Mechanisms of Cell Protection by Heme Oxygenase-1

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Abstract

Heme oxygenases (HO) catabolize free heme, that is, iron (Fe) protoporphyrin (IX), into equimolar amounts of Fe^{2+}, carbon monoxide (CO), and biliverdin. The stress-responsive HO-1 isoenzyme affords protection against programmed cell death. The mechanism underlying this cytoprotective effect relies on the ability of HO-1 to catabolize free heme and prevent it from sensitizing cells to undergo programmed cell death. This cytoprotective effect inhibits the pathogenesis of a variety of immune-mediated inflammatory diseases.

INTRODUCTION

HO: heme oxygenase
Fe: iron
CO: carbon monoxide
FtH: ferritin H chain
BVR: biliverdin reductase

Heme oxygenases (HO; encoded by *HMOX* genes) are evolutionarily conserved enzymes that catabolize heme, that is, iron (Fe) protoporphyrin (IX), into equimolar amounts of labile Fe, carbon monoxide (CO), and biliverdin (1). The ubiquitous expression of *HMOX* genes in most living organisms suggests that this enzymatic reaction appeared early during evolution (2, 3). The substrate of HO activity, that is, heme, exists essentially as a prosthetic group of hemoproteins (**Table 1**), an evolutionarily conserved strategy that allows the incorporation of Fe^{2+} into the tertiary structure of proteins.

Free heme can catalyze the production of free radicals through Fenton chemistry (4). Under homeostasis this pro-oxidant effect is tightly controlled by the insertion of heme into the heme pockets of hemoproteins, which control the rate of electron exchange between Fe-heme and a variety of ligands. Under oxidative stress, however, some hemoproteins can release their prosthetic heme groups, producing free heme that can catalyze the production of free radicals in an unfettered manner. We use the term free heme to refer to heme molecules that are not contained within the heme pockets of hemoproteins. This does not preclude the association of free heme with proteins or lipids in a manner that does not control its pro-oxidant activity.

Under oxidative stress, cells can avoid the pro-oxidant effects of free heme through a variety of mechanisms. These rely in large measure on the rapid induction of the heme oxygenase-1 (HO-1) isoenzyme, which increases the rate of free heme catabolism, preventing it from inducing programmed cell death in response to proinflammatory agonists (154). Whereas the constitutive HO isoenzyme, that is, HO-2, might afford some level of protection against free heme, its expression is not inducible in response to oxidative stress, which probably makes HO-2 less likely to play a central role in affording cytoprotection against free heme. We argue that the cytoprotective effect of HO-1 against free heme probably underlies the broad salutary effects of this stress-responsive enzyme against a variety of immune-mediated inflammatory diseases (5). We discuss the molecular mechanisms mediating the cytoprotective effects of HO-1 and how these might be used therapeutically to overcome some of the major causes of morbidity and mortality worldwide.

HEME OXYGENASE-1

Humans and rodents have two HO isoenzymes, namely HO-1 [~32 kDa, enzyme classification (EC) 1.14.99.3] and HO-2 (~36 kDa, EC 1.14.99.39) encoded by the *HMOX1* and *HMOX2* genes, respectively. HO-1 expression is induced ubiquitously in response to oxidative stress, whereas HO-2 is constitutively expressed and not inducible. *HMOX* genes are expressed by most living organisms including bacteria, algae, plants, insects, and mammals, suggesting that the need for heme catabolism by HO occurred early during evolution (reviewed in 2, 3). The existence and chemical properties of free heme may explain the evolutionary pressure leading to expression of *HMOX* genes. Heme is a protoporphyrin IX ring that contains in its center an Fe^{2+} atom that can act as a Fenton reactor to produce highly toxic hydroxyl radicals derived from hydrogen peroxide (H_2O_2) (4). In its simplest form, heme catabolism by HO enzymes can be viewed as a process that allows the extraction of Fe from the protoporphyrin IX ring (**Figure 1**). This reaction produces labile Fe that induces the expression of the ferritin H chain (FtH) (6), which combines with the ferritin L chain to form a multimeric (24-subunit) complex with high Fe-storing capacity (4500 Fe atoms per ferritin) (reviewed in 7) (**Figure 1**). This heteropolymer oxidizes Fe^{2+} into Fe^{3+}, a property attributed to the ferroxidase activity of FtH (reviewed in 7). In addition, heme catabolism by HO-1 produces biliverdin (**Figure 1**), which can be converted into the antioxidant bilirubin (8) by biliverdin reductase (BVR) (9) (reviewed in 10). Heme catabolism by HO-1 also

Table 1 Hemoproteins

	Function	Hemoprotein	Specific Biological function
Heme acts as a stable prosthetic group	Gas carriers	Hemoglobin	O_2 carrier in red blood cells
		Myoglobin	O_2 storage and delivery in muscles
		Neuroglobin	Uncertain function in neuronal cells
		Cytoglobin	Uncertain function in fibroblasts and related cells
		Nitrophorins	NO delivery in saliva (blood-feeding insects)
	Electron transporters	Cytochrome c	Electron transfer between complex III and IV in the mitochondria electron transport chain
		Cytochrome c oxidase (EC 1.9.3.1)	Converts molecular O_2 to H_2O_2 using electrons from cytochrome c
		Cytochrome c reductase (EC 1.10.2.2)	Catalyzes the reduction of cytochrome c by oxidation of coenzyme Q
		Cytochrome b5	Electron donor
		Cytochrome b558	Catalytic subunit of NADPH oxidase
	Catalysts of biodegradation or biosynthesis	Cytochrome P450 superfamily (CYP, P450) (EC 1.14.14.1)	External monooxygenases that catalyze the incorporation of one atom of O_2 into a substrate
		Indoleamine 2,3-dioxygenase (EC 1.13.11.52)	Catalyzes of oxidative metabolism of tryptophan
		Tryptophan 2,3 dioxygenase (EC 1.13.11.11)	Initiates oxidative metabolism of tryptophan
		Catalases (EC 1.11.1.6)	Decomposes H_2O_2 to H_2O and O_2
		Myeloperoxidase (EC 1.11.1.7)	Produces HOCl from H_2O_2 and Cl^-
		Cytochrome c peroxidase (EC 1.11.1.5)	Uses H_2O_2 to oxidize Fe^{2+}-cytochrome c
		Eosinophil peroxidase (EC 1.11.1.7)	Produces ROS
		Lactoperoxidase (EC 1.11.1.7)	Uses H_2O_2 to oxidize thiocyanate
		NO synthase 1 (NOS1)	NOS1-3 catalyze the oxidation of L-arginine to L-citrulline with a concomitant production of NO
		NO synthase 2 (NOS2)	
		NO synthase 3 (NOS3)	
		Cystathione b-synthase (EC 4.2.1.22)	Catalyzes the condensation of L-serine and L-homocysteine to form cystathionine
	Heme-based gas sensors	FixL*	Regulates microaerobic adaptation (bacteria)
		DOS*	Oxygen sensor (bacteria)
		AxPDEA1*	Regulates cellulose excretion (bacteria)
		Neuronal PAS2*	Mammalian CO-regulated transcription factor implicated in circadian rhythm regulation
		hemATs**	Aerotaxis transducers (bacteria)
		GRegs**	Gene regulator (bacteria)
		CooA protein	CO-sensing transcription factor (bacteria)
		Soluble guanylyl cyclase (EC 4.6.1.2)	Synthesis of cGMP from GTP
Heme acts as a cellular messenger	Heme-sensing Heme-regulated proteins	Heme activator protein (HAP1)	Transcription factor (yeast)
		Bach1	Transcriptional repressor
		δ−aminolevulinate synthase 1 (ALAS1)	Rate-limiting enzyme of heme synthesis
		Iron regulatory protein 2 (IRP2)	Regulates Fe metabolism; heme binding induces oxidation, ubiquitination, and degradation
		DiGeorge critical region-8 (DGCR8)	RNA-binding protein involved in microRNA processing; heme binding required for function

(*Continued*)

Table 1 (*Continued*)

	Function	Hemoprotein	Specific Biological function
Heme acts as a cellular messenger	Heme-regulated proteins	Eukaryotic initiation factor 2a kinase	Controls protein synthesis in response to heme availability; inhibited by heme
		Slo1 BK (big potassium) channel	Transmembrane movement of K^+; inhibited by heme
		HOIL-1	Heme-responsive E3 ubiquitin-protein ligase

*PAS domain-containing proteins.
**Globin-coupled sensors.

produces CO (**Figure 1**), a gasotransmitter that modulates cellular signal transduction (reviewed in 11). Our current understanding of the heme/HO system assumes that its biological effects reflect those of the end products produced via heme catabolism (reviewed in 5, 12). This notion is, however, challenged by the finding that cleaved forms of HO-1 that lose enzymatic activity can exert biologic activities that most probably include cytoprotection (13). We argue that, in addition, heme catabolism per se is a critical component mediating the biological effects attributed to HO-1 and in particular its cytoprotective action.

