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Review

30 some years of heme oxygenase: From a "*molecular wrecking ball*" to a "*mesmerizing*" trigger of cellular events ☆

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Abstract

In the beginning, the microsomal HO system was presumed to be made of one isozymes, now known as HO-1, which was cytP450-dependent; and, was thought to be of physiological significance solely in the context of catalysis of hemoglobin heme to bile pigments and CO. A succession of discoveries including characterization of the system as an independent mono-oxygenase, identification of a second form, called HO-2, free radical quenching activity of bile pigments, analogous function of CO in cell signaling to NO, and characterization of the system as HSP32 cognates has led to such an impressive expansion in the number of reports dealing with the HO system that surpass anyone's expectation. This review is a compilation of certain older findings and recent events that together ensure placement of the HO system in the mainstream research for decades to come.

Keywords: HO-1 and HO-2; Biliverdin reductase; HSP32 cognates; Intracellular gas sensor; Biliverdin/bilirubin; Carbon monoxide; Hypoxia/heme regulated enzymes

Introduction and background

Historically, enzymes that catalyze a degradation process within the cell are not necessarily held in as high regard as, and therefore do not attract the attention that is lavished upon the anabolic catalysts. The heme oxygenase (HO) system, which oxidatively cleaves heme (Fe-protoporphyrin IX) to produce CO, biliverdin, and free iron, fell into the low interest category for nearly two decades following two rather brief periods of notoriety: the initial excitement that it generated when it was described as a new member of the cytP450 family [1] and the subsequent finding that it is a rapidly and transiently inducible mono-oxygenase that functions independent of cytP450 [2]. Expansion of the number and categories of stimuli that could enhance heme degradation activity in subsequent years was all viewed in terms of their relevance to hemoglobin, cytP450 turnover, and drug metabolism [3].

The image began to change when, in rapid succession, a number of discoveries were made: a second and constitutive form of the enzyme, *HO-2*, was identified [4,5], with prominent presentation in the brain, which prompted the suggestion that "*HO in the brain has functions other than heme degradation*" [6]. Meanwhile nitric oxide, a known toxic gas, was dominating the stage as the result of recognition of its ability to activate soluble guanylate cyclase (sGC) and consequently, modulate a wide spectrum of cellular functions [7]; activation of sGC was shown to require NO-heme binding [8]. CO that, like NO, is a toxic heme-Fe binding gas was shown to activate sGC and inhibit platelet aggregation [9]; the catalyst for its production, HO-2, was found to be abundantly expressed in neurons [10]. The likelihood that CO generated in course of degradation of heme could itself function in vivo as an activator of sGC and hence cGMP production was hypothesized and was extended to apply to neurons [10,11]. The concept of CO functioning as a signal for cGMP production in brain

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and the cardiovascular system was upheld by experimental data [12–16]. The initial reports prompted an editorial writeup in *Science* [17] entitled "*Carbon Monoxide: killer to brain messenger in one step*" and predicted "*the gas is likely to provide fuel to run plenty of laboratories*," a prediction that has been validated beyond expectations. As the CO secrets were unraveling, bilirubin, the product of biliverdin reductase (BVR), activity was generating interest of its own when it was described as a chain-breaking inactivator of oxygen free radicals [18]. Bilirubin too had until then been studied primarily in the context of its neurotoxicity to the newborn.

In light of these developments, on-demand, rapid, and transient increase in CO and bilirubin production came to be considered important to cytoprotection, and hence redefining the HO enzymes as members of the HSP family of proteins [19].

Throughout these developments, BVR remained in the shadow of HO enzymes, and only a handful of laboratories showed interest in the enzyme. The unique dual pH/cofactor requirement of the reductase [20], however had remained the backdrop for uncovering its functions as a regulator of HO-1 activity and a link between heme degradation and cell signaling [21–24]. Recent investigations have identified several paths by which BVR regulates *HO-1* expression. BVR is an evolutionarily conserved enzyme that has a multitude of functions in the cell, not limited to catalyzing the final step in the heme metabolic pathway. Being a member of select group of dual specificity [25] kinases, i.e., a serine/threonine/tyrosine kinase and proximal to insulin receptor (IR), enables BVR to have input in signaling by both the insulin and MAPK signaling pathways [22].

The potential role of BVR in phosphorylation of HO proteins is consistent with reports on the occurrence of protein:protein interaction between *HO-1* and BVR [26–28]. Because HO-1 and HO-2 are phosphoproteins [29], it is most likely that BVR is not only a transcription factor for *HO-1* oxidative stress response but is also a component of the cascade of kinases involved in phosphorylating HO isozymes. Phosphorylation status of a protein can influence its stability and/or activity.

