

FORUM REVIEW ARTICLE

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## New Insights into Intracellular Locations and Functions of Heme Oxygenase-1

Louise L. Dunn,<sup>1,2</sup> Robyn G. Midwinter,<sup>3</sup> Jun Ni,<sup>1,2</sup> Hafizah A. Hamid,<sup>1,2</sup>  
Christopher R. Parish,<sup>4</sup> and Roland Stocker<sup>1,2</sup>

### Abstract

**Significance:** Heme oxygenase-1 (HMOX1) plays a critical role in the protection of cells, and the inducible enzyme is implicated in a spectrum of human diseases. The increasing prevalence of cardiovascular and metabolic morbidities, for which current treatment approaches are not optimal, emphasizes the necessity to better understand key players such as HMOX1 that may be therapeutic targets. **Recent Advances:** HMOX1 is a dynamic protein that can undergo post-translational and structural modifications which modulate HMOX1 function. Moreover, trafficking from the endoplasmic reticulum to other cellular compartments, including the nucleus, highlights that HMOX1 may play roles other than the catabolism of heme. **Critical Issues:** The ability of HMOX1 to be induced by a variety of stressors, in an equally wide variety of tissues and cell types, represents an obstacle for the therapeutic exploitation of the enzyme. Any capacity to modulate HMOX1 in cardiovascular and metabolic diseases should be tempered with an appreciation that HMOX1 may have an impact on cancer. Moreover, the potential for heme catabolism end products, such as carbon monoxide, to amplify the HMOX1 stress response should be considered. **Future Directions:** A more complete understanding of HMOX1 modifications and the properties that they impart is necessary. Delineating these parameters will provide a clearer picture of the opportunities to modulate HMOX1 in human disease. *Antioxid. Redox Signal.* 20: 1723–1742.

### Introduction

**H**EME OXYGENASES (HMOX) are rate-limiting enzymes that degrade heme (iron protoporphyrin IX) to carbon monoxide (CO), ferrous iron ( $\text{Fe}^{2+}$ ), and biliverdin IX $\alpha$ . Biliverdin IX $\alpha$  is, subsequently, converted to bilirubin IX $\alpha$  by biliverdin reductase (BVR). HMOX enzymatic activity consumes three moles of molecular oxygen ( $\text{O}_2$ ) per mole heme oxidized with electrons originating from NADPH and supplied by cytochrome P450 reductase (CPR) (164). The catabolism of heme is schematically represented in Figure 1. Notably, HMOX use heme as both a substrate and a prosthetic group (195). As HMOX degrade heme, the major source of iron in our body, they play a key role in whole body iron recycling/homeostasis. In addition, HMOX are implicated in vascular biology and cellular protection against stress (155). More recently, HMOX

has been reported to activate the transcriptional machinery that drives the induction of antioxidant genes (27, 101), likely in part, independent of its enzymatic activity (27, 65). The convergence of these different properties stresses the importance of HMOX as a key agent that protects the cell.

The HMOX family is represented by two distinct enzymes: heme oxygenase-1 (HMOX1) and heme oxygenase-2 (HMOX2). Human HMOX1 and HMOX2 are paralogs, sharing ~42% similarity in their amino-acid sequences (29). Both proteins possess a common 24-amino-acid sequence known as the “heme-binding pocket” or “HMOX signature” that facilitates the catabolism of heme (110). While both proteins utilize the same substrate and cofactor, they are different in their physiological properties and regulation. For example, HMOX1 is induced in response to a variety of external stimuli, while HMOX2 is ubiquitously expressed.

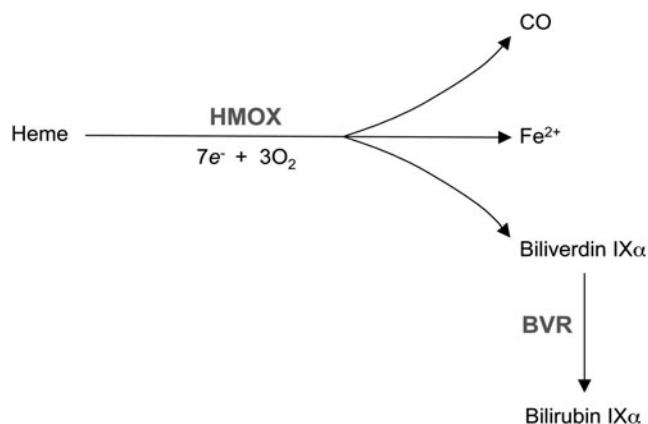
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<sup>1</sup>Vascular Biology Division, The Victor Chang Cardiac Research Institute, Darlinghurst, Australia.

<sup>2</sup>Faculty of Medicine, The University of New South Wales, Sydney, Australia.

<sup>3</sup>Sydney Medical School, University of Sydney, Camperdown, Australia.

<sup>4</sup>John Curtin School of Medical Research, The Australian National University, Canberra, Australia.



**FIG. 1. Pathway of heme catabolism.** HMOX enzymes catalyze the initial step in heme catabolism. HMOX oxidizes heme (Fe protoporphyrin IX) to biliverdin IX $\alpha$ . This reaction consumes three molecules of molecular oxygen (O $_2$ ) and seven electrons donated from NADPH by CPR, and it produces ferrous iron (Fe $^{2+}$ ), CO, and biliverdin IX $\alpha$  as the products. Biliverdin IX $\alpha$  is then reduced to bilirubin IX $\alpha$  by BVR. BVR, biliverdin reductase; CO, carbon monoxide; CPR, cytochrome P450 reductase; HMOX, heme oxygenase.

HMOX1 is a 32 kDa protein that is anchored to the endoplasmic reticulum (ER) by a single hydrophobic transmembrane segment (TMS) in the C-terminus (146, 196). HMOX1 colocalizes with CPR, and their interaction is required for maximal HMOX enzymatic activity (69, 103). In addition, HMOX1 has been demonstrated to localize to other organelles, including caveolae, mitochondria, and the nucleus (53, 83, 152), raising the possibility that HMOX1 may play a role in addition to heme degradation.

HMOX1 is typically expressed in mononuclear phagocytes of the spleen, liver, and bone marrow (164), although its expression and activity has been detected across almost all tissues assessed to date. HMOX1 is strongly induced by a number of chemical and physical stresses, including heat shock, heme and hemin, cytokines, lipopolysaccharide (LPS), growth factors, oxidative stress and hydrogen peroxide (H $_2$ O $_2$ ), hypoxia, CO, and Fe starvation [reviewed in ref. (135)].

HMOX2 has a molecular mass of 36 kDa and is expressed ubiquitously, with particularly high levels in the brain (156). Unlike HMOX1, HMOX2 contains heme regulatory motifs that act as a thiol/disulfide redox switch regulating the  $K_d$  for heme (193). To date, only corticoids are known to induce HMOX2 (110). HMOX2 is implicated in oxygen sensing and the regulation of the vascular tone of at least some vascular beds (183). CO acts as a vasodilator in peripheral vessels, whereas CO derived from HMOX2 acts as a vasoconstrictor in the cerebral circulation by preventing cystathionine  $\beta$ -synthase and forming the vasodilator hydrogen sulfide (117).

This review will focus on HMOX1, elaborating on the protein chemistry, subcellular localization, and therapeutic utility of this enzyme in cardiovascular diseases and diabetes mellitus.

### Post-Translational and Structural Modifications of HMOX1

Little is known about the potential regulation of HMOX1 by post-translational modifications, although there is in-

creasing appreciation that structural modifications, for example, truncation, underpin non-canonical functions of HMOX1 (27, 101). An *in silico* analysis of the human HMOX1 protein predicts a number of potential sites for post-translational modifications. These and those determined by the mining of mass spectrometry data and/or experimentally confirmed by *in vitro* or *in vivo* experiments are listed in Figure 2. Care needs to be taken, however, when interpreting data solely based on *in silico* analyses until these observations are confirmed. In the next section, we discuss the current knowledge of post-translational and structural modifications of HMOX1.

#### Phosphorylation

Phosphorylation, the addition of a phosphate group onto highly conserved, specific tyrosine, serine, or threonine residues, is a well-recognized post-translational modification. HMOX1 contains a strong consensus sequence for serine/threonine phosphorylation by the protein kinase, Akt. Akt phosphorylates recombinant human HMOX1 at serine 188 as determined by studies in the human embryonic kidney cell line, HEK293T (137). Phosphorylation at S188 leads to a modest increase in HMOX activity when compared with the non-phosphorylated HMOX1 protein. Fluorescence resonance energy transfer experiments demonstrated that a serine to asparagine point mutation at residue 188 in HMOX1 resulted in a lower  $K_d$  for the interaction between CPR and BVR than that observed for wild-type HMOX1. This suggests that the negative charge produced by phosphorylation at S188 increases the affinity of HMOX1 for these proteins. Therefore, any increase in Akt activity, as is observed in response to a range of stimuli (135), could conceivably lead to an increase in HMOX activity.