REGULATION OF HO-1 EXPRESSION

Expression of HO-1 is regulated essentially at the transcriptional level, with some exceptions, including mRNA stabilization by hypoxia (14) or acidosis (15). Moreover, HO-1 enzymatic activity can also be regulated, such as demonstrated for hyperoxia (16). *HMOX1* transcription can be induced by a variety of signal transduction pathways that activate different transcription factors. These recognize specific DNA-binding elements in the proximal (−0.3 kb) and distal (−4 kb-E1 and −10 kb-E2) regions of the *HMOX1* promoter, inducing *HMOX1* transcription (17, 18; reviewed in 19) (**Figure 2**). The signal transduction pathways and transcription factors regulating *HMOX1* transcription share as a common denominator their activation in response to oxidative stress (reviewed in 20) (**Figure 2**). This suggests that most, if not all, forms of oxidative stress are associated with a rapid increase in the rate of cellular heme catabolism, through the induction of *HMOX1* transcription and HO-1 expression. The reason why cells respond to oxidative stress by increasing their ability to catabolize free heme is not clear. Oxidative stress can lead to heme release from some hemoproteins (21) (reviewed in 22) (**Table 1**), an effect that produces cytotoxic free heme. By coupling oxidative stress to the induction of HO-1, cells would ensure that the free heme produced in response to oxidative stress does not act in a cytotoxic manner (23). This notion is strongly supported by the observation that the cytotoxic effects of oxidative stress are exacerbated in cells that lack HO-1 (*Hmox1*$^{-/-}$) (24–26) and thus cannot increase the rate of heme catabolism in response to oxidative stress. Whether this effect is directly related to the accumulation of free heme in these cells remains to be established.

Oxidative stress suppresses the activity of Bach1 (~82 KDa; human; O14867), a transcriptional repressor that binds to several stress-responsive elements (StREs) in the *HMOX1* promoter and inhibits its transcription (27, 28). Bach1 has heme-binding sites (29), via which heme can induce Bach1 conformational modifications. These inhibit Bach1 binding to StREs, inducing Bach1 nuclear export, polyubiquitination by the heme-responsive E3 ubiquitin-protein ligase HOIL-1, and subsequent degradation by the 26S proteasome pathway (28) (**Figure 2**). Reactive oxygen species can also target directly the sulfhydryl groups of Bach1 to inhibit its binding to StREs, thus promoting nuclear export and degradation (30). Release of Bach1 from

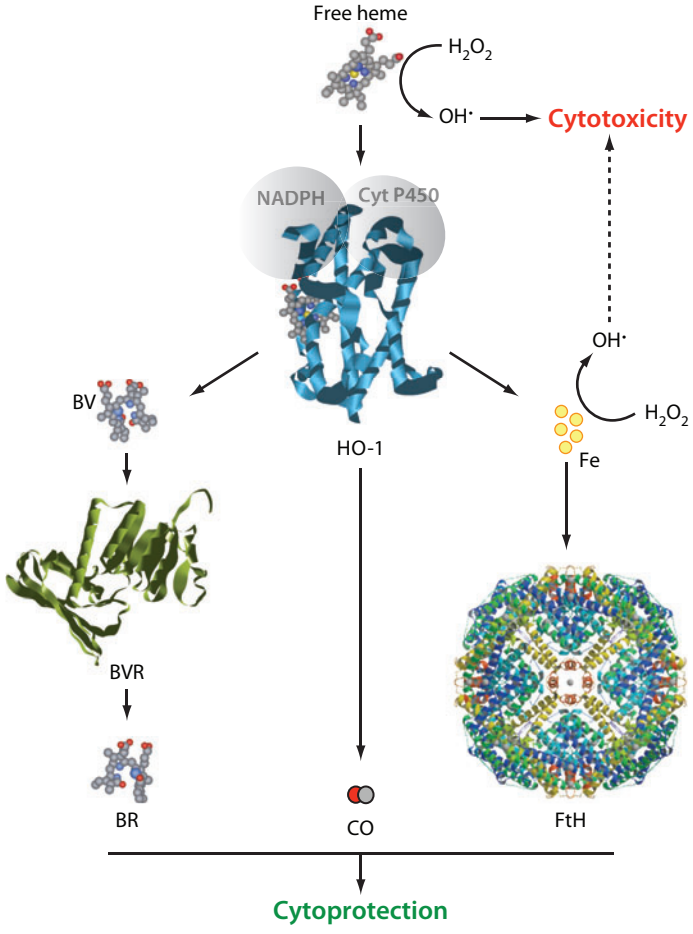

Figure 1

The heme/HO-1 system. Free heme is a reactive Fe compound that can catalyze, through the Fenton reaction, the formation of cytotoxic hydroxyl radical (OH•) from hydrogen peroxide (H_2O_2). Elimination of the reactive heme-Fe by HO-1 involves cleavage of the protoporphyrin IX ring of heme with production of biliverdin (BV) and CO as well as release of Fe. The Fe (*yellow circles*) released from the protoporphyrin IX ring of heme is then stored by the ferritin H chain (FtH). BV is converted by biliverdin reductase (BVR) into the antioxidant bilirubin (BR). All three end products of heme catabolism, that is, biliverdin/bilirubin, CO, and Fe/FtH, are cytoprotective.

the StREs in the *HMOX1* promoter allows the oxidative-stress responsive transcription factor NF-E2-related factor-2 (Nrf2) to access these StREs and induce *HMOX1* transcription (31, 32). Given the pro-oxidant activity of free heme, its production in response to oxidative stress might also act through this pathway to inhibit Bach1 activity and promote *HMOX1* transcription. (**Figure 2**).

Whether inhibition of Bach1 is required to support *HMOX1* transcription driven by other transcription factors is not clear. Binding of Bach1 to StREs can form long-range DNA loops that repress gene transcription (33). Because the *HMOX1* promoter has several StREs (34), Bach1 may act in a similar manner to repress *HMOX1* transcription. Such an effect should inhibit *HMOX1* transcription by several transcription factors, other than Nrf2 (**Figure 2**). This notion is supported

Nrf2: NF-E2–related factor-2

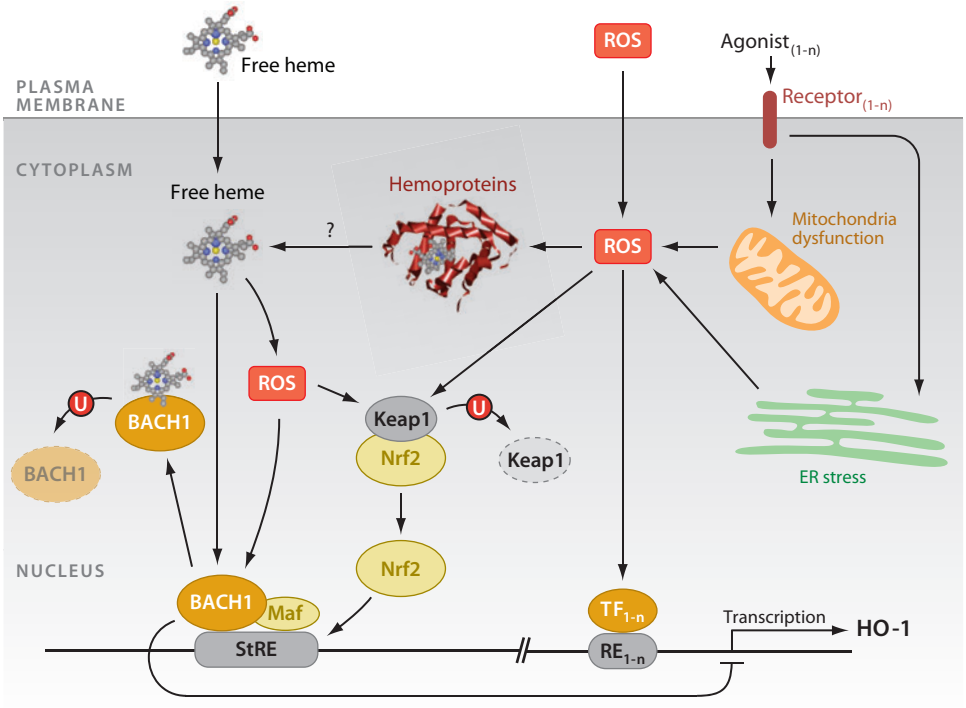

Figure 2

Transcriptional regulation of *HMOX1*. The rate of *HMOX1* transcription can be induced by heme as well as by a variety of other agonists (1-n) recognized by specific receptors (1-n). These trigger signal transduction pathways that are associated with the production of reactive oxygen species (ROS) by the mitochondria and/or the endoplasmic reticulum. ROS can elicit heme release from hemoproteins, leading to oxidative stress. The *HMOX1* promoter contains multiple DNA-responsive elements $(RE)_{1-n}$ recognized by specific transcription factors (TF_{1-n}) activated in response to oxidative stress. Under homeostasis Bach1/small Maf dimers bind constitutively to stress responsive elements (StREs) in the *HMOX1* promoter and inhibit *HMOX1* transcription. In response to oxidative stress, Bach1 is exported from the nucleus, ubiquitinated (*red circled u*) and degraded (*dotted lines*), releasing transcriptional repression. Oxidative stress also induces Keap1 ubiquitination-degradation (*u/dotted line*), allowing the transcription factor NF-E2-related factor-2 (Nrf2) to translocate into the nucleus. Nrf2 /small Maf protein heterodimers bind to StRE and promote *HMOX1* transcription. Most probably the Bach1/Nrf2 transcriptional system interacts functionally with other transcription factors to regulate *HMOX1* transcription.

by the observation that *Bach1*-deficient ($Bach1^{-/-}$) cells express constitutive high levels of *Hmox1* mRNA, an effect that is not ablated in cells also lacking Nrf-2 ($Bach1^{-/-}Nrf2^{-/-}$) (32). These observations suggest that oxidative stress, leading to heme release from hemoproteins or not, is a common denominator via which Bach1 activity is suppressed, allowing several stress-responsive transcription factors to induce *HMOX1* transcription (**Figure 2**).