This review is not intended to be a comprehensive account of the developments in the past 30 some years in the field. The space limitation and the expansion of developments in the field are prohibitive to referencing many scholarly contributions that have been made in those years. In the following, certain older, basic concepts and recent findings are highlighted in tandem, with a good deal of emphasis on the role of BVR in regulation of *HO-1* oxidative stress response.

What is a "heme oxygenase"?

To date, protein or proteins in any form of life that possess a sequence similar to those of the 23 amino acids that was first defined in the rat HO-1 and HO-2 as "heme-binding pocket" and is now known as the "heme oxygenase signature" [5,30,31] are classified as a member of the HO family (Fig. 1). The basic sequence, which is made of mostly hydrophobic residues with a conserved histidine residue [32], is preserved among animal species. The protein need not be catalytically active, as is the case with a variant of *HO-2*, known as *HO-3*, that has been described in the rat brain [33]; no homologue of this gene is found in either humans or mice and it is best characterized as a processed pseudogene [34,35]. Outside the "heme-binding pocket," the extent of sequence identity between HO-1 and HO-2 is limited to less than 50%. This dramatically decreases when plants and animals are compared (Fig. 2). The HO sequences in plants show conservation of the "heme-binding pocket" among themselves. However, the plant and vertebrate consensus sequences do show considerable divergence from each other, as might be expected given the ancient separation of these lineages.

Oxidation of heme by the HO proteins requires concerted activity of NADPH-cytP450 reductase (Fig. 3) for providing reducing equivalents for supporting the reduced state of iron (Fe^{2+}) and activation of molecular oxygen. The specific orientation of bound heme is crucial to specificity of cleavage of the porphyrin ring at the α meso carbon bridge. The reaction requires 3 mol of oxygen and 7 electrons. The rate-limiting step in oxidation of heme is the release of product, biliverdin [36].

Regulation of *HO-1* gene expression

Great strides have been made in defining cellular events that govern *HO-1* regulation. Arguably, *HO-1* gene transcription is activated by far a greater number of stimuli than that of any gene [3]; in fact, only a handful of agents, among them being its own products, reduce *HO-1* transcription. The overwhelmingly vast number of stimuli that activate *HO-1* reflects the presence of multiple response elements within its promoter that bind activated factors and the multiplicity of interactions between components of the cell signaling cascades. Characterization of these interactions has culminated in emergence of a highly integrated profile for regulation of *HO-1*. A number of eloquent reviews and reports have been published on this topic [37–45].

MAP kinase pathway was the first recognized signaling pathway for linking extracellular stimuli to stress-mediated induction of *HO-1*. MAP kinases form one arm of the insulin IGF-1 signaling pathway and various components of the three main paths of MAP kinase signaling—JNK, P38, and ERK—are involved in a vast network of interactions among themselves and with an assortment of serine/threonine/tyrosine kinases. Within the MAP kinase pathway, the majority of factors that control oxidative stress response of *HO-1* expression are members of basic "leucine zipper" (bzip) transcription factors. Because these factors can form heterodimeric complexes with other protein partners, as well as forming homodimers, a rather complex picture for regulation of the *HO-1* gene expression emerges; indeed, much of the picture is unclear at this time. The