More recently, HMOX1 phosphorylation at serine/threonine residues was detected in human brain samples (8, 19). It was reported that basal HMOX activity was significantly inhibited by brief treatment of neuron/glia cell cultures with inhibitors of the MEK and ERK signaling pathways (19). While there are several potential ERK phosphorylation sites on HMOX1, many of these are not conserved among divergent species (Fig. 2). Furthermore, there is no evidence that ERK directly phosphorylates HMOX1. Interestingly, and similar to BVR, HMOX1 has a conserved docking site/motif for ERK FXF (DEF motif) that is important for BVR to form a complex with MEK/ERK (95). On activation, MEK is released from ERK and the BVR/ERK complex enters the nucleus. Thus, it appears possible that the DEF motif in HMOX1 may act in a manner similar to that reported for BVR and be used as one mechanism to shuttle HMOX1 into the nucleus.

#### Palmitoylation

Palmitate is a 16-carbon saturated fatty acid that can be covalently attached to a number of eukaryotic proteins. There is no clear consensus sequence motif for palmitoylation, with the modification occurring at any one or more cysteine residues through a thioester linkage. The thioester bond is cleaved readily, enabling palmitoylation to play a significant role in cell signaling, subcellular trafficking, and protein-protein interactions. With regard to palmitoylation of HMOX1, only the murine and chicken proteins contain a cysteine residue (Fig. 2), suggesting that HMOX1 in these

Human	1	MERPQP---DSMPQDLSEALKEATKEVHTQAENAEFMRNFQKGGVTRDGFKLVMASLYHIYVALEEEIERNK	ESPVFAPVYFP	80
Mouse	1	MERPQP---DSMPQDLSEALKEATKEVHIQAENAEFMKNFQKGGVSREGFKLVMASLYHIYTALEEEIERNK	QNPVYAPLYFP	80
Rat	1	MERPQL---DSMQDLSEALKEATKEVHIRAENSEFMRNFQKGGVSREGFKLVMASLYHIYTALEEEIERNK	QNPVYAPLYFP	80
Chicken	1	METSQPHNAESMSQDSELLKEATKEVHEQAENTPFMKNFQKGGVSLHEFKLVLTASLYFIYSALEEEIERNK	DNPVYAPVYFP	83
Human	81	EELHRKAALEQDLAFWYGPWRQEVIPYTPAMQRYVKRLHEVGRTEPELLVAHAYTRYLGDLSGGQVLKKIAQK	KALDLPSS	160
Mouse	81	EELHRRAALEQDMAFWYGPWHQEIPCTPATQHYVKRLHEVGRTHPELLVAHAYTRYLGDLSGGQVLKKIAQK	KAMALPSS	160
Rat	81	EELHRRAALEQDMAFWYGPWHQEIPYTPATQHYVKRLHEVGGTHPELLVAHAYTRYLGDLSGGQVLKKIAQK	KAMALPSS	160
Chicken	84	MELHRKAALEKDLEFYGSNWRAEIPCPEATQKYVERLHVVGKHPPELLVAHAYTRYLGDLSGGQVLKKIAQK	KALQLPST	163
Human	161	GGLAFFTFPNIASATKFKQLYRSRMNSLEMTPAVRQRVIEEAKTAFLLNIQLFEELQELLTHDTKD-QSP	RAPGLRQRA	240
Mouse	161	GGLAFFTFPNIDSPTKFKQLYRARMNTLEMTPEVKHRVTEEAKTAFLLNIELFEELQVMLTEEKHD-QSP	QMASLRQRP	240
Rat	161	GGLAFFTFPSIDNPTKFKQLYRARMNTLEMTPEVKHRVTEEAKTAFLLNIELFEELQALLTEEKHD-QSP	QTEFLRQRP	240
Chicken	164	GGLAFFTFDGVSNATKFKQLYRSRMNALEMDHATKRVLEEAKAFLLNIQVFEALQKLVSKQENGHVAQ	PKAELRTRS	244
Human	241	SNKVQDSAPV-----ETPRGKPLNT-RSQAPLLRWLTLTSFLVATVAVGLYAM		288
Mouse	241	ASLVQDTAPA-----ETPRGKPKQISTSSSQTPLLQWLTLTSFLLATVAVGIYAM		289
Rat	241	ASLVQDTTSA-----ETPRGKSKQISTSSSQTPLLRWLTLTSFLLATVAVGIYAM		289
Chicken	245	VNKSHPNSPAAGKESERTSRMQADMLT-TS--PLVRWLLALGFIATTVAVGLFAM		296

**FIG. 2. Potential post-translational modifications of HMOX1.** Human, mouse, rat, and chicken HMOX1 protein sequences were aligned using COBALT:Multiple Alignment Tool (<http://ncbi.nlm.nih.gov/tools/cobalt>). Modifications reported in the literature and/or elucidated from mass spectrometry data mining are indicated as follows: green shading = acetylation, red box = ubiquitination, orange shading = palmitoylation, blue shading = phosphorylation, and gray shading = orthologous residues. To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)

species could be palmitoylated. Indeed, murine HMOX1 was found to be palmitoylated in the murine B16 melanoma cell line (107). It is not clear under what circumstances murine HMOX1 may be palmitoylated *in vivo*, and there is no indication as to whether palmitoylation affects HMOX activity. Surprisingly, despite a lack of cysteine residues, human HMOX1 has been reported to be palmitoylated in platelets (39), although to date, the effect of such palmitoylation on HMOX1 function remains unknown.

#### Acetylation

Acetylation of lysine residues is a reversible modification that plays an essential role in regulating gene expression. Lysine acetylation has also been shown to be important for cell cycle, nuclear transport, and chromatin remodeling. When assessing the residues that are important for the interaction between rat HMOX1 and CPR, nine (*i.e.*, K18, K22, K39, K48, K69, K149, K153, K179, and K196) out of a total of 15 lysine residues were identified by mass spectrometry (MALDI-TOF) as acetylated (61). Interestingly, K149 and K153 were protected from acetylation in the presence of CPR. However, in the absence of CPR, rat HMOX1 was acetylated at all nine lysine residues, and this led to a reduction in HMOX activity. These data suggest that at least in rats, CPR may modulate HMOX activity *via* inhibition of K149 and K153 acetylation. HMOX1 was also acetylated at K39 in human cancer cell lines (24) and at K18 in human liver tissue (199). The significance of acetylation of human HMOX1 at these residues remains unclear. In the instance of the K18 or K39, acetylation may confer nuclear localization to HMOX1, which may be associated with changes in gene transcription (27, 101). Indeed, acetyla-

tion of other transcription factors has been shown to promote gene transcription; for example, acetylation of Nrf2 at K588 and K591 facilitates binding to the HMOX1 promoter and induces gene transcription *in vitro* (81).