HO-1 IS PROTECTIVE AGAINST IMMUNE-MEDIATED INFLAMMATORY DISEASES

HO-1 has a broad healing effect, as demonstrated by several lines of evidence: First, *Hmox1* deletion in mice increases the severity of many experimental diseases (reviewed in 5). Second, the incidence

and/or severity of many diseases in humans are associated with a microsatellite polymorphism in the *HMOX1* promoter that regulates HO-1 expression (reviewed in 35). Third, pharmacological induction of HO-1 or administration of the end products of heme catabolism can exert therapeutic effects on many diseases (reviewed in 5, 12, 36, 37). Fourth, several molecules known to exert protective effects against a broad spectrum of diseases appear to do so via a mechanism that relies on the induction of HO-1, a phenomenon referred to as the HO-1 therapeutic amplification funnel (38; reviewed in 5).

HMOX1 Deletion

Studies comparing the outcome of several experimental pathologic conditions in mice that can express HO-1 ($Hmox1^{+/+}$) or not ($Hmox1^{-/-}$) demonstrate that when the expression of HO-1 is impaired, the pathologic conditions studied are exacerbated and return to homeostasis is severely compromised, resulting in high incidence of mortality (reviewed in 5). Pharmacological inhibition of HO activity in wild-type ($Hmox1^{+/+}$) mice mimics the phenotypes associated with *Hmox1* deletion, suggesting that exacerbation of the diseases studied in $Hmox1^{-/-}$ versus $Hmox1^{+/+}$ mice is not due to compensatory mechanisms acting independently of HO-1 (reviewed in 5).

Studies using mouse strains carrying *Hmox1* deletions (24, 39) have been hampered by the low yield of viable $Hmox1^{-/-}$ progeny compared with the expected Mendelian segregation ratios (24, 39). Female $Hmox1^{-/-}$ mice are infertile, and therefore $Hmox1^{-/-}$ mice can only be obtained from breeding of $Hmox1^{-/+}$ mice, which depending on the genetic background yields 1–8% viable $Hmox1^{-/-}$ progeny, compared with the expected 25%. More than a technical hurdle, this partial embryonic lethality reveals a critical involvement of HO-1 in sustaining successful pregnancy (reviewed in 40, 41). This notion is supported by a series of recent observations made in collaboration with the laboratory of Ana Claudia Zenclussen. Female $Hmox1^{-/-}$ mice have significantly lower levels of oocyte maturation and fertilization as well as lower ratios of successful fetus implantation compared with wild-type ($Hmox1^{+/+}$) mice (A. C. Zenclussen et al., unpublished observations). In addition, $Hmox1^{-/-}$ mice have severe defects in placentation as well as significantly decreased rates of postimplantation fetal acceptance compared with $Hmox1^{+/+}$ mice, resulting in increased abortion rates (A. C. Zenclussen et al., unpublished observations). Assuming that these observations can be extrapolated to humans, which is likely based not only on the evolutionarily conserved nature of the heme/HO-1 system (2) but also on the high level of HO-1 expression during human pregnancy (reviewed in 40, 41), one would predict that *HMOX1* deletion must be a rare event in humans. Presumably, *HMOX1* deletions are purged from human populations through a variety of clinically silent events, including lower female fertility and/or the occurrence of idiopathic recurrent miscarriages (reviewed in 40, 41). In keeping with this notion, only one clinical case study has described a functional disruption of the *HMOX1* locus in humans resulting in early lethality (42).

HMOX1 Polymorphisms

A number of polymorphisms in the human *HMOX1* locus, including a (GT)n microsatellite polymorphism in the *HMOX1* promoter that alters the levels of HO-1 expression, have been associated with the incidence and/or progression of a variety of diseases (43, 44; reviewed in 35). Based on the number (n) of GT repeats, ranging from 15 to 40, individuals with fewer repeats have higher HO-1 expression, and individuals with more GT repeats have lower HO-1 expression (reviewed in 35). This microsatellite (GT)n polymorphism might regulate HO-1 expression by modulating *HMOX1* transcription and/or translation. Even if few studies have addressed in a quantitative manner the influence of this (GT)n polymorphism on *HMOX1* transcription and/or translation (44),

several studies suggest that individuals with fewer (GT)n repeats and presumably stronger induction of HO-1 expression are less likely to develop a number of pathologies than individuals with a higher number of (GT)n repeats (reviewed in 35). These pathologies overlap broadly with those in which the outcome is exacerbated in mice that lack HO-1 (*Hmox1*$^{-/-}$) or in wild-type (*Hmox1*$^{+/+}$) mice in which HO activity is inhibited pharmacologically (reviewed in 5, 35). This (GT)n polymorphism has been associated with incidence of idiopathic recurrent miscarriages (45), and placentas from spontaneous abortions express lower levels of HO-1 compared with normal pregnancies (46), suggesting again that HO-1 expression is required to support successful pregnancy in humans.

IRI: ischemia reperfusion injury

Therapeutic Effects Associated with the Modulation of HO-1 Expression

Pharmacological induction of HO-1 or administration of the end products of its activity can exert therapeutic effects in a variety of immune-mediated inflammatory diseases (reviewed in 5, 47). It is possible that inhibition of HO-1 expression might be an intrinsic component of the pathogenesis of some of these diseases, as demonstrated for ischemia and reperfusion injury (IRI), where signaling via the proinflammatory toll-like receptor 4 contributes to disease progression via a mechanism associated with the inhibition of HO-1 expression (48, 49). Given that pharmacological induction of HO-1 or administration of end products of its activity can afford protection against IRI, this would argue that suboptimal expression of HO-1 is one of the factors involved in the pathogenesis of IRI. Whether the same is true for other immune-mediated inflammatory diseases remains to be established.

The HO-1 Therapeutic Amplification Funnel

Several molecules that exert salutary effects against immune-mediated inflammatory diseases might act via a common mechanism, namely, the induction of HO-1. Synthetic compounds that act in such a manner include sialic acid/aspirin (50), statins (51–53), and rapamycin (54). In addition, several molecules produced under physiologic conditions might act in a similar manner, including the anti-inflammatory cytokine interleukin-10 (55), 15-deoxy-12, 14-prostaglandin J2 (56), vascular endothelial growth factor (57, 58), stromal cell-derived factor 1 (59), nitric oxide (NO) (60), and nerve growth factor (61; reviewed in 36). The mechanisms via which these molecules induce the expression of HO-1 are not clear. It is possible, however, that they share as a common denominator the inhibition of Bach1 activity and the activation of transcription factors that promote *HMOX1* transcription (**Figure 2**). If this proves to be the case, then Bach1 might be a therapeutic target to suppress the pathogenesis of a broad range of immune-mediated inflammatory diseases.

CYTOPROTECTION AS A COMMON DENOMINATOR UNDERLYING THE SALUTARY EFFECTS OF HO-1 AGAINST IMMUNE-MEDIATED INFLAMMATORY DISEASES

Having established that HO-1 exerts protective effects against a wide variety of pathological conditions, it becomes interesting to understand the mechanisms underlying these unusually broad salutary effects. It has been assumed that when expressed in innate immune cells, HO-1 exerts anti-inflammatory effects that limit the damaging consequences of inflammation and immunity (reviewed in 12, 37). However, HO-1 is not anti-inflammatory when expressed under physiologic conditions in innate immune cells, such as monocyte/macrophages (Mø) (24, 62, 63; reviewed in 64). In a similar manner, HO-1 also fails to exert immunoregulatory effects when expressed under physiologic conditions in adaptive immune cells, such as T cells (65). Moreover, expression of

HO-1 in Mø promotes the production of proinflammatory cytokines such as type I interferon β (66) and interleukin-1β (R. Larsen and L. O. Otterbein, unpublished observation). We argue that the salutary effects of HO-1 are due to its cytoprotective action, a notion strongly supported by the observation that when challenged by proinflammatory agonists, HO-1 deficient (*Hmox1*$^{-/-}$) mice succumb from unfettered oxidative tissue injury rather than exacerbated inflammation (24, 67; reviewed in 5, 64).

TNF: tumor necrosis factor

TNFR-1: TNF receptor 1

c-IAP-2: cellular inhibitor of apoptosis protein 2

MECHANISMS UNDERLYING THE CYTOPROTECTIVE EFFECT OF HO-1

The cytoprotective effect of HO-1 was first revealed by the work of R. Tyrrell and colleagues, demonstrating that induction of HO-1 expression mediates an adaptive cytoprotective response to oxidative stress in cultured human fibroblasts (68, 69). Shortly thereafter, G. Balla and colleagues reported that HO-1 induction in cultured endothelial cells also affords an adaptive cytoprotective response against oxidative injury (23). Although instrumental in defining the cytoprotective role of HO-1, these studies have not addressed its mechanisms of action.