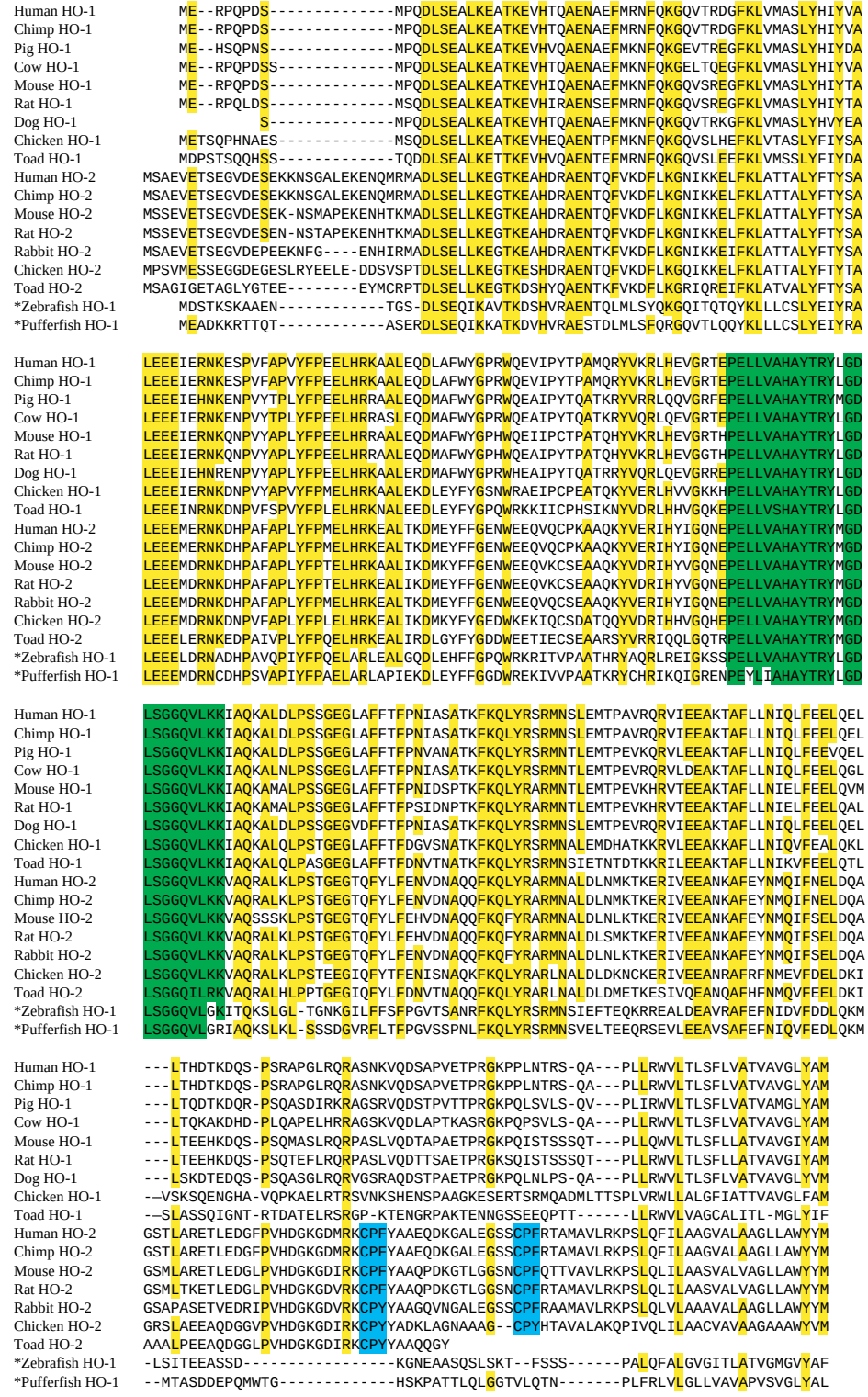

Fig. 1. Alignment of vertebrate HO sequences. Two consensus sequences (for HO-1 and HO-2) were derived in preliminary alignments—where residues in the combined alignment are identical to those in both consensus sequences, they are highlighted in yellow. The "heme-binding pocket/HO signature" motif is highlighted in green. The heme-binding sites specific to HO-2 are indicated in blue. Asterisks indicate two fish species (*Danio rerio* or *Tetraodon nigroviridis*), neither of which appears to have an HO-2 gene, despite virtually complete genome sequences. The "toad" sequences are from *Xenopus laevis*.

availability of the dimeric partner of a bzip factor determines its preference for DNA-binding site. Therefore, through exchange of heterodimerization partner the bzip transcription factors can control regulation of the expression of a far greater number of genes than controlled by a given homodimer.

BVR is a most recently identified member of the bzip transcription factor family [46] and a component of the

Human HO-1	PELLVAHAYTRYLGDLSGGQVL-KK
Human HO-2	PELLVAHAYTRYMGDLSGGQVL-KK
Pea HO-1	PQAFICHFYNIYFAHSAGGRMIGKK
Tomato HO-1	PQAFICHFYNTYFAHSAGGRMIGKK
Arabidopsis HO-1	PQAFICHFYNIYFAHSAGGRMIGRK
P. taeda HO-1	PPAFLCHFYNVYFAHTAGGRMIGRK
Arabidopsis HO-3	PQAFICHFYNIYFAHSAGGQMIGTK
Arabidopsis HO-4	PPAFICHFYNINFAHSAGGRMIGTK
Soybean HO-1	PQAFICHFYNIYFAHSAGGRMIGKK
Soybean HO-3	PQAFICHFYNIYFAHSAGGRMIGKK
Tomato HO-2	PRLFLSHFYNIYFSHIAGGQVIAKK
Sorghum HO-2	APAFLSHYYNIYFAHITGGVAIGNK
Sorghum HO-1	PQAFICHFYNVYFAHTAGGRMIGKK

Fig. 2. Alignment of "heme-binding pocket/HO signature" motif of animal and plant heme oxygenases. The residues in the 23 amino acid motif that are common to all vertebrate heme oxygenases are highlighted in green, those found typically in vertebrate HO-1 are indicated in gray, those typical of HO-2 in blue, and those conserved in plants in yellow.

signaling pathway that functions in both major arms of insulin/IGF-1 signaling (MAP kinase and PI3-K) [22,23,46]. It is becoming increasingly evident that BVR may serve as a mechanism for integrating both arms of the insulin/IGF signaling pathway for regulation of *HO-1*.