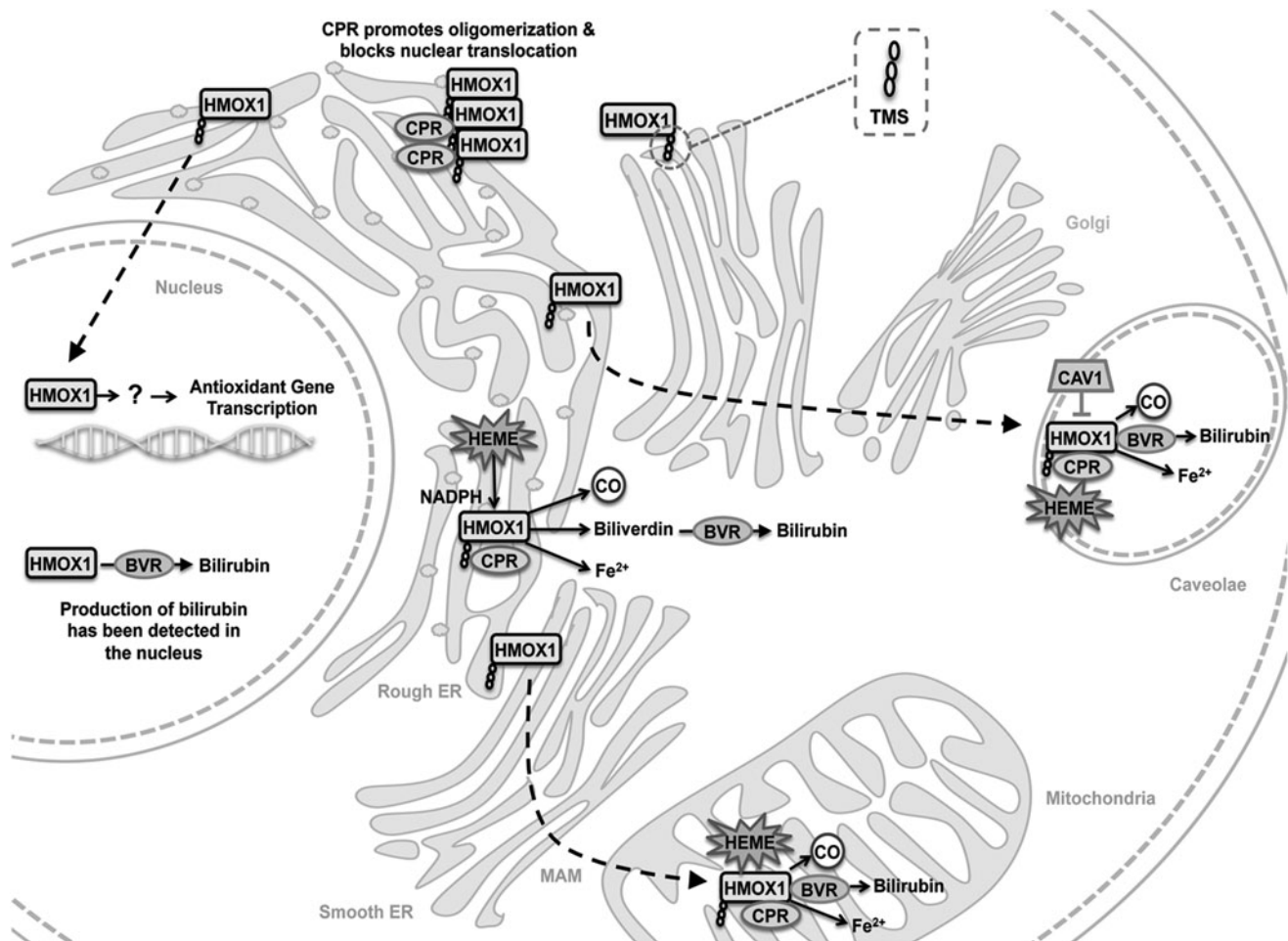
#### Ubiquitination

Degradation of proteins by the ubiquitin-proteasome pathway enables cells to respond to a changing environment. Signal-dependent ubiquitination frequently results in the complete degradation of the targeted protein. HMOX1 protein turnover occurs *via* the ubiquitin proteasome system in the ER-associated membrane and has been shown for vascular smooth muscle cells (SMCs), HEK293 cells (100), and rat adrenal pheochromocytoma PC12 cells (189). In HEK293T cells, ER-resident E3 ubiquitin ligase is responsible for HMOX1 degradation *via* an interaction with the TMS region (100). However, it remains unclear how truncated HMOX1 protein in which the TMS sequence has been lost is turned over. Interestingly, treatment of the human colon adenocarcinoma cell line, HCT116, with the proteasome inhibitor bortezomib resulted in K39, K69, K86, K148, K153, and K243 residues of HMOX1 becoming ubiquitinated; while treatment with the proteasome inhibitor epoxomicin led to ubiquitination of K179 and K256 (85). These data suggest that not all ubiquitination events are regulated identically.

#### Dimerization and oligomerization

A lack of cysteine residues in HMOX1 led to the assumption that HMOX1 acts as a monomer. However, Hwang *et al.* demonstrated that in the ER of HEK293 cells, HMOX1 gives





**FIG. 3. Subcellular localization of HMOX1.** HMOX1 localizes to different subcellular compartments. HMOX1 is tethered to the ER membrane by a trans-membrane sequence (TMS). CPR colocalizes with HMOX1 on the ER to facilitate heme degradation to CO, Fe<sup>2+</sup>, and biliverdin. Biliverdin is then converted to bilirubin by BVR. HMOX1 may traverse the ER and MAM compartments to the mitochondria. In the mitochondria, HMOX1 is anchored to the inner mitochondrial membrane, where it may detoxify mitochondrial heme. HMOX1 may be transported to caveolae and the plasma membrane through the ER and Golgi apparatus, where it similarly detoxifies heme. The activity of HMOX1 in caveolae may be modulated by CAV1 that binds to and decreases HMOX activity. CPR can promote oligomerization of HMOX1 to increase its stability and enzymatic activity and to prevent nuclear translocation. Cleavage of the TMS enables truncated HMOX1 to enter the nucleus, where it can induce the transcription of antioxidant response genes. CAV1, caveolin-1; ER, endoplasmic reticulum; MAM, mitochondrial membrane associated; TMS, transmembrane segment.

rise to dimers and oligomers, the formation of which were essential for HMOX activity (70). Using mutant HMOX1 constructs in fluorescence resonance energy transfer and co-immunoprecipitation experiments, the TMS region was shown to be the interface for protein-protein interaction. HMOX1 dimers have also been observed in preparations of lipid vesicles (112). The TMS region is also responsible for binding CPR, which maximizes the catalytic activity of HMOX1 (69). Moreover, in HEK293 cells, CPR promotes HMOX1 oligomerization, which can prevent hypoxia-induced translocation of HMOX1 to the nucleus (103).

#### Truncation

Microsomal full-length HMOX1 (32 kDa) is trypsinized easily, resulting in a water-soluble truncated protein that typically lacks a 23–55-amino-acid hydrophobic C-terminal TMS (194). Purified recombinant full-length HMOX1 can also

be cleaved by thrombin (69). In these and other studies, protein cleavage has given rise to at least three types of truncated HMOX1, including 27, 28, and 30 kDa isoforms. The 23-amino-acid truncated HMOX1 (HMOX1<sub>Δ23</sub>) has been crystallized, and this led to the identification of histidine 25 within the proximal alpha helix as the ligand for heme Fe (144). Truncated forms of HMOX1 have been reported to localize to the nucleus (see next), with cysteine proteases having been implicated in proteolytic cleavage (101). While the TMS of HMOX1 enhances its interaction with CPR and BVR (see earlier), there is convincing evidence that HMOX1 lacking the C-terminus retains catalytic activity. Thus, earlier studies established unambiguously that isolated purified human HMOX1 with approximately 67 of its C-terminus amino-acid residues deleted retains ~50% of the enzymatic activity of full-length HMOX1 when purified CPR is supplied as the source of electrons [see, e.g., refs. (143, 144, 181)]. In addition to CPR, ascorbate is an established alternative source of

electrons for truncated HMOX1, yielding biliverdin IX $\alpha$  as the stereospecific product (168, 177).

### Subcellular Localization of HMOX1

Since its discovery on microsomes (163), the location of HMOX1 has been assigned traditionally to the ER. More recently, however, HMOX1 has been reported to be present in other cellular compartments, including caveolae, mitochondria, and the nucleus (Fig. 3). In the ER, caveolae and mitochondria HMOX1 appear to be anchored by the TMS and to co-localize with CPR and BVR, suggesting heme degradation as its primary role. However, we are unaware of evidence for the presence of CPR in the nucleus, where truncated HMOX1 likely regulates gene transcription. In this section, we will review the localization of full-length and truncated HMOX1 within different cellular compartments.

#### Endoplasmic reticulum

The ER is the site of protein, lipid, and carbohydrate synthesis, and for the packaging of these molecules into vesicles for delivery-to-end organelles. The ER is also important for the regulation of cellular calcium, glycosylation, insertion of integral membrane proteins, disulfide bond formation and protein folding, and drug metabolism. Disturbances in redox regulation, glucose deprivation, cellular calcium, and viral infections can lead to ER stress and the unfolded protein response. Interestingly, HMOX1 and CO play integral roles in many disease processes in which ER stress is implicated. These include cardiovascular and metabolic diseases (84, 104). As previously discussed, HMOX1 is anchored to the ER *via* a hydrophobic C-terminus TMS (146). It has also been ascertained that the orientation of ER-bound HMOX1 is toward the cytosol (56). Within the ER or in response to stress such as hypoxia, the TMS may be cleaved, resulting in truncated HMOX1 that may then translocate to the cytoplasm and the nucleus (70, 101).

#### Caveolae

At the plasma membrane, there are specialized microdomains that are enriched in cholesterol and glycosphingolipids, and these are known as lipid rafts and caveolae. Cell signaling proteins localize to these lipid-rich areas for vesicular transport and rapid induction of signaling cascades in response to external stimuli. Caveolins (CAVs) are membrane proteins that are predominantly found in caveolae. CAVs form scaffolds on which signaling molecules can assemble, thereby facilitating rapid cell signaling responses.

One of the first reports of non-ER compartmentalization of HMOX1 was in the caveolae of rat pulmonary artery endothelial cells (ECs) (83). When treated with LPS, heme, or hypoxia, a proportion (25–40%) of HMOX1 protein was found to localize to a detergent resistant fraction that contained CAV-1. Co-immunoprecipitation studies confirmed that HMOX1 directly interacted with CAV1 to reduce HMOX activity (83), as was previously seen with endothelial nitric oxide synthase (48). When cells were depleted of cholesterol, HMOX1 disappeared from the detergent-resistant fraction. Moreover, as CAV1 was decreased, HMOX activity increased. Similarly, LPS treatment of isolated murine peritoneal macrophages resulted in HMOX1 translocation to caveolae, while

exogenous CO enhanced the binding of CAV1 to toll-like receptor 4 and inhibited pro-inflammatory signaling (179). Cadmium has also been shown to cause HMOX1 and CAV1 association in mouse mesangial cells (78), and it is now appreciated that CAV1 is involved in the regulation of HMOX activity (159).

#### Mitochondria

Mitochondria are the energy powerhouses of the cell, producing energy in the form of ATP. In addition, mitochondria play key roles in signaling, apoptosis, cell survival, cellular growth, and heme synthesis. As such, "mitochondrial dysfunction" is implicated in many aspects of cardiovascular diseases, diabetes complications, and aging.

Mitochondrial HMOX activity was initially reported in the southern multimammate mouse (*Mastomys coucha*) (152), although that study did not discriminate between HMOX1 and HMOX2 activity. More recently, treatment of rat pulmonary EC with heme or LPS has been reported to result in a proportion of HMOX1 protein becoming associated with cell fractions containing cytochrome *c* (83). In fact, HMOX1 appears to be constitutively expressed in mitochondria of rat liver, where it colocalizes with BVR in the inner mitochondrial membrane (28). This suggests that mitochondrial HMOX1 may play a role in the detoxification of mitochondrial heme (see next).

