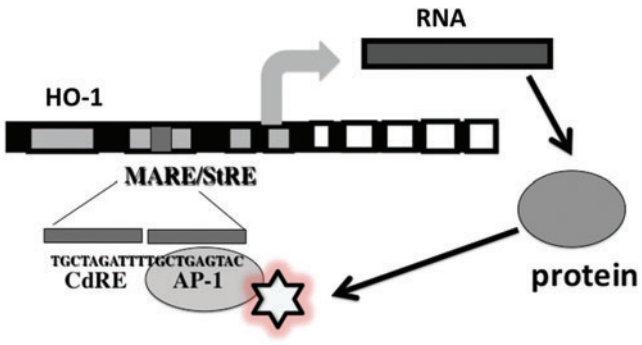

FIG. 7. Self-regulation of HO-1. The HO-1 protein can regulate its own expression *via* activation of AP-1 (star). Since the HO-1 gene contains antioxidant response elements on its distal enhancers, which contain the AP-1 consensus binding site, this leads to enhanced HO-1 transcriptional activation. AP-1, activator protein-1. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

mechanism by which the HO-1 protein modulates eNOS phosphorylation is not yet clear, because HO-1 is not a kinase. Nevertheless, there are multiple putative phosphorylation sites on the HO-1 protein that may be involved and, as mentioned in a previous section, recombinant human HO-1 protein can be phosphorylated by Akt/PKB at Ser188 (78). How this affects cellular signaling needs to be further understood.

Clinical Relevance of HO-1 Signaling

Examples of how modulation of HO-1 can have clinically relevant effects continue to amass. Several investigators have also shown that HO-1 is preferentially found in the nucleus in a variety of cancer models (Fig. 8). In prostate cancer, metastatic cells demonstrate nuclear localization of HO-1, whereas this is not the case in benign prostatic hypertrophy (40). Similarly, in head and neck cancer, with malignant progression, HO-1 immunoreactive protein localizes to the nucleus (36). The mechanism by which tumor progression is mediated by nuclear localization of HO-1 is not addressed in these publications; however, more recently, Elguero *et al.* (30) demonstrated that HO-1 protein can bind to the transcription factor STAT3, thereby enhancing its cytoplasmic retention and altering signaling in tumor cells of the prostate. Since STAT3 regulates cell proliferation, migration, and invasion *via* downstream genes that are associated with inflammation and angiogenesis, binding to HO-1 and subsequent inhibition of STAT3 signaling (30) may help explain how the binding of HO-1 and STAT3 could mediate tumor progression. Another mechanism by which HO-1 would modulate tumor proliferation and progression may be through the inhibition of NFκB activation as seen in prostatic cancer cells, thus preventing angiogenic activity (30). These observations were interpreted to mean that preferential distribution of HO-1 in the nucleus is a cytoprotective response to prevent further metastatic transformation. Nevertheless, the up-regulation of factors that enhance proliferation and angiogenesis may result in enhanced tumorigenesis. In fact, investigators have documented that nuclear HO-1 enhanced cytoprotection of myeloid leukemia cell against a chemotherapeutic agent by mitigating the formation of reactive oxygen species (90). In addition, in non-small cell lung cancer, high expression of nuclear HO-1 was also associated with tumor invasiveness and poor clinical outcome (93), further contradicting the suggestion that nuclear HO-1 is protective to cancer cells, enabling their proliferation. In other preliminary experiments, HO-1 was seen to bind to an NrF2 immunoreactive fragment and through this binding, altered cellular metabolism through preferential induction of certain downstream genes of NrF2 such as G6PDH. This was associated with a change in the ability to withstand glucose deprivation, suggesting a preferential utilization of the hexose monophosphate shunt pathway (Biswas, C., unpublished observations). This is another mechanism by which cancer cells are protected against death and continue to proliferate (17). Overall, the nuclear localization of HO-1 could have significant physiologic effects in tumor progression.