Most studies addressing the molecular mechanisms responsible for the cytoprotective effects of HO-1 have done so in the context of apoptosis, a type of programmed cell death induced by cytotoxic agonists such as tumor necrosis factor (TNF), lymphotoxin, or Fas, among others (reviewed in 70, 71). These cytotoxic agonists trigger several signal transduction pathways leading to the activation of caspases (72), a group of cysteine proteases that include the initiator caspases-2, -8, -9, and -10 and the effector caspases-3, -6, and -7 (reviewed in 70, 71). Caspase activation is responsible for the major biochemical and morphological events defining apoptosis, including plasma membrane blebbing, mitochondrial dysfunction, chromatin condensation, and nuclear fragmentation (reviewed in 70, 71, 73, 74). We found that HO-1 protects endothelial cells from undergoing TNF-mediated apoptosis (75), an effect later expanded to many other proapoptotic agonists (76) as well as cell types (reviewed in 77). The subsequent finding that when applied exogenously to endothelial cells in vitro, CO can mimic the cytoprotective effect of HO-1 (78) (**Figure 3**) led to the notion that the cytoprotective effect of HO-1 is mediated via the production of CO.

The proapoptotic effect of TNF is exerted mainly via the p55/tumor necrosis factor receptor 1 (TNFR-1) (79) (**Figure 3**). Neither HO-1 nor CO modulate p55/TNFR-1 expression or its ability to recognize soluble TNF (80). Engagement of TNFR-1 induces the recruitment of the TNFR-1-associated death domain (TRADD), tumor necrosis factor receptor (TNFR)-associated factor 2 (TRAF2), receptor-interacting protein 1 (RIP1), and the cellular inhibitor of apoptosis protein-1 and -2 (c-IAP-1, c-IAP-2) into complex I (81, 82), leading to the activation of the nuclear factor kappa B (NF-κB) family of transcription factors (82). NF-κB consists of five family members: NF-κB1/p105, NF-κB2/p100, RelA/p65, RelB, and c-Rel, which form active dimers (reviewed in 83). These are constitutively repressed by the cytoplasmic inhibitors of NF-κB (IκB), that is, IκBα (84), IκBβ, IκBγ, IκBε, and Bcl-3 (reviewed in 83). Signaling via the TNFR-1 complex I activates the IκB kinase (IKKα, β, γ) complex, targeting IκB molecules for phosphorylation (85), ubiquitination (86) and subsequently for proteolytic degradation by the 26S proteasome pathway (87). This allows for NF-κB nuclear translocation and binding to DNA κB motifs in the promoters of a variety of genes (reviewed in 83), including pro-inflammatory as well as antiapoptotic genes that suppress TNF-mediated apoptosis (88–90). HO-1 down-modulates NF-κB activation (80) without interfering with the expression of cytoprotective genes (80, 91). Moreover, the cytoprotective effect of HO-1 against TNF-mediated apoptosis requires the activation of NF-κB (91) (**Figure 3**). Ectopic expression of a subset of NF-κB-dependent genes

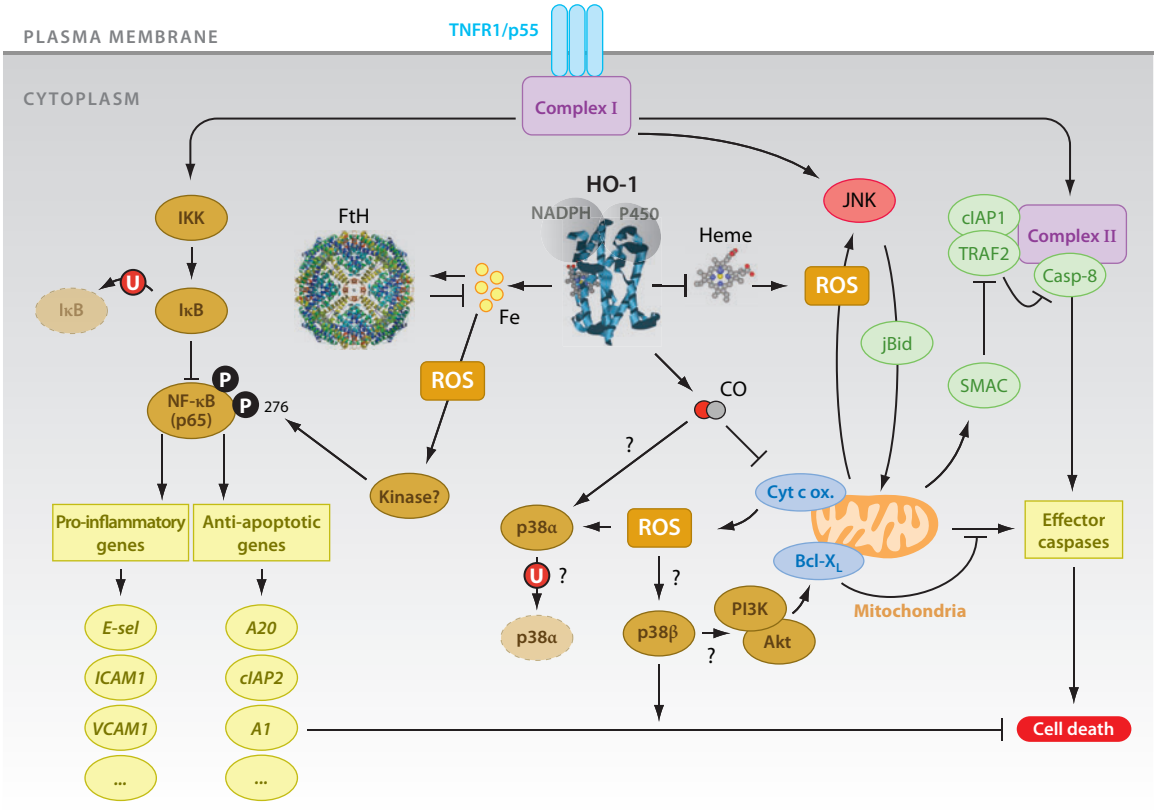

Figure 3

Cytoprotective effects of HO-1. Upon engagement, tumor necrosis factor receptor 1 (TNFR-1) induces the formation of multiprotein complex I, which activates the nuclear factor kappa B (NF-κB) signal transduction pathway. This consists of the activation of inhibitor of NF-κB (IκB) kinases (IKK), release of NF-κB (e.g., p65/RelA) from IκB molecules, NF-κB (e.g., p65/RelA) phosphorylation (e.g., Ser 276), nuclear translocation, and transcription of proinflammatory (e.g., E-selectin, ICAM-1, VCAM-1) as well as antiapoptotic genes (e.g., A20, cellular inhibitor of apoptosis protein 2 c-IAP-2 and -A1). TNFR-1 triggers the formation of a second cytosolic multiprotein complex, complex II, that activates caspase-8 and leads to apoptosis. TNFR-1 also activates the c-jun-N-terminal kinase (JNK) signaling transduction pathway, which can activate caspase-8 through a mitochondrial amplification loop in which the second mitochondria-derived activator of caspases (SMAC) disrupts the TNFR-associated factor 2 (TRAF2)/c-IAP-1 complex to promote sustained caspase-8 activation. Heme catabolism by HO-1 induces the expression of FtH and controls in this manner the pro-oxidant activity of labile Fe, an effect which inhibits p65/RelA phosphorylation (i.e., Ser 276), limiting the transcription of proinflammatory genes. The CO produced through heme catabolism by HO-1 targets cytochrome *c* oxidase to produce a transient oxidative burst. In addition, CO leads to the degradation of the p38α isoforms (*dotted circles*), activating the antiapoptotic p38β isoform, which interacts functionally with A1 and c-IAP-2 to suppress caspase activation and apoptosis. Moreover, HO-1 induces Bcl-x_L expression through activation of the phosphatidylinositol-3-kinase signal transduction pathway, an effect that inhibits the mitochondrial intrinsic apoptotic cell death pathway. HO-1 and FtH also decrease the cellular pools of free heme and free Fe, respectively, an antioxidant effect that limits the extent of JNK activation, thus acting in a cytoprotective manner. The putative cytoprotective effects of biliverdin and bilirubin are not illustrated in this figure.

(i.e., A1 and c-IAP-2) restores the cytoprotective effect of HO-1, under inhibition of NF-κB (91), suggesting that HO-1 interacts functionally with these genes to prevent apoptosis (91) (**Figure 3**).

The mechanism via which HO-1 down-modulates NF-κB activation without compromising its cytoprotective effect is not clearly established (**Figure 3**). HO-1 does not modulate NF-κB nuclear translocation or NF-κB binding to DNA κB motifs in the promoter of NF-κB-dependent

genes (92). Instead, it inhibits the phosphorylation of at least one NF-κB family member, that is, p65/RelA. It does so at Ser276, a phosphoacceptor residue that others have shown to modulate p65/RelA activity (93, 94) and to determine its specificity toward different NF-κB-dependent genes (95; reviewed in 96). Presumably, this explains how HO-1 modulates the NF-κB signal transduction pathway without interfering with the expression of c-IAP-2 and -A1, which are probably required to support its cytoprotective effect (91, 92).

MAPK: mitogen-activated protein kinase

Engagement of TNFR-1 forms a second protein complex that translocates into the cytoplasm, that is, complex II (**Figure 3**) (82). Recruitment of Fas-associated death domain (FADD) and caspase-8 into complex II promotes caspase-8 activation and triggers the activation of the extrinsic and/or intrinsic apoptotic pathways (reviewed in 73). The extrinsic pathway acts through direct activation, by caspase-8, of effector caspases, for example, caspase-3 (reviewed in 73). The intrinsic pathway requires an additional mitochondrial amplification loop triggered by activated caspase-8, involving the cleavage of cytosolic BH3 interacting domain (Bid) death agonist protein (97; reviewed in 98). Truncated Bid translocates into the mitochondria, where it interacts with the proapoptotic Bcl-2 family members Bax and Bak (reviewed in 99), leading to permeabilization of the outer mitochondrial membrane and release of several intermembrane mitochondrial proteins into the cytosol. These include cytochrome *c* (a hemoprotein) and the second mitochondria-derived activator of caspases (SMAC/DIABLO) (reviewed in 98, 100). Binding of cytochrome *c* to the apoptotic peptidase-activating factor-1 (Apaf-1) leads to the formation of the apoptosome, a cytosolic multiprotein complex in which caspase-9 is activated (reviewed in 100), which in turn induces the activation of effector caspases, for example, caspase-3 (71).