A large number of genes that factor into the regulation of *HO-1* gene expression appear to be subjected to regulation by BVR [21]. The target genes were identified by over-expressing the enzyme in human kidney cells (h293A cells) using an adenovirus construct of the human BVR and analyzing the expression profile of cell signaling genes by microarray [21]. The identified target genes included ATF-2/CREB-2 (cAMP response element-binding protein), c-Jun, and HSF1 (heat shock transcription factor 1), Bcl2, Cox-2, PKCα, heat shock proteins 90 and 27, and WISP3 (inducible signaling pathway protein 3).

Because ATF-2 has a central role in cell signaling whereby it functions as a common transcription factor for expression of cAMP responsive genes, its confirmed regulation by BVR [23] was of particular importance for defining potential interactions among cell signaling pathway components in regulation of *HO-1* gene expression. ATF-2 is a constitutive transcription factor whose expression, unlike that of c-Jun (which is inducible), is not dependent on extracellular signals [47–49]. ATF-2, like BVR and the transcriptional factors c-Fos (Fos-like), c-Jun, c-Myc, Bach-1, Maf, NF-E2, is a bzip protein, and binds DNA in a dimeric and heterodimeric form. BVR regulates ATF-2 activity by increasing the levels of its transcript, protein, and phosphorylation. A direct interaction of BVR with the *ATF-2* promoter region cAMP response element (CRE) site underlies the mechanism of transcription and activation of *ATF-2* [23]. The CRE-binding site differs from the 7 base pair AP-1 site (TGACTCA) by one additional base; BVR in a homodimeric form also binds to

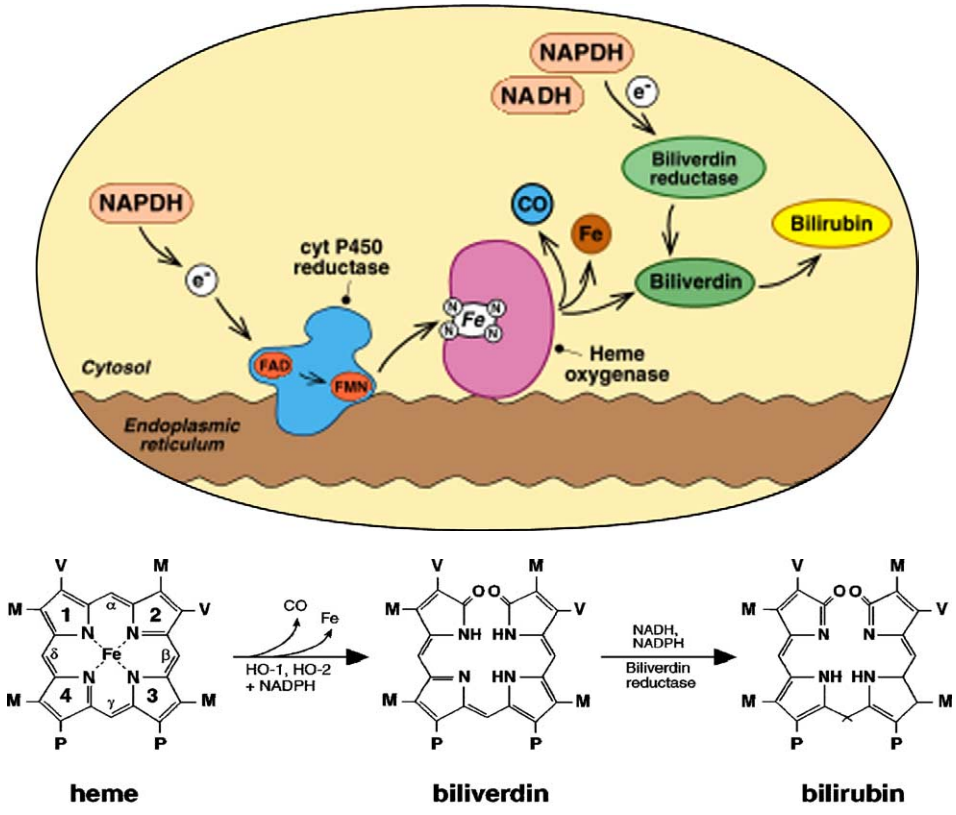

Fig. 3. The pathway of heme degradation in mammalian cells.