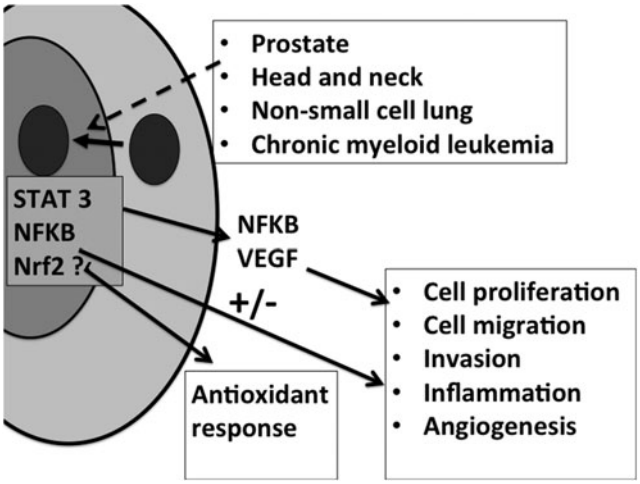

FIG. 8. Putative role of nuclear HO-1 in cancer. In various cancer models, HO-1 migrates to the nucleus, where it can activate transcription factors, which then regulate the expression of genes that are involved in cell proliferation, migration, and invasion as well as inflammation, angiogenesis, and antioxidant response. There is still a debate as to whether this provides cytoprotection to the cancer cell or to the host.

In Alzheimer disease, there was induction of HO-1 and increased serine phosphorylation on the HO-1 protein, suggesting that it may be subjected to oxidative damage itself and undergo post-translational modifications in brain tissue (15). Further, HO-1 is cytoprotective in cerebral ischemic injury, as it can preserve NO bioavailability and prevent oxidative stress (22). These data suggest that strategies which modulate HO-1 could be clinically used in brain neuroprotection.

Although not yet used in clinical practice, there is great interest in the beneficial effects of CO against organ transplant rejection, as it can act on the host to suppress rejection, on the graft to enhance engraftment, and on the donor to modulate immunoreactivity (8).

HO provides protection against acute lung injury and acute respiratory distress syndrome, which are major causes of morbidity and mortality. It acts as an inducible defense mechanism that can protect lung cells and tissues against injury. Lung protection by HO-1 has been demonstrated in several models of acute lung injury and sepsis. The protective effects of CO therapy have been demonstrated in acid-induced lung injury, ventilator-induced lung injury, endotoxin challenge, and cecal ligation and puncture-induced sepsis. Pretreatment of mice subjected to pneumonia-induced sepsis with zinc deuteroporphyrin 2,4-bis glycol, a nonspecific HO inhibitor, increased neutrophils in the bronchoalveolar spaces, reduced the bacterial load at the site of infection, prevented the modulation of cytokines and neutrophil chemoattractants, and decreased alveolar collapse, thereby improving pulmonary mechanics and survival rate (26). Despite some evidence of therapeutic benefit in animals, there are also reports of detrimental effects, and the efficacy of CO and/or modulation of HO activity in humans with sepsis-related lung dysfunction remains unclear. Controlled clinical trials will be needed.

During pregnancy, HO appears to play a key role in proper placentation and fetal growth. In placentas from patients with pregnancy complications such as recurrent miscarriages, spontaneous abortions, and pre-eclampsia, HO-1 expression was reduced (10). In addition, End-tidal breath CO levels were lower in women with pregnancy-induced hypertension compared with controls (16). These effects are not only attributable to the vasodilatory effects of CO but may also be due to the effects of HO on vascular endothelial growth factor and the soluble vascular endothelial growth factor receptor-1

receptor Flt-1 (2). Even a partial deficiency of HO-1, as seen in the heterozygote mice, was associated with delayed placental and embryonic development (105).

The examples cited earlier demonstrate that HO signaling, either through its byproducts or perhaps *via* the protein itself, can modulate clinically relevant disease processes.

Conclusions

In summary, HO-1 is a pleiotropic protein with multiple roles, including a heme degradation function leading to the formation of antioxidant bile pigments and of the important signaling gas CO as well as a nonenzymatic signaling function, resulting in the regulation of gene expression, modulation of protein translation, and binding to DNA repair proteins. Understanding these roles and finding therapeutic tools to specifically alter them could lead to clinical interventions that prevent oxidative injury and repair, tumor progression, and cell proliferation.

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Abbreviations Used

AP-1 = activator protein-1
AREs = antioxidant response elements
ATP = adenosine tri-phosphate
BVR = biliverdin reductase
CO = carbon monoxide
eNOS = endothelial nitric oxide synthetase
GFP = green fluorescent protein
H_2O_2 = hydrogen peroxide
hnRNPK = heterogeneous nuclear ribonucleoprotein K
HO = heme oxygenase
Keap-1 = kelch-like ECH-associated protein 1
MAM = mitochondrial-associated membrane
MAP = mitogen-activated protein
LC/MS/MS = liquid chromatography/tandem mass spectrometry
NFκB = nuclear factor kappa B
NO = nitric oxide
NrF2 = nuclear factor (erythroid-derived 2)-like 2
PARP = poly (ADP-ribose) polymerase
PEST = proline (P), glutamate (E), serine (S), and threonine (T)
STAT = signal transducer and activator of transcription
TLR = toll-like receptor