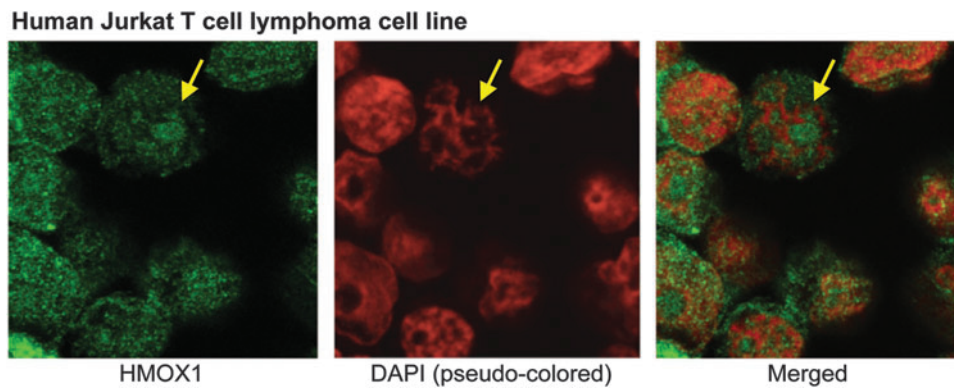
#### Nucleus

Critical functions that occur within the nucleus include regulation of gene transcription and cell cycle progression. HMOX1 has been detected in the nucleus of various cell types, including brown adipose tissue (53), prostate cancer cells (136), astroglial cells (97), EC (83), squamous cell carcinoma, (51), Hepa, NIH3T3 cell lines (101), dendritic cells (52), and cerebral cortex tissue (117). Our laboratory has shown that the yeast homolog *Hmx1* may translocate to the nucleus and regulate gene transcription, although the possibility of ER-associated *Hmx1* contamination has not yet been ruled out (27). However, we have shown that hemin treatment leads to nuclear translocation in two mammalian cell types, namely human Jurkat T-cell lymphoma cells (Fig. 4) and rat aortic SMC (unpublished data).

Heme and hypoxia have also been shown to cause translocation of truncated HMOX1 to the nuclei (101). In that study, it was demonstrated that loss of the TMS was required for nuclear translocation. As noted earlier, truncation decreases (but does not eliminate) CPR-dependent activity of HMOX1. Indeed, overexpression of human HMOX1 $\Delta_{24}$  in HEK293T cells was reported to significantly lower HMOX activity compared with cells transfected with full-length HMOX1 (1.2-fold increase in HMOX activity for HMOX1 $\Delta_{24}$  truncated protein compared with 6.0-fold increase for full-length HMOX1) (70). As mentioned earlier, this could be due to truncated HMOX1 being located primarily in the nucleus where CPR was absent (101, 103, 182).

### Proposed Modes of Action of HMOX1 at Different Cellular Locations

The primary role of HMOX1 across all species is undoubtedly the degradation of heme, which has led to the retention of



**FIG. 4. Nuclear localization of HMOX1.** Human Jurkat T-cell lymphoma cells were treated with 30  $\mu$ M hemin for 24 h; the nuclei were isolated by fractionation and stained for HMOX1 (SPA-895, Stressgen and AlexaFluor 488; Life Technologies) and DAPI. Fluorescent photomicrographs were taken using a Leica SPEII confocal microscope. HMOX1 staining is shown in green. DAPI images were pseudo colored from blue to red, and HMOX1 and pseudo-colored DAPI images were merged using Leica LAS AF Lite software. Yellow *arrows* indicate nuclei. Magnification = 100 $\times$ . DAPI, 4',6'-diamidino-2-phenylindole. To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)

the HMOX genes throughout evolution. However, variations in the HMOX1 protein, post-translational modifications, and subcellular localization indicate an expanded repertoire of function (Fig. 3). Recent studies also indicate that HMOX1 shuttles between subcellular compartments, as summarized in Table 1. Therefore, it may be appropriate to consider HMOX1 as a "dynamic" protein.

The association of HMOX1 with the ER and its orientation toward the cytosol (56) is not random. Rather, it is an efficient way to deal with the redox-active  $Fe^{2+}$  generated as a result of heme degradation. Free iron derived from heme commonly induces expression of the heavy chain of ferritin (43) and the

$Fe^{2+}$  exporter ferroportin (33). Together, ferritin and ferroportin efficiently sequester and remove redox-active  $Fe^{2+}$ , thereby minimizing Fenton chemistry-induced oxidative damage. Moreover, colocalization of BVR and CPR with HMOX1 ensures efficient conversion of biliverdin to bilirubin. In caveolae, the proximity of HMOX1, BVR, and CPR (83) as well as ferroportin would be expected to increase the efficiency of heme degradation and Fe export.

It has been shown that in isolated murine peritoneal macrophages, the LPS-induced translocation of HMOX1 from the ER to the caveolae proceeds by a p38 MAPK-dependent mechanism (179). In addition, this translocation was blocked

TABLE 1. TRANSLOCATION OF HEME OXYGENASE-1 TO NON-ENDOPLASMIC RETICULUM COMPARTMENTS

<i>Treatment</i>	<i>Cell type</i>	<i>Compartments</i>	<i>References</i>
15-deoxy- $\Delta^{12,14}$ -prostaglandin J2	Murine cortical neurons	Cytoplasm, microsomes	(86)
Cigarette smoke extract	A549 alveolar cell line	Mitochondria, cytoplasm	(147)
	Beas-2b bronchial epithelial cell line	Mitochondria, cytoplasm	(147)
Co-PP IX	Rat renal cells	Mitochondria	(172)
Heme	NIH3T3 fibroblast cell line	Nucleus	(101)
Hemin	Head and neck squamous cell carcinomas	Nucleus	(51)
	A549 alveolar cell line	Mitochondria, cytoplasm	(147)
	Beas-2b bronchial epithelial cell line	Mitochondria, cytoplasm	(147)
	PC3 prostate cancer cell line	Nucleus, cytoplasm	(136)
	LnCAP prostate cancer cell line	Nucleus, cytoplasm	(136)
Hypoxia	Rat liver	Mitochondria, microsomes	(28)
	NIH3T3 fibroblast cell line	Nuclear	(101)
	HEK293T embryonic kidney cell line	Nuclear	(103)
	Rat pulmonary artery endothelial cells	Caveolae	(83)
Indomethacin	Rat gastric mucosa	Mitochondria	(10)
LPS	Murine bone marrow derived macrophages	Cytoplasm	(34)
	Murine peritoneal macrophages	Caveolae	(179)
	A549 alveolar cell line	Mitochondria, cytoplasm	(147)
	Beas-2b bronchial epithelial cells	Mitochondria, cytoplasm	(147)
	Rat liver	Mitochondria, microsomes	(28)
Palmitoylation	Murine B16 melanoma cell line	Mitochondria associated membrane	(107)

LPS, lipopolysaccharide.



by brefeldin-A that disrupts Golgi complexes, suggesting that Golgi processing is also involved (Fig. 3). As mentioned earlier, CAV1 is enriched in caveolae, where it has been shown to bind to and inhibit the activity of HMOX (78, 83, 159). Indeed, deletion of CAV1 increases, while CAV1 overexpression inhibits LPS-induced HMOX activity in EC (83). CAV1 interferes with the binding of hemin to HMOX1 (159). Thus, CAV1 may be considered a cellular regulator of HMOX activity.

Electron microscopy studies indicate that mitochondrial HMOX1 is localized to the inner mitochondrial membrane (28), although HMOX1 lacks a typical mitochondria-targeting sequence as assessed by *in silico* analysis. Mitochondria are linked to the ER *via* mitochondrial-associated membranes (MAM) (54) that represent a likely route for HMOX1 trafficking between the two organelles. In the mouse melanoma cell line B16, inhibition of palmitoylation has been reported to result in translocation of HMOX1 from the MAM to the ER (107). Whether palmitoylation is a requisite for HMOX1 shuttling, however, is questionable, given that this modification does not occur in all species. Nevertheless, these data suggest that HMOX1 may shuttle between these organelles.

As mentioned earlier, mitochondrial HMOX1 colocalizes with BVR and CPR (28). Thus, one function of mitochondrial HMOX1 appears to be the regulation of mitochondrial heme content, in concert with heme synthesis that also occurs in mitochondria (129). Mitochondrial HMOX1 also plays a role in apoptosis. Treatment of rat renal cells with the HMOX1 inducer cobalt protoporphyrin IX results in translocation of HMOX1 to mitochondria and attenuation of the release of cytochrome *c* (172). HMOX1 induction was further associated with increased transcription and phosphorylation of the antiapoptotic protein Bcl2. Hemin and cigarette smoke extract also increase mitochondrial HMOX1 expression in A549 alveolar and Beas-2b bronchial epithelial cell lines (147) that was associated with preservation of ATP production.

The mode of action of nuclear HMOX1 appears to depart somewhat from the membrane-associated forms of HMOX1 discussed so far. Known inducers of HMOX1, such as hypoxia and hemin, are associated with nuclear translocation of HMOX1 (Table 1). While it remains uncertain whether protease-mediated cleavage of the TMS region of HMOX1 is essential for nuclear translocation, nonspecific inhibition of cysteine proteases blocked nuclear translocation of HMOX1 in one study (101). Studies of truncated rat HMOX1 indicate that a highly conserved leucine-rich nuclear shuttling sequence enables nuclear translocation (101) (Fig. 2). This leucine-rich sequence is partially conserved between human and mouse, and, to a lesser extent, chicken.