The cytoprotective effect of HO-1 is dependent on the p38 mitogen-activated protein kinase (MAPK) signal transduction pathway, as revealed by the observation that pharmacologic inhibition of the p38α and p38β MAPK isoforms suppresses the antiapoptotic effect of HO-1 (78). Activation of the p38α MAPK isoform essentially works in a proapoptotic manner (101), whereas activation of the p38β MAPK isoform is antiapoptotic (reviewed in 102). The finding that HO-1 specifically targets the p38α MAPK isoform for degradation by the 26S proteasome pathway, sparing p38β (103), suggests that HO-1 controls the ratio of these two isoforms so that the action of the cytoprotective p38β predominates over the cytotoxic p38α (**Figure 3**). Activation of p38β MAPK is probably involved in the mechanism by which HO-1 interacts functionally with c-IAP-2, -A1, and possibly other cytoprotective genes to suppress TNF-mediated apoptosis (91) (**Figure 3**).

Activation of p38 MAPK by HO-1 also induces the expression of Bcl-x_L via the phosphatidylinositol-3-kinase (PI3K/Akt) signal transduction pathway (104, 105), an effect that should inhibit the intrinsic (mitochondrial) apoptotic pathway (**Figure 3**). In addition, activation of the PI3K/Akt signal transduction pathway induces the expression of HO-1 (106) and modulates its activity via Ser188 phosphorylation (107). This suggests that some of the protective effects of HO-1 are mediated via the PI3K/Akt signal transduction pathway, which induces the expression of the antiapoptotic genes such as Bcl-x_L, Bcl-2, or HO-1 itself while inhibiting the expression of proapoptotic genes such as Bax and Bak (108, 109). Presumably, this effect should contribute to inhibition of the intrinsic (mitochondrial) apoptotic pathway (**Figure 3**).

CYTOPROTECTIVE MECHANISMS OF THE END PRODUCTS OF HO-1 ACTIVITY

The cytoprotective effect of HO-1 is thought to be exerted essentially via its enzymatic activity (78), that is, via the catabolism of free heme and presumably therefore through the production of the end products of this reaction, that is, CO, Fe, and/or biliverdin. It is possible, however, that

HO-1 might exert cytoprotective effects via mechanisms that act independently of its enzymatic activity (13).

ROS: reactive oxygen species

The Gasotransmitter CO

The finding that inhaled CO is protective against hyperoxic (110) or ischemic (111) lung injury as well against the rejection of cardiac transplants (112), together with the finding that CO is cytoprotective (78), leads to the notion that when produced endogenously, this gasotransmitter mediates, or at the very least can mediate, the (cyto)protective effects of HO-1 (78, 112, 113). Presumably, the low reactivity of CO, compared to other gasotransmitters such as NO or hydrogen sulfide (H_2S), favors the ability of CO to act as a gasotransmitter under physiologic conditions (reviewed in 11, 114, 115). The cytoprotective effect of CO (78) relies most probably on its ability to interact with divalent metals such as Fe^{2+} in the prosthetic heme groups of hemoproteins (reviewed in 11, 114, 115) (**Table 1**). One of these hemoproteins is probably the terminal acceptor of the mitochondrial electron transport chain, cytochrome *c* oxidase (116). When exposed to CO, cells release within seconds or minutes a small burst of mitochondrial reactive oxygen species (ROS) (116), presumably owing to the binding of CO to the heme group of cytochrome *c* oxidase, which remains to be demonstrated unequivocally (116, 117). This ROS burst can activate the p38 MAPK signal transduction pathway (116, 117), which we have shown to be critically involved in the cytoprotective effect of HO-1 (78, 103) as well as that of CO (78) (**Figure 3**). When applied exogenously to cells, CO triggers the proteolytic degradation of the proapoptotic p38α MAPK isoform (103), favoring signaling via the antiapoptotic p38β MAPK isoform (103). This effect of CO, which mimics that of HO-1, probably involves the ubiquitination of p38α via a mechanism that is not established. Activation of p38β MAPK by CO is involved in the mechanism via which HO-1 interacts functionally with c-IAP-2 and A1 to suppress TNF-mediated apoptosis (91) (**Figure 3**).

The mitochondrial ROS burst produced in response to CO can trigger the activation of other signal transduction pathways, including one leading to the activation of the hypoxia-inducible factor 1 alpha (118). This transcription factor induces the expression of transforming growth factor beta, which can mediate some of the cytoprotective effects of CO (118).

Most studies done so far have analyzed the biological effects of exogenous CO in wild-type (*Hmox1*$^{+/+}$) cells that can express HO-1. The finding that CO mimics the cytoprotective effects of HO-1 (78) suggests that CO acts independently of HO-1 to exert these cytoprotective effects. However, one should consider the possibility that the cytoprotective effects of CO might be mediated, at least in some cases, via the induction of HO-1. Exposure of cultured endothelial cells to CO, and more so to CO-releasing molecules, has been shown to induce *HMOX1* transcription through a mechanism involving the transcription factor Nrf2 (119). This occurs via activation of the upstream protein kinase R-like endoplasmic reticulum kinase (119), a member of the eukaryotic translation initiator factor (eIF)-2α kinase family that regulates cellular responses to endoplasmic reticulum stress (reviewed in 120). According to this observation, CO might participate with HO-1 in a positive forward feedback loop in which CO induces the expression of HO-1, which produces more CO, which induces further the expression of HO-1. This mechanism is reminiscent of other positive forward feedback loops underlying the salutary effects of HO-1 (57, 121). The involvement of protein kinase R-like endoplasmic reticulum kinase in the cytoprotective action of HO-1 also suggests that HO-1 and CO might afford cytoprotection against endoplasmic reticulum stress (119).

CO can diffuse within cells, and as such its cytoprotective effects can be exerted in a bystander manner (78). Because tissue Mø are probably the cell type that can express the highest levels of

HO-1, it is possible that the cytoprotective effects of CO might be exerted via the expression of HO-1 in tissue Mø. Such an effect would allow Mø to produce high levels of proinflammatory mediators upon activation (66) while preventing their cytotoxic effects on bystander cells, via the production of CO (78). However, the overall contribution of Mø to the cytoprotective effect of HO-1 needs further study.

JNK: jun N-terminal kinase

Labile Fe

Heme catabolism releases Fe from the protoporphyrin IX ring, which might seem counterintuitive (122) because the labile Fe produced in this manner can catalyze the production of free radicals through the Fenton chemistry (4) and thus act as a cytotoxic pro-oxidant. However, one should consider that, contrary to the Fe atom contained in the protoporphyrin IX ring of heme, the free Fe released from heme can be neutralized by a variety of Fe metabolic pathways, induced by Fe itself (6, 123). These include, but are probably not restricted to, the induction of a Fe efflux pump (123, 124) as well as the induction, at the translational level (and thus very quick), of FtH expression, a Fe sequestering protein (6; reviewed in 7, 125). Both mechanisms neutralize the pro-oxidant activity of free Fe and are cytoprotective (124, 126–129).

Expression of FtH protects cells from undergoing TNF-mediated apoptosis (126–129) by preventing the pro-oxidant effect of labile Fe from sustaining the activation of the c-jun-N-terminal kinase (JNK) signal transduction pathway (130). Briefly, the pro-oxidant effect of labile Fe inhibits the action of ROS-sensitive phosphatases that control JNK activation (131; reviewed in 132). This promotes the sustained activation of JNK in response to TNF, which targets the caspase-8 (FLICE; FADD-like IL-1β-converting enzyme) inhibitor protein [c-FLIP (FLICE-like-inhibitor-protein)] for ubiquitination and proteolytic degradation (133), leading to caspase-8 activation and apoptosis (reviewed in 132). When FtH neutralizes the pro-oxidant effect of labile Fe, the JNK signal transduction pathway is arrested, which explains the cytoprotective effect of FtH.

Reduction of cellular labile Fe content associated with the expression of HO-1 also regulates the NF-κB signal transduction pathway in a manner that supports the cytoprotective effects of HO-1 (91). As mentioned above, HO-1 inhibits the phosphorylation of p65/RelA at Ser276, an effect mediated via its ability to reduce cellular labile Fe content (92). This effect, which inhibits the expression of proinflammatory genes regulated by NF-κB, does not impair the expression of antiapoptotic genes such as A1 and c-IAP2, which support the cytoprotective effects of CO (91). That the cytoprotective effect associated with the reduction of cellular labile Fe contributes to the salutary effects of HO-1 is strongly suggested by the observation that FtH can mimic the protective effects of HO-1 against immune-mediated inflammatory diseases, as demonstrated for IRI (129).

Biliverdin

Biliverdin can be converted by BVR (9) into bilirubin, a potent antioxidant (8). Bilirubin has been suggested to form with biliverdin a positive forward amplification loop, whereby bilirubin can be oxidized into biliverdin and then recycled back into bilirubin by BVR (134). This mechanism, involving the oxidation of bilirubin into biliverdin, has been recently questioned (135) and therefore its physiologic relevance remains to be established.