AP-1 site [46]. c-Jun binding to AP-1 site activates expression of oxidative stress-response genes; *c-Jun* is among those genes whose transcriptional activation by BVR has been confirmed [23]. This may also play an important role in the regulation of *c-Jun* gene expression by influencing the known autoregulatory transactivation by the c-Jun homodimer. The members of the ATF/CREB family were originally described to specifically bind to a palindromic sequence, TGACNTCA.

Although activation of the MAPK signaling pathway and c-Jun/c-Fos-AP-1 binding is a key mechanism for the induction of *HO-1*, the latter is also responsive to cAMP [50]. Accordingly, an increase in ATF-2 activity could directly affect *HO-1* expression through activation of CRE and/or AP-1 sites. Biliverdin, the substrate for BVR, both impairs AP-1-BVR complex formation in vitro [23] and inhibits HO enzyme activity in vivo [51]. Biliverdin is the only normal cell constituent identified to date to inhibit HO activity both in vivo and in vitro.

When levels of ATF-2 are increased, it effectively competes with c-Fos, the usual dimer partner of c-Jun. The ATF-2/c-Jun heterodimer preferentially binds to the 7 base AP-1 sites rather than ATF-2's usual site [48]. Moreover, the ATF-2/c-Jun dimer–DNA complex is more stable than c-Fos/c-Jun dimer–DNA complex [52,53].

Heterodimerization not only influences the binding site of ATF-2 on DNA and its binding affinity [53], but also gene regulation activity of the dimeric partner. The ability of BVR to induce *ATF-2* gene expression therefore is likely to change the gene expression profile in the cell. For instance, heterodimerization with c-Jun will likely result in sustained changes in expression pattern of genes that control growth factors, chemokines or cytokines all of which are inducers of *HO-1* expression [47]. Furthermore, members of the ATF/CREB family of transcription factors also bind constitutively to the hypoxia-inducible factor-1 DNA recognition site (HIF-1) [54]. By extension, a function for BVR in regulation of hypoxia-inducible expression of *HO-1* can be predicted. Similarly, it is reasonable to suggest that binding to enhancer elements of target genes for the two classes of NF-κB, and their family of homo- or heterodimeric forms, would be affected by an increase in ATF-2 in the cell. Activity of NF-κB, a regulator of iNOS expression and a component of the signaling pathways that lead to conditions such as vascular inflammation and atherogenesis [55,56], is also influenced by ATF-2 [57]. NO radicals are among known inducers of *HO-1*.

An early observation with regard to the regulation of *HO-1* expression was that a common denominator for agents that induce *HO-1* expression is the ability to lower the intracellular thiol/disulfide (SH/S–S) ratio [58,59]. The molecular basis of this observation can now be explained by identification of the role of the redox-sensitive transcription factors—inducers of gene expression, AP-1 and NF-E2 (nuclear factor erythroid 2-related factor-2, Nrf2), and repressor factor, Bach-1—all of which are bzip factors, in mediating *HO-1* response to oxidative stress and hypoxia. Bach-1 is a hypoxia-inducible gene. The ratio of GSH/GSSG in the cell dictates NF-E2 binding to ARE (antioxidant response element) or STRE and activation of HO-1. NF-E2 is retained in the cytoplasm through binding to Keap1, but enters the nucleus when released from Keap1 after cells are exposed to oxidative stress with the ensuing oxidation of Keap1 cysteine residues [41,60,61].

In all likelihood BVR influences *HO-1* expression by controlling gene repressor activity of Bach-1. Bach-1 is a heme-regulated transcription repressor that forms a heterodimeric complex with a small Maf protein, an activator of gene expression from AP-1/CRE recognition sites [62]. The heterodimer prevents Maf from recognizing the MARE sequence motif in DNA [63]. Because, as noted above, BVR also binds to AP-1/CRE elements [23,46], and Maf proteins heterodimerize with the AP-1 family of transcription factors [62], formation of a BVR-Maf complex, should it occur, would block the repressor activity of Bach-1 and allow induction of *HO-1*, consistent with that seen in ischemia/reperfusion injury [64].

Regulation of *HO-2* gene expression

In contrast to the structure and organization of the *HO-1* gene, which has a simple composition of five exons and four introns, that of mammalian *HO-2* is among the most complex gene structures [65,66]. Five or more transcripts for *HO-2*, ranging in size from 1.3 to 2.4 kbp, are present in mammalian tissues, with tissue-dependent patterns of display and abundance [65]. Two transcripts of 1.3 and 1.9 kbp are the most ubiquitous species. The transcripts arise from use of two different poly(A) signals that are separated by 560 nucleotides and by alternative splicing of the first exon. The minus strand of intron 1 contains a nested sequence of 1046 nt with 87% identity to the cDNA encoding human non-histone chromosomal protein, HMG17. As such, *HO-2* remains to date the only mammalian gene to harbor a nested gene. Interestingly, the glucocorticoid element (GRE) is the only demonstrated functional response element in the promoter sequence of *HO-2* [67–69]. Opiates also increase *HO-2* protein levels [70], however, the molecular basis for the increase is not known.