Once in the nucleus, truncated HMOX1 may protect against stress by different mechanisms, including the activation of transcription factors (52, 101). Consistent with this, we reported that yeast cells transfected with human HMOX1 and treated with oxidants showed nuclear translocation of HMOX1 and increased expression of antioxidant genes such as  $\gamma$ -glutamylcysteine synthetase, glutathione peroxidase, catalase, and methionine sulfoxide reductase (27). Whether the biological functions of nuclear HMOX1 require enzymatic activity is a matter of debate. As previously mentioned, truncated human HMOX1 retains enzymatic activity (143, 144, 181). Evidence against nuclear HMOX1 being enzymatically active comes from studies performed by Dennerly and coworkers. These authors reported HMOX activity in 3T3 fi-

broblasts transfected with human *EGFP-HMOX1* $\Delta_{23}$  to be similar to that in non-transfected control cells, whereas transfection with full-length *EGFP-HMOX1* increased enzymatic activity  $\sim 10$  fold (101). In addition, transfection of cells with an enzymatically inactive HMOX1 mutant significantly altered the expression of various transcription factors (101). Thus, enzymatic activity does not appear to be required for HMOX1 to affect gene transcription in model systems, although the biological relevance of this remains to be established.

If enzymatically active, the resulting CO itself may modulate antioxidant gene transcription *via* the activation of the Nrf2 transcription factor that is a key initiator of *HMOX1* gene transcription (91). Thus, the HMOX1 response can be amplified. This amplification may be kept in check by CPR, which promotes oligomerization of HMOX1 and prevents cleavage and nuclear translocation of the truncated protein. In addition, HMOX1 protein has been shown to auto-regulate itself, independent of enzymatic activity (102). HMOX1 and CO can induce the transcription factor Yin Yang 1 (YY1) (9) that suppresses SMC proliferation without affecting EC proliferation (138). Up-regulation of YY1 by the HMOX1 inducer probucol was necessary for the inhibition of intimal hyperplasia in a rat model (9). Moreover, overexpression of YY1 can up-regulate HMOX1 expression (9). Similarly, both vascular endothelial growth factor (VEGF) and the pro-angiogenic chemokine stromal cell-derived factor-1 (SDF-1) can induce HMOX1 or, in turn, be induced by HMOX1 (37, 99). Exogenous administration of CO has also been shown to activate DNA repair signaling in HEK293 cells (126). This coordinate response to stress involving HMOX1, its associated machinery, and metabolites underlies the importance of heme degradation in cellular homeostasis.

The possibility of enzymatically active HMOX1 being located in the nucleus raises a number of intriguing questions, including the source of the substrate heme and how it is transported into the nucleus; the source and mode of transfer of reducing equivalents required for enzymatic activity; the fate of the  $\text{Fe}^{2+}$  released as a consequence of heme catabolism; and whether biliverdin IX $\alpha$  is converted to bilirubin IX $\alpha$  within the nucleus. There are some data to address these questions. Of note,  $\text{Fe}^{2+}$ -sequestering ferritin has been observed in the nucleus in a number of cell types, including astrocytes, where it was shown to protect DNA from  $\text{Fe}^{2+}$ -induced oxidative damage (165). Maines and coworkers also reported the presence of BVR in the nucleus, and they proposed that BVR might act as a transporter for heme into the nucleus (95). While CPR is not expected to be present in the nucleus, ascorbate could conceivably donate electrons to truncated HMOX1 in the nucleus. These data and our own that demonstrate the presence of bilirubin IX $\alpha$  in the nucleus of hypoxia-treated rat SMC as assessed by immunohistology (J Ni, pers. comm.) would seem to indicate that in addition to the activation of transcription factors, nuclear HMOX1 may retain some heme detoxification activity.

The effect of hypoxia on the regulation of HMOX1 is complex. For example, hypoxia increases HMOX1 expression in human dermal fibroblasts (127), while hypoxia is associated with decreased HMOX1 expression in human coronary artery EC, human umbilical vein EC, and immortalized human microvascular EC (88, 106, 119). The findings in human tissues are reminiscent of the tissue-specific effects of HMOX1 induction on cell proliferation, for example, inhibition in SMC

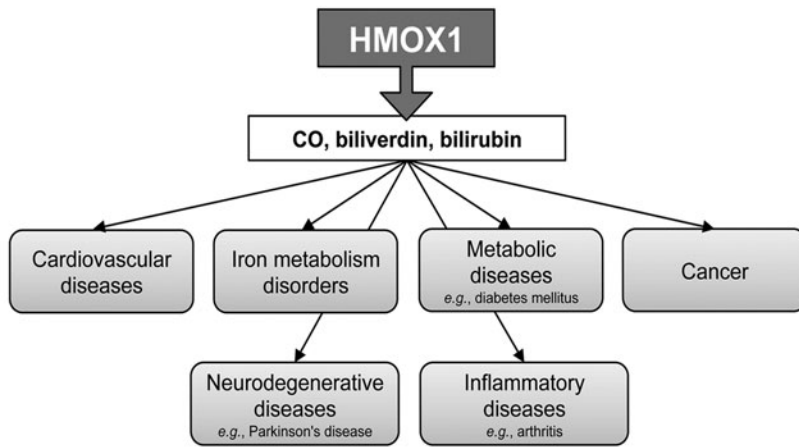
(9, 42) and stimulation in EC growth (36). By contrast, in rodents, hypoxia increases HMOX1 expression in rat pulmonary aortic EC (83), immortalized mouse EC (55), and rat aortic SMC (92). These species-specific differences in hypoxia-mediated HMOX1 induction may be explained, in part, by the presence or absence of Bach1 (hypoxia-induced repressor of Nrf2 and HMOX1 gene transcription) (106) or other transcription factors in human EC.

**HMOX1 As a Therapeutic Target for the Treatment of Human Disease**

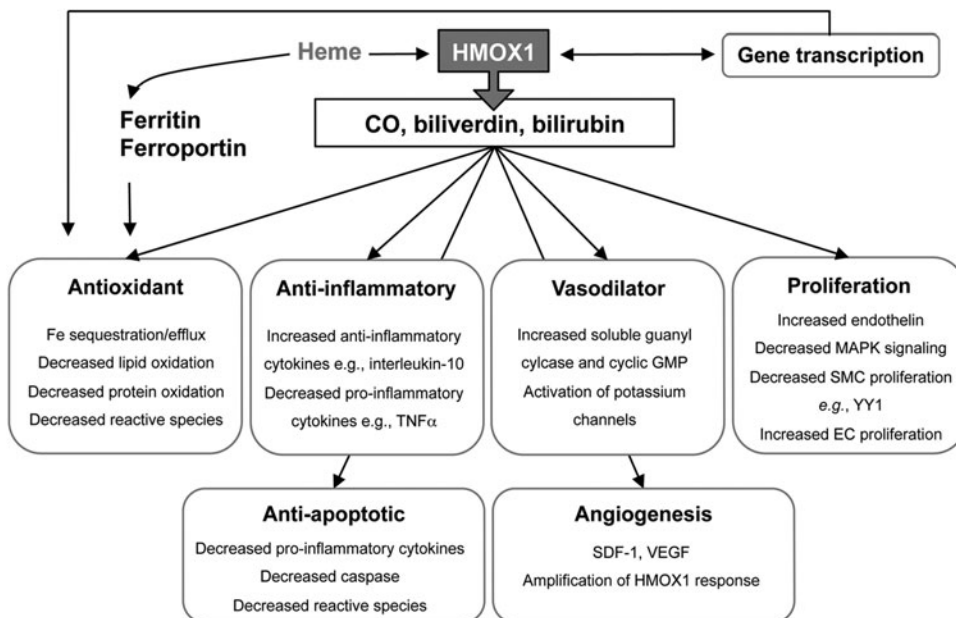
HMOX1 is recognized as a promising therapeutic target for a broad range of conditions, including cardiovascular, metabolic, neurodegenerative, and other inflammatory diseases (Fig. 5A). The next section will focus on cardiovascular diseases. The catabolism of heme provides pro-

tection to cells *via* multiple avenues, including the induction of ferritin to store redox-active Fe (43), the antioxidant actions of biliverdin and bilirubin (154), and the anti-inflammatory and anti-apoptotic effects of CO (151). Avenues relevant to cardiovascular diseases are summarized in Figure 5B. Potential treatment modalities include pharmacological induction, gene delivery of *HMOX1*, or direct delivery of CO, biliverdin, and bilirubin. The ability of progenitor cells to specifically home to sites of vascular injury (187) also raises the possibility that HMOX1 or heme degradation products could be delivered by autologous stem cell therapy. In this section, we will discuss insights amassed from the *Hmox1*<sup>-/-</sup> mouse and the therapeutic utility of HMOX1 and heme degradation products as determined from animal models of human disease. Evidence for the association of HMOX1 with cardiovascular disease and diabetes is summarized in Table 2.