Biliverdin affords cytoprotection against necrosis (134), a form of programmed cell death induced by unfettered oxidative stress and characterized by irreversible swelling of cellular organelles, intracellular release of lysosomal enzymes, mitochondrial calcium overload, activation of cytosolic calpains and phospholipases, and disruption of plasma membrane integrity and release

of intracellular content (73, 136). Contrary to apoptosis, necrosis is driven by a series of signal transduction pathways that do not require the activation of caspases (reviewed in 136). These signal tranduction pathways can be triggered by a variety of cytotoxic agonists associated with the production of high levels of free radicals (reviewed in 136, 137). In addition, engagement of death receptors such as Fas (137) or TNFR-1 can induce a form of programmed cell death related to necrosis but that shares some biochemical features of apoptosis, that is, necroptosis (138, 139). This composite form of programmed cell death occurs via the production of high levels of free radicals through the activation of receptor interacting protein 1 (RIP1) (140) and nicotinamide adenine dinucleotide phosphate (NADPH), a free-radical-producing enzyme (141). As described above, production of free radicals in response to TNF sustains JNK activation, ultimately leading to programmed cell death (131, 141). The biliverdin-bilirubin system can afford cytoprotection against necrosis induced by NO (143), H_2O_2 (134, 144), or hypoxia (145), but whether this cytoprotective effect can be extended to Fas- or TNF-driven necroptosis remains to be established. Given its potent antioxidant activity, bilirubin should contribute in a predominant manner to the cytoprotective effect of the biliverdin-bilirubin system. However, one should also consider that BVR might also act in a cytoprotective manner, independently of its ability to convert biliverdin into bilirubin (reviewed in 10).

The cytoprotective effects of biliverdin and bilirubin are likely to contribute to the salutary effects of HO-1 against a variety of immune-mediated inflammatory diseases. This is strongly suggested by the observation that when applied exogenously biliverdin and bilirubin afford protection against IRI (146), rejection of transplanted organs (147, 148; reviewed in 149), and severe sepsis (150, 151), as well as vascular lesions associated with the development of intimal hyperplasia (152).

CYTOTOXIC EFFECT OF FREE HEME

The cytotoxic effect of TNF (reviewed in 153) is repressed via the induction of several immediate-early responsive cytoprotective genes, regulated at the transcriptional level by the transcription factor NF-κB (89). To the best of our knowledge, no molecules produced under pathophysiologic conditions can override this cytoprotective effect. We found that free heme acts in such a manner (**Figure 4*a***), sensitizing a variety of nonhematopoietic cell types including hepatocytes, oligodendrocytes, and pancreatic β-cells to undergo TNF-mediated programmed cell death (**Figure 4*b***) (154). This cytotoxic effect is also observed when cells are exposed to anti-Fas antibodies, H_2O_2, or peroxynitrite (R. Gozzelino, unpublished observation).

When exposed to free heme and immediately thereafter to TNF, hepatocytes, oligodendrocytes, and pancreatic β-cells undergo programmed cell death by apoptosis (**Figure 4**) (154). This is revealed by the appearance of membrane blebbing, caspase activation, nuclear shrinking, and fragmentation, as well as formation of apoptotic bodies and chromatin condensation, all of which are hallmarks of apoptosis (74). We cannot exclude, however, that free heme might sensitize these cells to undergo necroptosis in response to TNF because (*a*) pharmacologic inhibition of caspases does not fully suppress this type of programmed cell death (154), (*b*) antioxidants suppress this type of programmed cell death (154), (*c*) heme plus TNF induce the release of high-mobility-group box 1 (R. Gozzelino and R. Larsen, unpublished observation), a marker of necrosis (156), and (*d*) free heme can induce necroptosis in microglial cells (157).

The cytotoxic effect of free heme does not involve modulation of TNF-responsive genes because (*a*) inhibition of mRNA synthesis by actinomycin D, (*b*) inhibition of protein synthesis by cycloheximide, and (*c*) inhibition of NF-κB activation using an IκBα dominant negative (R. Gozzelino, unpublished observation) do not modulate heme plus TNF-mediated

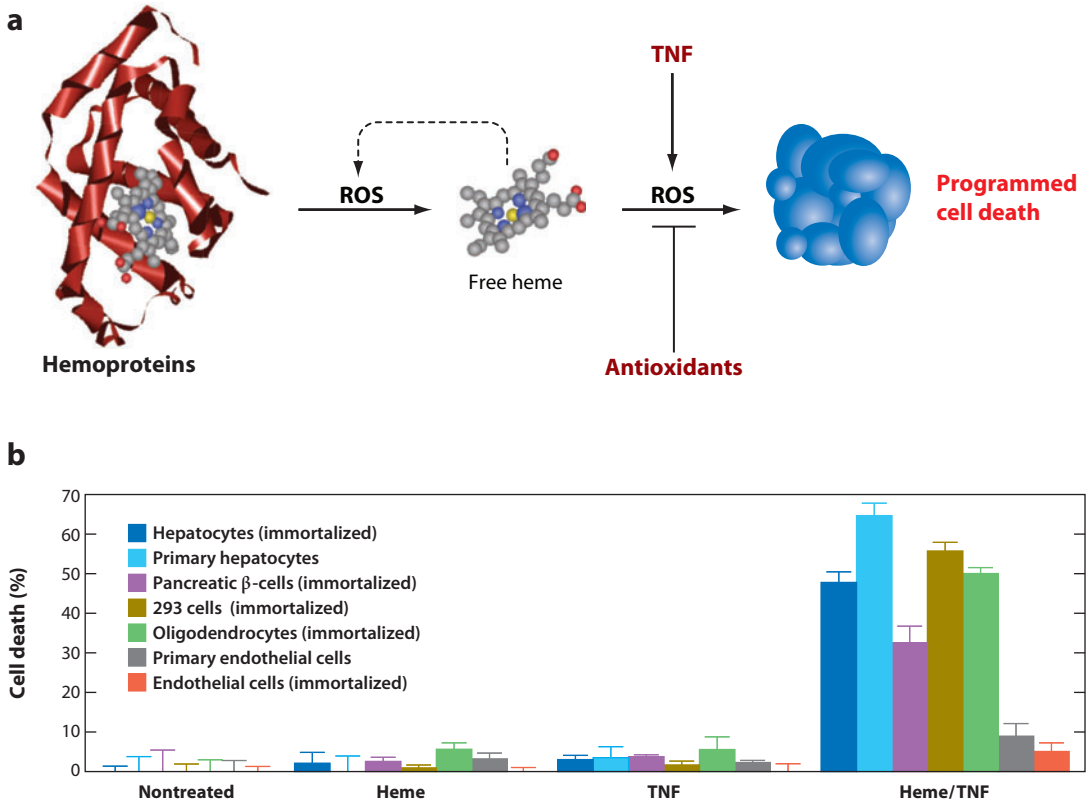

Figure 4

Free heme sensitization to TNF-mediated programmed cell death (154). (*a*) When exposed to ROS, some hemoproteins can release their prosthetic heme groups. These act as pro-oxidant catalysts to promote further ROS production. When exposed to free heme and to the proinflammatory cytokine TNF, a variety of cell types undergoes programmed cell death via a mechanism that is dependent on the production of ROS. This cytotoxic effect can be suppressed by antioxidants (154). (*b*) Cells were exposed to heme in Hanks balanced salt solution, washed in phosphate buffered saline (PBS) and exposed to TNF in complete medium. Mouse hepatocytes [Hepa 1-6; American Type Culture Collection (ATCC)] were exposed to heme (40 μM; 1 h) plus TNF (50 ng/ml; 6 h). Primary mouse (C57BL/6) hepatocytes were exposed to heme (5 μM; 1 h) plus TNF (5 ng/ml; 16 h). β-pancreatic cells (TC3, ATCC) were exposed to heme (40 μM; 1 h) plus TNF (50 ng/ml; 24 h). Human embryonic kidney 293 cells (ATCC) were exposed to heme (40 μM; 1 h) plus TNF (50 ng/ml; 24 h). Mouse oligodendrocytes (Oli-Neu, kind gift from J. Trotter, University of Mainz; Germany) were exposed to heme (5 μM; 1 h) plus TNF (50 ng/ml; 6 h). Primary bovine aortic endothelial cells were exposed to heme (40 μM; 1 h) plus TNF (50 ng/ml; 6 h). Mouse endothelial cells (EC240) were exposed to heme (40 μM; 1 h) plus TNF (50 ng/ml; 24 h). Cytotoxicity was assessed by crystal violet, vital staining. Mean cytotoxicity ± standard deviation ($n = 6$) in one out of three independent experiments.

programmed cell death (154). Instead, free heme acts in a cytotoxic manner through the production of free radicals in response to TNF, a pro-oxidant effect catalyzed by its Fe atom (154). This causes lipid peroxidation and sustained JNK activation in response to TNF (R. Gozzelino, unpublished observation), inducing caspase-8 and -3 activation and leading to programmed cell death (131; reviewed in 136) (R. Gozzelino, unpublished observation). The cytotoxic effect of free heme is inhibited by water-soluble (N-acetylcysteine) or lipid-soluble (butylated hydroxyanisole) antioxidants (154) (**Figure 4*a***). Considering that free heme and TNF can be produced simultaneously during the pathogenesis of several immune-mediated inflammatory diseases, the ability of free heme to sensitize a variety of cell types to undergo programmed cell death in response to TNF could contribute in a critical manner to the pathogenesis of these diseases.