For preservation of the gene pool of a species, expression of those genes that play a pivotal role in cellular homeostasis must be tightly controlled. Given that *HO-2* is by far the prominent form of HO protein in the brain and testis, the refractory response of *HO-2* to stimuli that induce *HO-1* is not surprising [71]. In addition to activation of GREs, the cellular levels of HO-2 are regulated through the control of mRNA stability and change in the ratio of transcripts; it is also noteworthy that those transcripts that use the second poly(A) signal are not effectively translated [6].

There is an extensive pattern of secondary structures (stem/loops) between the two HO-2 poly(A) signals

[66,72]. Stem/loops provide binding sites for proteins that control RNA stability and translation [73] as well as the use of poly(A) sites. In *HO-2*, the first poly(A) site is at the base of a stem/loop and the second is in the loop of another. Several of the stem/loop structures and a consensus sequence of TTTTTGCA are strategically placed between the 2 poly(A) sites. The latter is 100% identical to the oxygen/nitrogen sensing sequence that is also found in the erythropoietin gene [74].

HO-2 transcription levels are sensitive to hypoxia and redox status of the cell [12,65] and in all instances the ratio of the 1.3/1.9 kbp transcripts is affected. Accordingly, the dominant mechanism of regulation of HO-2 protein is through control of transcript species, rather than modulation of gene expression, as befits a protein whose function is the intracellular sensor of gaseous heme ligand signal molecules, O_2, NO, and CO [75–77]. We have detected the presence of one or two nuclear proteins that bind to the nucleotide sequence between the two poly(A) signals and their binding to this region is diminished in cells exposed to hypoxia, with a marked increase in the levels of the inefficiently translated 1.9 kbp *HO-2* mRNA. In transformed astroglial cells, the level of 1.9 kbp species is also markedly elevated [65]. These findings point to a pivotal role of nuclear proteins in tight control of HO-2 levels in the cell.

Function of HO-2 as the intracellular sensor of O_2, NO, and CO

To date, nearly 50 heme-based sensors for gaseous signaling molecules-O_2, CO, and NO, have been identified in bacteria, plants, and animals [78]. The sensors are predominantly present in bacteria; familiar examples include CooA, involved in CO metabolism, and Rhizobial FixLS, involved in nitrogen fixation. Aside from HO-2, few other gas sensors in mammals have been identified, including sGC and (N)PAS2 (PAS = first letter of 3 founding members of the family: PER, ARNT, and SIM, where PER is the product *Drosophila* period gene; ARNT is the AHR nuclear translocator; SIM is the product of the *Drosophila* single-minded locus).

Although both HO-1 and HO-2 actively catalyze the same heme degradation reaction [3], they are quite dissimilar in other molecular and biochemical properties. Aside from the overall differences in amino acid composition, a major difference is the presence of cysteine residues in all HO-2s and their absence in all HO-1s [31,79–82]. Cysteine is an amino acid that has high affinity for heme iron. To date, seven proteins have been identified to contain the "heme regulatory motif (HRM)," and these proteins have a heme/oxygen regulatory function in the cell [83]. An HRM consists of cysteine–proline dipeptide core flanked upstream by positively charged residues and downstream by the hydrophobic residue phenylalanine [83]. HO-2 has two copies of the HRM that tightly bind heme [81,82,84] giving the hemoprotein character to the enzyme, but are not required for heme oxidation activity of HO-2 [82]. Heme gaseous ligands, CO, O_2, and NO, have high affinity for the chelated iron of heme; of these, NO has the highest affinity [76,85,86]; hemoproteins, including hemoglobin, can also be nitrosylated [87]. Cysteine is the axial ligand for the heme prosthetic moiety in cytP450's and NO synthase isozymes. The initial indication that HO-2 functions as an intracellular gas sensor was provided by detection of high affinity NO binding to its HRM-bound heme [76]. This observation led to the proposal that HRMs function as "sinks" for the heme ligand and was extended to suggest function of HO-2 as an intracellular sensor of oxygen [75]. In fact, a recent report confirms this function of HO-2 for a calcium-sensitive potassium channel [77].