**A HMOX1 AS A THERAPEUTIC TARGET FOR HUMAN DISEASES**



**B HMOX1 IN CARDIOVASCULAR DISEASE**



**FIG. 5. Protective properties of HMOX1 in cardiovascular diseases.** (A) HMOX1 is a therapeutic target for a broad range of human diseases. (B) In cardiovascular diseases, HMOX1 and heme catabolism products have antioxidant, anti-inflammatory, anti-apoptotic, vasodilatory, and anti-proliferative properties. The antioxidant effects of heme include the activation of transcriptional machinery that induces a range of antioxidant genes.



TABLE 2. EVIDENCE FOR THE PROTECTIVE EFFECT OF HEME OXYGENASE-1 IN CARDIOVASCULAR DISEASE AND DIABETES

Evidence	References
<b>Cardiovascular disease</b>	
Longer GT repeats associated with increased risk of coronary artery disease and coronary events	(38, 79)
Longer GT repeats associated with increased atherosclerosis burden and plaque rupture	(20)
Longer GT repeats associated with increased restenosis	(38)
Longer GT repeats associated with increased inflammation	(141)
Longer GT repeats or low bilirubin associated with increased risk of cerebral ischemia	(87)
Low bilirubin concentrations associated with increased coronary artery disease	(145)
Low bilirubin concentrations associated with endothelial dysfunction, increased carotid intima-media thickness, or arterial stiffness	(45, 200)
High bilirubin concentrations associated with coronary collateral growth	(200)
<b>Diabetes mellitus</b>	
Long GT repeats and low serum bilirubin concentrations in diabetes mellitus associated with increased risk of coronary artery disease	(150)
High levels of HMOX1 reduce the risk of gestational diabetes mellitus	(131)
High bilirubin concentrations are associated with reduced HbA1c or lower incidence of diabetes	(21)

### HMOX1 deficiency

To date, only two cases of human *HMOX1* deficiency have been reported (133, 188). In the first reported case, a 6-year-old boy had severe retardation, Fe loading in the kidneys and liver, vascular injury, and hyperlipidemia (188). Similarly, in the second case, a 2-year-old girl presented with reduced growth, Fe metabolism disorders, asplenia, hepatomegaly, nephritis, leukocytosis, and vascular injury (133, 134).

The apparently low penetrance of this gene deficiency may be explained by the fact that *Hmox1* gene deletion in mouse embryos is lethal in most instances (130). This embryonic lethality could be explained by defects in the placental vasculature. Recent studies using *Hmox1*<sup>+/-</sup> heterozygous mice demonstrated that partial *Hmox1* deficiency was associated with malformed vasculature and impaired spiral artery remodeling in the placenta (198). In these investigations, the maternal allele was identified to be responsible for the placental defects. These observations may account for the reduced numbers of *Hmox1*<sup>-/-</sup> pups born. It is also possible that partial *HMOX1* deficiency in a mother may underpin repeated failures at pregnancy or early miscarriage, and this could be the reason that only two cases of human *HMOX1* deficiency have been reported to date.

Many of the features of human *HMOX1* deficiency are reflected in the *Hmox1*<sup>-/-</sup> mouse. The latter displays a profound inflammatory phenotype with features of human iron overload syndrome, including tissue iron deposition, splenomegaly, hepatomegaly, hepatic fibrosis, growth retardation, and premature death (130). Moreover, *Hmox1*<sup>-/-</sup> mice have

increased numbers of leukocytes, activated CD4<sup>+</sup> T cells, proinflammatory cytokines, and oxidized proteins and lipids (80, 124).

### HMOX1 promoter variation in cardiovascular diseases and diabetes mellitus

There is some variation in the transcriptional activity of the *HMOX1* gene by virtue of a microsatellite of GT repeats within the promoter. *In vitro* studies in human SMC have shown that shorter GT repeats lead to increased *HMOX1* transcriptional activity (18). In human EC, shorter GT repeats lead to reduced oxidative stress and proinflammatory cytokines, and increased responsiveness to VEGF-induced proliferation (158). In humans, longer GT repeats have been associated with increased inflammation after balloon angioplasty (46, 141) and increased in-stent restenosis (38). These data are reflected in the seminal observation that low plasma bilirubin levels are associated with an increased risk of coronary artery disease (66, 145). Not all studies have shown an association between the microsatellite and restenosis or coronary disease (44, 166). However, in two of these studies, shorter GT repeats were associated with higher bilirubin levels and a healthy lipid profile (44), or reduced inflammation (141). The underlying reasons for this disparity could include patient ethnicity and variations in the severity of the diseases examined.

There is continued debate regarding the association of longer GT repeats with an increased risk of developing Type 2 diabetes mellitus (5, 6, 23). Similar to cardiovascular diseases, the overall inconsistent results may be explained by differences in ethnicity and disease severity. *HMOX1* expression is reduced in peripheral blood mononuclear cells of diabetes patients (150). In contrast, high serum levels of *HMOX1* in early pregnancy may reduce the risk of developing gestational diabetes mellitus (131).

### HMOX1 in vascular health and disease

As mentioned earlier, *Hmox1*<sup>-/-</sup> mice are not born in Mendelian ratios and partial *Hmox1* deficiency is associated with malformed placental vasculature (198), implicating *HMOX1* in angiogenic processes. *Hmox1*<sup>-/-</sup> mice produce higher levels of the angiogenic inhibitors soluble VEGF and soluble endoglin (30). They also have impaired wound healing and wound neovascularization compared with wild-type littermates (57). *Hmox1* deficiency is associated with more damage from myocardial ischemia-reperfusion injury (77, 197). Conversely, cardiac-specific overexpression of the human *HMOX1* transgene in the mouse led to improved cardiac function and increased numbers of newly formed blood vessels (104). Similarly, adenoviral overexpression of rat *Hmox1* resulted in improved blood flow recovery and limb function in a rat hind limb ischemia model (157). In *ex vivo* aortic ring sprouting angiogenesis assays, *Hmox1* deficiency impairs VEGF- and SDF-1-induced angiogenesis (37). This impairment in angiogenesis could be attenuated by administration of exogenous CO.

*HMOX1* is strongly implicated in vascular diseases such as atherosclerosis. *HMOX1* protein is expressed in atherosclerotic lesions in both *apolipoprotein E (ApoE)* and *low-density lipoprotein receptor-deficient mice*, where it is thought to protect from disease (72, 178). This interpretation is supported by

studies demonstrating that adenoviral overexpression of human HMOX1 is associated with decreased atherosclerosis in *Apoe*<sup>-/-</sup> mice (76). Moreover, mice deficient in *Hmox1* and *Apoe* have increased atherosclerosis and vein graft stenosis compared with *Apoe*<sup>-/-</sup> mice (191).

HMOX1 is also associated with vascular remodeling and endothelial function. In a pig model of arterial injury, adenoviral overexpression of *Hmox1* led to a decrease in SMC proliferation and improved vascular reactivity (42). In rats, *Hmox1* gene delivery or HMOX1 induction *via* hemin treatment attenuates vascular remodeling and neointimal hyperplasia after balloon injury (169, 170). Adenoviral overexpression of HMOX1 has also been demonstrated to attenuate the development of graft arteriosclerosis in a rat aortic transplant model (40), and to improve graft survival in a rat aorta chronic rejection model (12). Further, HMOX1 induction by heme arginate leads to decreases in proinflammatory cytokines and improved endothelial function in *low-density lipoprotein receptor-deficient mice* (82). Outside the systemic circulation, HMOX1 is implicated in diseases of the pulmonary circuit such as pulmonary hypertension. Reduced expression of HMOX1 was found in lung tissues of newborns suffering from congenital diaphragmatic hernia and pulmonary hypertension (149). Compared with wild-type littermates, *Hmox1*<sup>-/-</sup> mice exposed to chronic hypoxia have features that are consistent with pulmonary hypertension, including exaggerated right heart hypertrophy, ventricular infarcts, and thrombi (192). Conversely, tissue-specific overexpression of human HMOX1 in the lungs of transgenic FVB/N mice decreases pulmonary hypertension in response to chronic hypoxia (115). In rats, the administration of CO (41) or the induction of HMOX1 by hemin or nickel chloride (25) inhibits the development of pulmonary hypertension. Data in humans are lacking at this point in time but are important to obtain, particularly as sufferers of pulmonary hypertension have a poor diagnosis. Inhalation of CO is currently undergoing clinical trials in idiopathic pulmonary hypertension (*e.g.*, <http://clinicaltrials.gov/ct2/show/NCT01214187>) and severe pulmonary arterial hypertension (*e.g.*, <http://clinicaltrials.gov/ct2/show/NCT01523548>).

#### HMOX1 in diabetes

There is increasing evidence for a role of HMOX1 in experimental diabetes and associated complications. In diabetic mice, vascular injuries are exacerbated compared with nondiabetic controls; HMOX1 up-regulation is beneficial, although the impact of diabetes on the expression and activity of HMOX1 is inconsistent between different tissues (47, 132). *Hmox1*<sup>-/-</sup> mice treated with streptozotocin have increased oxidative stress and infarct size after myocardial ischemia reperfusion compared with nondiabetic *Hmox1*<sup>-/-</sup> mice (104). In addition, in the *db/db* mouse model, impaired wound healing and wound neovascularization is ameliorated by adenoviral overexpression of rat *Hmox1* (57). Similarly, overexpression of murine *Hmox1* attenuates the immune response in NOD mice and slows the progression to diabetes *via* a mechanism that involves CO (67). Furthermore, selective overexpression of murine *Hmox1* in the pancreas of NOD mice was associated with a decrease in proinflammatory mediators, and these mice were less likely to develop diabetes and

had improved graft survival after islet transplantation compared with control animals (68).