PATHOLOGIC EFFECTS OF FREE HEME

Hb: hemoglobin
Mb: myoglobin

Whereas most immune-mediated inflammatory diseases are associated with the production of proinflammatory cytokines such as TNF (reviewed in 153, 158, 159), whether the same is true for free heme remains to be established. Free heme is probably produced mainly through the oxidation of hemoproteins (**Table 1**). The two largest pools of hemoproteins in our body are hemoglobin (Hb) and myoglobin (Mb), expressed in red blood cells and muscle cells, respectively. Under homeostasis, heme release from these hemoproteins is controlled through a variety of processes that avoid the production of cytotoxic free heme. However, red blood cell lysis (that is, hemolysis) and/or muscle cell lysis (that is, rhabdomyolysis) can lead to Hb and/or Mb release into the extracellular space, respectively. Subsequent oxidation of these hemoproteins can lead to release of their prosthetic heme groups, as demonstrated for cell-free Hb (21; reviewed in 22). We have recently shown how heme release from oxidized cell-free Hb can contribute to the pathogenesis of immune-mediated inflammatory diseases, namely malaria (21, 22, 154).

Hb is a tetrameric ($\alpha_2\beta_2$) hemoprotein that accounts for 97% of the total dry content of red blood cells (reviewed in 160). When confined inside red blood cells, $\alpha_2\beta_2$ Hb tetramers are maintained in a reduced (Fe^{2+}) state (reviewed in 160). However, if released from red blood cells, Hb tetramers dissociate into $\alpha\beta$ dimers, which react with free radicals such as NO ($10^7\ M^{-1}s^{-1}$). This reaction can have two pathological consequences: (*a*) reduction of NO bioavailability (161) and (*b*) Hb oxidation into ferric (Fe^{3+}) Hb, that is, methemoglobin (162, 163), with subsequent release of heme. This chain of events is likely to contribute to the pathogenesis of several pathological conditions associated with hemolysis (**Table 2**). Probably the best example of one such pathological condition is malaria, caused by *Plasmodium* infection.

When infected by *Plasmodium* (i.e., *berghei* ANKA), C57BL/6 mice develop a lethal neurovascular inflammatory syndrome that resembles in many aspects cerebral malaria, a major cause of

Table 2 Pathologies associated with intravascular hemolysis

Cause of hemolysis	Disorder	Cell-free Hb	Free heme	Reference
Trauma	Burns	Yes	?	(219)
	Hemorrhage	Yes	?	(220)
Red blood cell disorders	Paroxysmal nocturnal hemoglobinuria	Yes	?	(221)
	Hereditary spherocytosis	Yes	Yes	(221)
	Sickle cell disease	Yes	Yes	(222)
	Thalassemias	Yes	Yes	(222)
	Pyruvate kinase deficiency	Yes	?	(223)
Hemodynamic stress	Microangiopathy	Yes	?	(224)
	Aortic stenosis	Yes	?	(225)
	Prosthetic heart valves	Yes	?	(226)
	Disseminated intravascular coagulation	Yes	?	(227)
	Extracorporal circulation during surgery	Yes	?	(228)
Antibody-mediated	Acute hemolytic transfusion reaction	Yes	?	(229)
	Paroxysmal cold hemoglobinuria	Yes	?	(230)
Infection-mediated	Malaria	Yes	Yes	(21)
	Dengue hemorrhagic fever	Yes	?	(231)
	Sepsis	Yes	Yes	(227)
Chemical-mediated	Lead poisoning	Yes	?	(232)
	Potassium dichromate poisoning	Yes	?	(233)
	Arsenic exposure	Yes	?	(234)

death associated with malaria in humans (164, 165). On the other hand, when infected with the same *Plasmodium* strain, BALB/c mice do not develop experimental cerebral malaria. We found that the onset of experimental cerebral malaria in C57BL/6 mice is associated with higher concentration of free heme in the plasma, compared with BALB/c mice (21, 22). The contribution of free heme to the pathogenesis of this disease is demonstrated by the observation that when administered to BALB/c mice after *Plasmodium* infection, free heme triggers the onset of experimental cerebral malaria in this otherwise resistant mouse strain (21, 22). The recent finding that free heme also accumulates in the plasma of *Plasmodium falciparum*-infected children developing cerebral malaria (M. do Rosario Sambo et al., unpublished observation) suggests that free heme might have a similar pathological effect in human malaria.

Plasmodium infection can also lead to the development of noncerebral forms of severe malaria that contribute significantly to the overall mortality associated with this infectious disease in humans (164, 165). There is a general consensus that TNF plays a critical role in the pathogenesis of both cerebral (166, 167) and noncerebral (168) forms of severe malaria. We found that free heme also contributes to the pathogenesis of noncerebral forms of severe malaria (154). When infected by *Plasmodium* (i.e., *chabaudi chabaudi*), DBA/2 mice display high concentrations of TNF as well as free heme in plasma (154), developing a lethal form of liver failure associated with widespread hepatocyte programmed cell death (154). Neutralization of circulating TNF or free heme using either anti-TNF antibodies or overexpression of HO-1 in the liver, respectively, protects hepatocytes from undergoing programmed cell death and suppresses the onset of liver failure (154, 155). These observations suggest that heme sensitization to TNF-mediated programmed cell death plays a critical role in the pathogenesis of this immune-mediated inflammatory disease. Whether the pathologic effect of free heme can be extended to other diseases remains to tested.

Heme release from oxidized hemoproteins (**Table 1**) might be involved in the pathogenesis not only of hemolytic diseases such as malaria (**Table 2**) but also of several immune-mediated inflammatory diseases. Severe sepsis caused by polymicrobial infection in mice is associated with the accumulation of high levels of free heme in plasma, which sensitizes cells in different tissues to undergo programmed cell death in response to proinflammatory agonists involved in the pathogenesis of this disease, that is, TNF as well as Fas, H_2O_2, or peroxynitrite (R. Larsen et al., unpublished observation). This cytotoxic effect of free heme should contribute to the development of multiorgan failure, a hallmark of severe sepsis. Based on these observations, it is tempting to speculate that if free heme contributed to the pathogenesis of other immune-mediated inflammatory diseases, this would explain the unusual salutary effects of HO-1 against these diseases.

CYTOPROTECTION AGAINST FREE HEME

We argue here that the cytoprotective effect of HO-1 is mediated, at least in part, via the neutralization of free heme, not only through its physical degradation but also by the action of some of the end products produced through its catabolism. In addition, we posit that other putative mechanisms might afford cytoprotection based on their ability to neutralize free heme.

Heme Catabolism by HO-1 Suppresses the Cytotoxic Effects of Free Heme

HO-1 can prevent the deleterious effects of free heme by several mechanisms. These include inhibiting (*a*) the release of free heme from hemoproteins, (*b*) the accumulation of free heme in cells, and/or (*c*) the pro-oxidant effects of free heme.

HO-1 can prevent heme release from hemoproteins (21, 22). Once bound to the heme groups of hemoproteins, CO inhibits Fe^{2+}-heme oxidation, thus limiting the oxidation of hemoproteins

and preventing heme release (reviewed in 22). We have demonstrated how this mechanism inhibits the accumulation of free heme in plasma following *Plasmodium* infection, thus preventing the onset of severe malaria in mice (21, 22). As for cell-free Hb, CO might target other hemoproteins and prevent the release of their heme groups. This could be particularly relevant in cells expressing high levels of hemoproteins, such as muscle cells that express Mb. Presumably, binding of CO to the heme groups of Mb should inhibit its oxidation and heme release. This effect might prevent muscle cells such as myocardial cells from accumulating free heme in response to oxidative stress and thus from undergoing programmed cell death in response to cytotoxic agonists such as TNF, Fas, H_2O_2, or peroxynitrite. Although speculative at this point, this hypothesis is consistent with the observation that CO can protect myocardial cells from undergoing programmed cell death in response to IRI (113).

Heme catabolism by HO-1 should also prevent the accumulation of free heme within cells. This cytoprotective mechanism must, however, be coupled to the induction of FtH expression to avoid the pro-oxidant effects of labile Fe produced via heme catabolism. This notion is consistent with the observation that FtH can mimic the cytoprotective effects of HO-1 (127–129).

Some of the end products of heme catabolism by HO-1 might also prevent the pro-oxidant effects of free heme. This is probably the case for biliverdin, which has antioxidant properties by itself but in addition can be converted by BVR into the potent lipid-soluble antioxidant bilirubin (8, 142). Owing to its lipophilic nature, free heme might act as a pro-oxidant primarily within cellular membranes. This deleterious effect might be inhibited by lipophilic bilirubin, which would explain the ability of HO-1 to inhibit (via the production of bilirubin) lipid peroxidation in cells exposed to free heme and TNF (154).

Regulation of Intracellular Free Heme Content Modulates its Cytotoxic Effects

Intracellular heme content must be regulated in a manner that presents heme for incorporation into newly synthesized apoproteins while avoiding its accumulation. This is ensured by several mechanisms controlling the rate of heme (*a*) synthesis, (*b*) incorporation into hemoproteins, (*c*) cellular import, (*d*) cellular export, and (*e*) binding to intracellular heme scavengers.