HO-2, a cytoneuroprotectant against NO and O_2 radicals

Because of the reactive nature of NO and oxygen radicals, they can readily cross biological membranes and target various cellular components. In the circulation, hemoglobin heme functions in binding and inactivating NO radicals. In the cell, a good case can be made for HO-2 functioning as a means to sequester and, hence inactivate free radicals. HO-2 is constitutively expressed in all cell types and most abundantly in neurons and testis Leydig cells and spermatocytes [10,88]. In addition, HO-2 colocalizes with NOS in neurons [89]. Low-level production of NO by the constitutive NOS functions as a signal molecule in neurons and endothelial cells [90–92]. However, brain cells are not devoid of the inducible form of NOS [68], which can produce a high-level burst of NO with formation of the highly reactive pro-oxidant NO radicals [93,94].

Aside from sequestering NO free radicals, HO-2 in three other ways can control these reactive species; both relate to its heme catalytic activity, thus sharing cytoprotective mechanisms with HO-1. First, by controlling the levels of heme and ergo, synthesis of NOS; second, by inhibiting iNOS expression and NO production by product of heme degradation, bilirubin [95]; and third, by CO inhibition of NOS activity. Of course, these functions are also carried out by HO-1, however, because, with exception of the spleen, under normal conditions *HO-1* is expressed at exceedingly low levels in all tissues and cell types [3], HO-2 activity becomes the primary source of defense against free radicals. It is noteworthy that transcriptional regulation of *HO-2* and *NOS* by glucocorticoids in brain shows a reciprocal relationship; *HO-2* is induced by glucocorticoids, *NOS* expression is repressed [68].

The same type of reasoning can be considered with respect to interaction between HO-2 and NPAS. NPAS, a transcription factor in the brain that possibly functions in neuronal signaling [96], binds heme, and formation of its complex with DNA is inhibited by low concentration of CO [78]. Therefore, again there are at least three mechanisms by which the HO system, particularly HO-2, can

influence NPAS transcriptional activity: control of heme levels needed for DNA-binding complex formation; production of CO to inhibit DNA binding of the complex and HO-2 HRMs competing with NPAS for heme and CO.

Function of the heme degradation products in cytoprotective capacity

Since the initial links between free radical quenching activity of bilirubin and function of CO as an activator of sGC were made [9,18], there has been an outburst of reports on the cytoprotective and signaling activities of heme degradation products. The outburst of interest in the HO system and its activity products are depicted by the exponential rate of increase in the number of publications dealing with the system, from a mere 8 in 1975 to 8729 to date. There are a number of eloquent reviews describing a wide range of functions of heme degradation products, particularly that of CO, in the cells that include their anti-inflammatory, proapoptotic, antioxidant activity, as well as participating in cell signaling, and maintenance of vascular homeostasis [45,97–102]. For the most part, studies have focused on heme degradation products resulting from induction of *HO-1*, a phenomenon that reflects its rapid and robust response to most stimuli. *HO-2*, of course, produces the same products and findings with regard to biological activity of CO, bile pigments, and Fe apply to both isozymes. As noted in the following, not all reports, however, uniformly associate induction of *HO-1* with a beneficial outcome to the cell.

On the balance, there is ample evidence for a cytoprotective function of bilirubin in the cell. The concept of a role of bilirubin as a cellular antioxidant, which was initially proposed by Stocker et al. [18], has been confirmed in a variety of settings. Indeed, low levels of bilirubin are reported to be associated with increased risk of coronary artery disease and bleaker outcome of heart attack in men [103]. Moreover, because of ability of bilirubin to inhibit immune effector function, the outcome of *HO-1* induction is considered as a potential means to suppress the inflammatory response and allograft rejection. The decrease in immune responses caused by bilirubin, however, can be viewed as either positive or negative depending upon the situation. Bilirubin inhibits IL-1 and -2 production, and decreases natural killer activity and antibody dependent cellular cytotoxicity, lymphokine-activated killing activity, and DNA synthesis; all desirable effects for suppression of inflammatory response. On the other hand, the same effects could be deadly in certain instances. For example, hyperbilirubinemia has been implicated as a prominent factor in mortality caused by sepsis in patients with obstructive jaundice and in neonatal jaundice [104]. Also, the time-honored concerns about cytotoxic effects of high levels of bilirubin on neurons and erythrocytes of the newborn persist [105,106]. Although increase in HO-1 is mostly considered as an antiapoptotic event, it also has been associated with apoptosis. The proapoptotic influence of HO activity can be construed as a negative effect, as is the case with bilirubin-induced apoptosis of developing neurons [107], or positively, in the case of chaperoning damaged neurons to the path of apoptotic death rather than necrosis [108]. Because in uninjured cells and/or transformed cells, increased HO-1 activity has antiapoptotic activity, in this instance inhibition of its activity can be effectively used as a target in anticancer treatment [109].