The induction of HMOX1 by cobalt protoporphyrin IX has been reported to improve insulin sensitivity and adipose remodeling in the Zucker diabetic rat (123). Cobalt protoporphyrin IX has also been shown to regulate adiposity in male mice (15). In both female and male mice, HMOX1 induction was further associated with lower blood pressure and proinflammatory cytokines, with increased serum adiponectin and expansion of insulin-sensitive adipocytes (15). Similarly, hemin-mediated induction of HMOX1 has been shown to increase insulin sensitivity and glucose metabolism in a range of models of diabetes, including the obese Zucker rat, Goto-Kakizaki rat, and streptozotocin-treated rats (120–122).

#### Pharmacological inducers of HMOX1 protein expression

A number of pharmacological agents induce HMOX1 expression, and those in use or trialed for the treatment of cardiovascular diseases or diabetes are listed in Table 3, along with the proposed mode of action for inducing HMOX1. Interestingly, this list includes three classes of lipid-lowering drugs, that is statins, probucol (and its analog succinobucol), and fenofibrate.

Simvastatin, pravastatin, atorvastatin, and fluvastatin were demonstrated to increase HMOX1 in RAW264.7 murine macrophages *via* protein kinase G, ERK, and p38 MAPK signaling (17). Simvastatin and pravastatin also increase HMOX1 in vascular EC (173) and renal epithelial cells (16). In human EC (63) as well as in human and rat vascular SMC (93), simvastatin increased HMOX1 *via* the PI3K-Akt pathway, although potential phosphorylation of HMOX1 at S188 by Akt (see above) was not explored. Similarly, fluvastatin increased the PI3K-Akt pathway in coronary artery SMC, resulting in enhanced HMOX1 expression *via* transcription factor Nrf2 (111). However, it remains to be clearly established whether this activity translates to pharmacologically relevant concentrations of statins [see *e.g.*, (105)].

Probuco increases HMOX1 expression in EC and vascular SMC *in vitro* and *in vivo*, and these are associated with a ~2-fold increase in HMOX activity in the arterial wall of animal models of atherosclerosis (15, 35, 160, 186). Increasing HMOX1 by a systemic administration of probucol has the dual benefit of inhibiting SMC proliferation while simultaneously increasing EC proliferation (9, 90, 186). This is associated with the inhibition of intimal hyperplasia and the promotion of re-endothelialization after arterial balloon injury in rabbits and rats, and in-stent re-endothelialization after femoral stenting in rabbits (160). Succinobucol, a more water-soluble mono-succinate derivative of probucol, increases HMOX1 in balloon-injured rabbit aortas and decreases neointimal hyperplasia (187). Similar to probucol, succinobucol increases HMOX1 expression in SMC *in vitro*, and this is associated with decreased cell proliferation. Unlike probucol, however, the anti-proliferative effect of succinobucol appears to be *via* the promotion of apoptosis rather than increased HMOX activity (114). Both probucol and succinobucol enhance the mobilization of progenitor cells to sites of vascular injury (167, 187). In clinical studies of atherosclerosis and restenosis, probucol and succinobucol have yielded mixed

TABLE 3. HEME OXYGENASE-1 INDUCERS AND HEME DEGRADATION PRODUCTS IN CARDIOVASCULAR DISEASES AND DIABETES

<i>Pharmacological agent</i>	<i>Model or cell type</i>	<i>Mode of HMOX1 induction</i>	<i>References</i>
<i>Lipid modulating drugs</i>			
<i>Fibrates</i>			
Fenofibrate	Human umbilical vein EC, human vascular SMC	PPAR $\alpha$ agonist	(89)
<i>Phenolics</i>			
Probucol	Rabbit aorta after balloon injury	Anti-inflammatory, antioxidant, and beneficial lipid profile, transcription factor Yin Yang 1	(9, 35, 186)
Succinobucol	Rat vascular smooth muscle cells after balloon injury	Apoptosis	(114)
	Human coronary events and new onset of diabetes	Anti-inflammatory and antioxidant mechanisms	(162)
<i>Statins</i>			
<i>Atorvastatin</i>			
	Murine RAW264.7 macrophages	PKG, ERK, and p38 MAPK signaling activation	(17)
	Human umbilical vein EC, human aortic EC	Induction of Krüppel-like factor 2	(3)
	Rat aortic SMC	Inhibition of NF- $\kappa$ B translocation/anti-inflammatory	(59)
<i>Fluvastatin</i>			
	Murine RAW264.7 macrophages	PKG, ERK, and p38 MAPK signaling activation	(17)
	Human coronary artery SMC	Increased Nrf2 <i>via</i> PI3K-Akt signaling	(111)
<i>Pravastatin</i>			
	Murine RAW264.7 macrophages	Protein kinase G, ERK, and p38 MAPK signaling activation	(17)
	Rat renal tubular epithelial cells	PPAR $\alpha$ binds to peroxisome proliferator response element in the <i>HMOX1</i> promoter	(16)
<i>Simvastatin</i>			
	Murine RAW264.7 macrophages	Protein kinase G, ERK, and p38 MAPK signaling activation	(17)
	Human and rat aortic SMCs, mouse aorta	p38 MAPK and PI3K/Akt signaling	(93)
	Human EC	PI3K/Akt signaling	(63)
<i>Anti-proliferative drugs</i>			
<i>Paclitaxel</i>			
	Drug-eluting stent inhibits rat vascular SMC proliferation	JNK, ERK, and p38 MAPK signaling activation	(22)
<i>Sirolimus (Rapamycin)</i>			
	Drug-eluting stent, rabbit endothelialization, and human aortic EC	Prevents binding of PPAR $\gamma$ to <i>HMOX1</i> promoter	(49)
<i>Anti-inflammatory drugs</i>			
<i>Aspirin</i>			
	Human umbilical vein EC	NO-dependent pathway	(58)
<i>Anti-diabetic drugs</i>			
<i>Rosiglitazone</i>			
	Rat cardiomyoblast cell line, rat model of pre-eclampsia	PPAR $\gamma$ agonist	(89)
<i>Agent</i>	<i>Indication</i>	<i>Status</i>	<i>References</i>
<i>Clinical trials</i>			
<i>Heme arginate</i>			
	Cardiac injury after myocardial ischemia	Unclear	<a href="http://clinicaltrials.gov/ct2/show/NCT00483587">http://clinicaltrials.gov/ct2/show/NCT00483587</a>
	Adenosine-induced vasodilation in atherosclerotic disease	Terminated	<a href="http://clinicaltrials.gov/ct2/show/NCT00856817">http://clinicaltrials.gov/ct2/show/NCT00856817</a>
	Ischemia-reperfusion injury in renal transplants	Recruiting	<a href="http://clinicaltrials.gov/ct2/show/NCT01430156">http://clinicaltrials.gov/ct2/show/NCT01430156</a>
<i>Hemin</i>			
	Gastroparesis in diabetic patients	Recruiting	<a href="http://clinicaltrials.gov/ct2/show/NCT01206582">http://clinicaltrials.gov/ct2/show/NCT01206582</a>
<i>Inhalants</i>			
<i>Carbon monoxide</i>			
	Idiopathic pulmonary hypertension	Recruiting	<a href="http://clinicaltrials.gov/ct2/show/NCT01214187">http://clinicaltrials.gov/ct2/show/NCT01214187</a>

(continued)



TABLE 3. Continued

Agent	Indication	Status	References
	Severe pulmonary arterial hypertension	Recruiting not commenced	<a href="http://clinicaltrials.gov/ct2/show/NCT01523548">http://clinicaltrials.gov/ct2/show/NCT01523548</a>
	Pulmonary inflammatory response to endotoxin	Local inflammatory response not altered by inhaled CO	<a href="http://clinicaltrials.gov/ct2/show/NCT00094406">http://clinicaltrials.gov/ct2/show/NCT00094406</a> (108)

CO, carbon monoxide; EC, endothelial cell; HMOX1, heme oxygenase-1; SMC, smooth muscle cell.

results [reviewed in ref. (153)]. For example, while probucol failed to provide benefits in patients with femoral atherosclerosis (161, 162, 175), the drug was reported to decrease atherosclerosis in carotid arteries and associated cardiac events (140). Of potential importance, long-term treatment of probucol in combination with other cholesterol-lowering drugs prevents secondary cardiovascular events in patients with heterozygous familial hypercholesterolemia (190). In preclinical studies, fenofibrate, a PPAR $\alpha$  agonist, was shown to increase HMOX1 in human umbilical vein EC and vascular SMC (89). Similarly, niacin increases HMOX1 and protects against vascular inflammation (184), similar to the manner in which apolipoprotein AI mimetic peptides have been shown to increase HMOX1 and to attenuate adipocyte dysfunction (174) and intimal hyperplasia, respectively (185).