A similar argument can be made with respect to heme iron, that is, depending on the cellular milieu, HO activity can be considered as a proponent of oxidative stress, by liberating chelated iron from the heme molecule; or, as a means to generate an antioxidant. Increase in HO-1, loss of complex IV, and iron accumulation in the mitochondria are observed in Alzheimer's disease and in the aging brain [110,111]. Both heme and iron are catalysts for generation of the reactive oxygen radical; provided that the cellular capacity to sequester free iron in ferritin and transferrin is not compromised [112,113], an increase in HO-1 activity would be considered a cytoprotective response by removing free heme with its high rather non-selective high affinity for cellular constituents such as proteins, lipids, and chromatin. On the other hand, catalytically active free iron causing formation of reactive oxygen species by Harler–Weiss chemistry would have devastating effects in a cell, such that its metabolic activity would be compromised.

Aside from catalyzing formation of free radicals, a previously unknown mechanism by which chelatable iron, hence HO, plays a central role in redox regulation of cellular function in macrophage or similar cell type has been described [114]. As reported, ferrous ion plays a role in a feedback loop for synthesis of NO: the ion activates IκB kinase (IKK), which is also induced by peroxynitrite ($ONOO^-$), with subsequent activation of NF-κB and induction of NOS. One mechanism by which NF-κB is activated involves degradation of IκBα, itself a consequence of activation of IKK, causing phosphorylation of key serine residues of IκBα, followed by polyubiquination and degradation [115]. Elucidation of the role of iron in IKK activation indicates an intimate and interwoven link between the HO, NOS, and NF-κB signaling pathways for gene regulation and response to oxidative stress. Because it is only the chelatable form (free) of iron that activates IKK/NF-κB, and the availability of free iron in the cell (i.e., redox active iron) is for the most part dependent on HO activity and its release from the porphyrin ring, then the availability and the size of the iron pool would be the rate-limiting factor. In turn, activation of *HO-1* gene expression by NO radicals [56] would enlarge the pool. Because NOS isozymes are hemoproteins [116], the accelerated rate of heme degradation would bring to a halt increased production of NO radicals, as well as inflicting inhibitory effects of CO and bilirubin on NOS activity and production that were discussed above.

It would appear then that the role of HO system in cytoprotection may be summarized as: "*it all depends on the*

cellular milieu whether increase of HO activity is beneficial or detrimental to the cell.'' This would also help to explain the not so infrequently encountered contradictory reports on the topic.

Of course, bilirubin production is dependent on the activity of BVR. And, aside from producing the antioxidant, the protein has cytoprotective activity on its own. As noted above, the human BVR is a dual specificity kinase [22]; as such, most likely it can influence a wide range of functions and signaling pathways in the cell that are involved in cell death/survival. Consistent with this assessment is finding that BVR is a major component of cellular defense against arsenite-mediated apoptosis and a key component of *HO-1* response to oxidative stress [23,46]. Known inducers of *HO-1* gene expression, such as inflammatory cytokines, oxidative stress, and free radicals [117], enhance BVR transcription [117].

Arsenite is an established human carcinogen that causes chromosomal aberrations, sister chromatid exchange, and micronucleus formation [118]. Attenuation of BVR expression markedly increases both the proportion of apoptotic cells in arsenite-treated preparations, and also increases the levels of factors associated with apoptosis, including extramitochondrial cytochrome *c*, TRAIL, death-receptor-5 (DR-5) mRNA, and PARP. This supports a role for BVR in cytoprotection against antioxidants that goes beyond degrading heme and CO production by HO-1.

Clearly, BVR does not affect all mechanisms by which *HO-1* stress response is evoked and cytoprotection is offered. For instance, whereas siHO-1 does not influence arsenite-induced apoptosis in 293A cells [23], lung-specific siHO-1 enhances ischemia/reperfusion-induced lung injury [119]. Arsenite induces production of oxygen radicals, whereas ischemia/reperfusion injury has an initial hypoxic component. And, those stimuli activate different factors [120,121]. Thus, the profile of genes activated by arsenite and ischemia/reperfusion could differentially affect pathways of cell death and survival.

Because oxygen radicals are suspected to underlie the etiology of a long list of pathophysiological conditions, it would be reasonable to suggest that BVR with its multidimensional input into the regulation of oxidative stress-responsive genes may find a useful place in therapeutic settings. This concept is reinforced when the wide range of cellular functions that are modulated by activity of *HO-1* [75,99,122,123] and the prominent role in regulation of its expression are considered.

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