Inducers of HMOX1 such as heme arginate and hemin are also the subjects of several clinical trials that are relevant to vascular disease (see Table 3). The indications include prevention of cardiac injury after myocardial ischemia, adenosine-induced vasodilation in atherosclerotic disease, ischemia-reperfusion injury in renal transplants (all heme arginate), and improvement of gastroparesis in diabetic patients (hemin).

#### Biliverdin, bilirubin, and CO

Biliverdin and bilirubin were originally considered waste products; however, these products of heme catabolism have antioxidant and cell-protective benefits in their own right (154). Low bilirubin levels are associated with an increased risk of coronary artery disease (66, 145) and an increased risk of metabolic diseases such as diabetes mellitus (21). In contrast, exogenous bilirubin has been shown to protect from ischemia-reperfusion injury (1) and to prevent neointimal hyperplasia in rats (128). In Gilbert syndrome, congenital hyperbilirubinemia is associated with a decrease in the development of diabetic vascular complications compared with non-Gilbert diabetic patients (71). The addition of bilirubin, or its precursor biliverdin, to culture media also prevents oxidant-induced cytotoxicity in vascular SMC (26) and the adhesion of leukocytes to EC (60).

More recently, HMOX1 induction or administration of heme degradation products has been found to have beneficial vascular effects, in part, *via* regulation of free heme and NADPH oxidases (116). NADPH oxidases generate superoxide and H<sub>2</sub>O<sub>2</sub> derived from it that not only play important roles in host defense and cell signaling but can also lead to oxidative stress and inflammation. However, hemin-mediated induction of HMOX1 decreases NADPH oxidase

activity in murine aorta *in vivo*, while bilirubin administration mimics the decrease of NADPH oxidase activity *in vitro* in rat vascular SMC (31) and human EC (73). In Spontaneous Hypertensive Rats, hemin treatment increased HMOX activity, improved endothelial function, and was associated with a decrease in NADPH oxidase-2 (96). In contrast, HMOX1 induction blocked vascular hypertrophy in *NADPH oxidase-4* null mice (142). These data suggest that HMOX1 may modulate individual NADPH oxidases independently of each other in different tissues or disease states.

CO is a signaling molecule with anti-inflammatory, anti-apoptotic, and anti-proliferative properties (135). CO inhibits LPS-induced increases in pro-inflammatory cytokines such as tumor necrosis factor- $\alpha$ , interleukin-1 $\beta$ , and macrophage inhibitory protein-1 $\beta$  (125). Furthermore, CO can induce the release of anti-inflammatory interleukin-10 *via* p38 MAPK signaling (94). Finally, low doses of CO protect cells against inflammation-induced oxidative stress (14). In addition to the clinical trials of CO inhalation for pulmonary disease previously mentioned, CO inhalation has also been assessed with regard to its effect on the pulmonary inflammatory response to endotoxin (see *e.g.*, <http://clinicaltrials.gov/ct2/show/NCT00094406>).

The administration of non-toxic concentrations of CO has been shown to be beneficial in a number of animal models. It attenuates neointimal hyperplasia in balloon-injured rat carotid arteries (171) and, similar to increasing HMOX1, attenuates infarct size in a cerebral model of ischemia-reperfusion injury (176). Similarly, CO administration induces HMOX1 and reduces ischemic lung injury in *Hmox1*<sup>-/-</sup> mice (50). In addition, in aortic tissues from *Hmox1*<sup>-/-</sup> mice, CO has been demonstrated to rescue impairment of angiogenesis that is induced by SDF-1 (37).

There is also evidence that HMOX activity and its products may be relevant for transplantation-related cardiovascular diseases (148). Thus, inhibition of HMOX activity by tin protoporphyrin has been reported to enhance the rejection of mouse-to-rat heart transplants (139); while human *HMOX1* (13) or rat *Hmox1* overexpression prolongs cardiac allograft survival (4). These effects appear to be attributable to CO and/or biliverdin, as their administration also leads to marked inhibition of ischemia-reperfusion injury, with improved donor graft survival and recipient animal survival (118, 139).

Of note, increasing HMOX1 or administration of heme degradation products may augment HMOX1 expression *via* a positive feedback loop. Thus, CO can activate the transcription factor Nrf2, which binds to antioxidant response elements and induces *HMOX1* gene transcription, and this, in turn, amplifies the HMOX1 response (91) (Fig. 6).

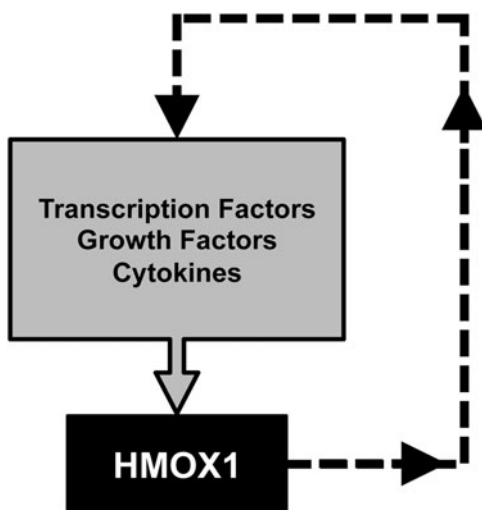


FIG. 6. HMOX1 may act downstream and upstream of transcription factors, growth factors, and cytokines. A new paradigm for HMOX1 as a cause and effect in gene transcription and cell signaling.

Amplification benefits have also been attributed to the biliverdin/bilirubin redox cycling (7) by BVR, but this notion has been questioned ever since (109, 113).

#### HMOX1 and cancer

While the potential benefits of inducing HMOX1 and heme degradation products to alleviate cardiovascular diseases are promising, due consideration to the multiple effects of HMOX1 is necessary. For example, HMOX1 expression is elevated in a variety of tumors and neoplasms [reviewed in ref. (180)]. HMOX1 may alternatively be over-expressed in cells supporting the immediate surroundings of the tumor, for example, macrophages (32). Within the tumor, or its microenvironment, HMOX1, CO, Fe, and bilirubin may encourage cell proliferation; while HMOX1 and CO have antiapoptotic effects that may improve tumor survival (180). In contrast to these observations, HMOX1 overexpression has been shown to block proliferation by increasing cell cycle arrest and apoptosis (62), and to prevent invasion (98) of the human breast cancer cell line MCF7 *in vitro*. Similar to the different effects of HMOX1 on EC and SMC proliferation, these data suggest that the role of HMOX1 in tumor growth may be complex. Nevertheless, HMOX1 inhibitors such as zinc protoporphyrin have shown promise in reducing tumor growth (64).

HMOX1 is a strong promoter of angiogenesis and neovascularization (75, 180) *via* its association with SDF-1 (37), a potent signal for progenitor mobilization and homing (2) supporting tumor growth. The promotion of angiogenesis may also facilitate metastasis. Furthermore, the rupture of malformed neovessels could lead to the release of hemoglobin (2), which, in turn, amplifies the HMOX1 response. Current treatment approaches such as UV irradiation and chemotherapy themselves induce HMOX1 expression, amplifying a pro-survival response that may reduce treatment efficacy (180). Moreover, HMOX1 and CO may inhibit dendritic cell effector responses that may impact T-regulatory cells and lead to immunosuppression [reviewed in ref. (11)]. Collectively, these data identify the potential obstacles to be overcome for

inducers of HMOX1 to be successful therapeutics against cardiovascular disease.

#### Conclusions

In addition to degrading and detoxifying heme, HMOX1 is increasingly recognized as a protein that protects cells *via* multiple pathways. Increasing our understanding of the functions and post-translational modifications of HMOX1 in different subcellular localizations is paramount for our ability to exploit HMOX1 for the treatment of human diseases. At present, the application of HMOX1 inducers as treatment modality for cardiovascular and metabolic diseases should be carefully considered. This is due to the multiple activities of the enzyme that includes the regulation of transcription *via* an interplay with transcription factors such as YY1 (9) and hypoxia-inducible factor-1 (92), or growth factors/chemokines such as VEGF (74) and SDF-1 (37). Intriguingly, at least in the cases of VEGF and YY1, HMOX1 has been reported to be both downstream and upstream of key regulators of cellular metabolism and action. This raises the possibility that this action of HMOX1 may extend to other factors (Fig. 6). HMOX1 is elevated in a variety of cancers, and it can be associated with tumor angiogenesis and metastasis (64). Therefore, it will be of utmost importance to pay due consideration to the relationship between HMOX1 and specific diseases, and/or overlapping disease states.

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Address correspondence to:

Prof. Roland Stocker

Vascular Biology Division

The Victor Chang Cardiac Research Institute

405 Liverpool St.

Darlinghurst, NSW 2010

Australia

E-mail: r.stocker@victorchang.edu.au

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#### Abbreviations Used

Apoe	= apolipoprotein E
BVR	= biliverdin reductase
CAV	= caveolin
CO	= carbon monoxide
CPR	= cytochrome P450 reductase
DAPI	= 4',6-diamidino-2-phenylindole
EC	= endothelial cell
ER	= endoplasmic reticulum
H <sub>2</sub> O <sub>2</sub>	= hydrogen peroxide
HMOX	= heme oxygenase
LPS	= lipopolysaccharide
MAM	= mitochondrial-associated membrane
SDF-1	= stromal cell-derived factor-1
SMC	= smooth muscle cell
TMS	= transmembrane segment
VEGF	= vascular endothelial growth factor
YY1	= Yin Yang 1