Heme synthesis is regulated by eight evolutionarily conserved genes encoding four mitochondrial and four cytoplasmic enzymes (reviewed in 169). The initial and rate-limiting step in heme synthesis is the production of δ-aminolevulinic acid (ALA) in the mitochondria (reviewed in 169). This reaction is catalyzed by two ALA synthase (ALAS) isoforms, namely, the ubiquitously expressed ALAS1 (molecular weight: 70.6 kDa) and the erythroid-specific ALAS2 (molecular weight: 64.6 kDa; EC: 2.3.1.37) (reviewed in 169). Heme synthesis is controlled in most cell types by a negative feedback loop in which heme inhibits ALAS expression by inducing *ALAS1* mRNA degradation (170) and/or by inhibiting ALAS mitochondrial import (171).

Accumulation of free heme can also be prevented by matching the rate of heme and hemoprotein synthesis, so that all the newly synthesized heme is readily incorporated into apoproteins. Free heme induces, at the transcriptional level, the expression of Hb in erythroid cells (172) as well as Mb in skeletal muscle cells (173) and neuroglobin, a hemoprotein expressed in neuronal cells (174). Heme can also induce at a post-transcriptional level the expression of some of these hemoproteins, as demonstrated for the regulation of Hb mRNA translation by the heme-regulated eukaryotic initiation factor 2 (eIF-2α) (175). Heme modulates eIF-2α phosphorylation in a manner that induces its activity and promotes *Hb* mRNA translation. Whether a similar mechanism regulates *Mb* or *Neuroglobin* mRNA translation has to the best of our knowledge not been reported.

The recent finding that intracellular heme content can be regulated by several heme transporters challenges the view that hydrophobic free heme can access cellular compartments by

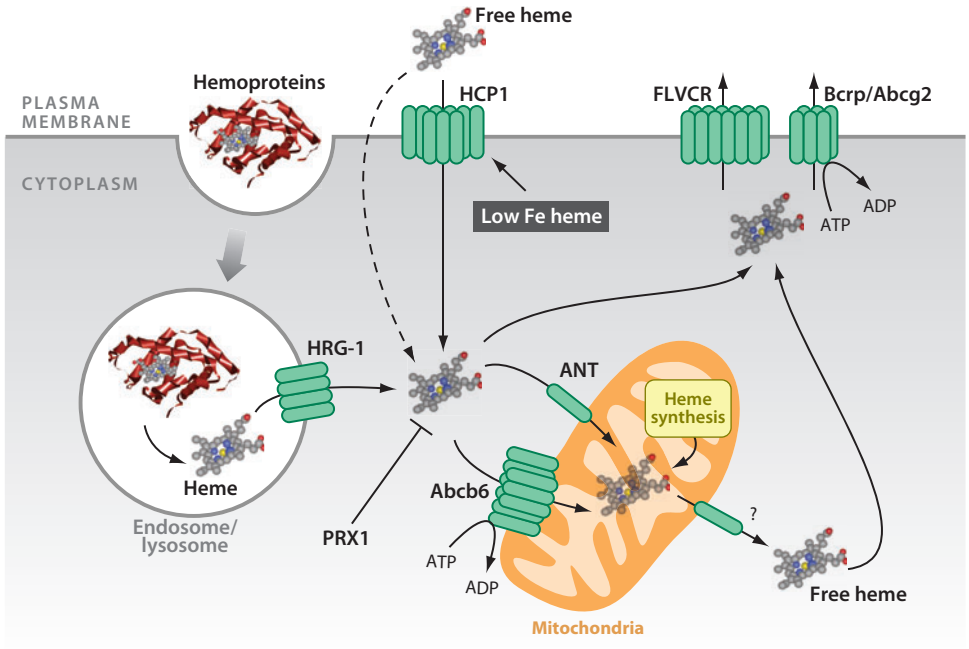

Figure 5

Cellular free heme regulation. Free heme can gain intracellular access via different mechanisms, including through endocytosis of hemoproteins, a process linked to intracellular heme import by the heme-responsive gene-1 (HRG1). The plasma membrane heme transporter heme carrier protein-1 (HCP1) can also import extracellular free heme. Cells are also able to secrete intracellular free heme via the feline leukemic virus receptor (FLVCR) and the Bcrp/Abcg2 plasma membrane transporters. Transit of intracellular free heme between the mitochondria and the cytosol is ensured by the mitochondrial heme transporters Abcb6 and adenine nucleotide translocator (ANT). Cytosolic PRX1 acts as a heme scavenger that neutralizes its pro-oxidant activity.

passive diffusion across cellular membranes (**Figure 5**). Among these cellular heme transporters are the mitochondrial adenosine triphosphate-binding cassette family member Abcb6 (molecular weight: ~94 kDa; human; Q9NP58) (176) and the adenine nucleotide translocator (molecular weight: ~33 kDa; human; P05141) (177) (**Figure 5**). Abcb6 and adenine nucleotide translocator can regulate heme synthesis by controlling the access of heme precursors to the mitochondria (177, 178). Whether these heme transporters can control the cytotoxic effects of free heme has not been established.

Access of extracellular free heme into cells is controlled by several heme transporters. These include the heme-responsive gene-1, which encodes a transmembrane protein (molecular weight: ~26 kDa; human), expressed mainly in endosomes and lysosomes, where it facilitates heme transport into the cytoplasm (179) (**Figure 5**). The heme carrier protein-1 (180, 181) is a cytoplasmic heme transporter (molecular weight: ~125 kDa; human; Q86VB7) that can translocate into the plasma membrane in response to heme-Fe depletion, promoting the import of extracellular free heme (181, 182) (**Figure 5**). Whether heme-responsive gene-1 or heme carrier protein-1 can modulate the cytotoxic effects of extracellular free heme remains to be established.

Cells can export free heme via at least two heme transporters, namely the adenosine triphosphate-binding cassette Abcg2/Bcrp (molecular weight: ~72 kDa; human; Q9UNQ0)

(183, 184) and the feline leukemic virus receptor (185–187) (molecular weight: ~60 kDa; human; Q9Y5Y0) (**Figure 5**). Expression of Abcg2/Bcrp is induced under hypoxia (178, 184) and affords cytoprotection against hypoxia as well as free heme (184; reviewed in 178). Feline leukemic virus receptor also controls intracellular free heme content (187) and affords cytoprotection against free heme (187, 188).

Hp: haptoglobin

LDL: low-density lipoproteins

HDL: high-density lipoproteins

Hpx: hemopexin

Another protective mechanism against intracellular free heme relies on the expression of the high-affinity ($K_d = 10^{-8}$ M) heme-binding protein peroxiredoxin I (Prx1) (i.e., HBP23; ~22 kDa; human; Q06830) (189, 190). Prx1 is a member of a larger family of peroxiredoxins (191, 192) that acts as a cytoplasmic heme scavenger that neutralizes the pro-oxidant activity of free heme (**Figure 5**). Expression of Prx1 is induced by heme (190, 193), suggesting that Prx1 is part of a protective stress-response that prevents the cytotoxic effects of intracellular free heme. PRX1 is cytoprotective against oxidative stress (194), presumably by neutralizing the cytotoxic effects of free heme produced in response to oxidative stress.

Systemic Regulation of Extracellular Free Heme Cytotoxicity

Cell-free Hb is probably one of the main sources of extracellular free heme (**Figure 6**). Under homeostasis, cell-free Hb is recognized ($K_M \sim 10^{-12}$ M) by haptoglobin (Hp; human; P00738) (**Figure 6**), which prevents its accumulation in plasma (~0.16–0.62 mmol/L). In addition, Hp inhibits the pro-oxidant effects of cell-free Hb (195) as well as heme release from cell-free Hb. Recognition of Hb by Hp forms Hb-Hp complexes recognized ($K_M \sim 2.10^{-11}$–10^{-12} M) by the Hp receptor (CD163; Q86VB7) (196), which promotes endocytosis of Hb-Hp complexes by hemophagocytic Mø and subsequently heme catabolism by HO-1 (197). There is a *Hp* polymorphism in humans (i.e., *Hp2-2*) associated with decreased Hp affinity toward cell-free Hb. This *Hp* polymorphism acts as an independent risk factor for several immune-mediated inflammatory diseases (reviewed in 198, 199), suggesting that neutralization of cell-free Hb by Hp exerts protective effects against these diseases. Presumably, this protective effect relies on the ability of Hp to prevent the accumulation of cell-free Hb in plasma as well as to inhibit heme release from cell-free Hb, thus avoiding the deleterious effects of free heme.

The protective effect of Hp can be overwhelmed under several pathologic conditions associated with extensive hemolysis (**Table 2**) (reviewed in 200). When this occurs, cell-free Hb accumulates in plasma and is oxidized, releasing its heme groups (21). The extracellular free heme is transferred into several plasma proteins (201), including hemopexin (Hpx) (202), albumin (203), α_1-microglobulin (204), and lipoproteins (205) such as low-density lipoproteins (LDLs) and high-density lipoproteins (HDLs) (**Figure 6**). Among these, Hpx appears to play a central role in preventing the deleterious effects of extracellular free heme.

Hpx is an acute-phase protein (molecular weight: ~63 kDa; human; P02790) that binds free heme in plasma, with the highest affinity ($K_d < 10^{-12}$ M in humans) of any protein described so far (202) (**Figure 6**). The resulting heme-Hpx complexes are removed via the Hpx receptor (CD91; molecular weight: ~85 kDa; human; Q07954), which promotes endocytosis of heme-Hpx complexes in Mø (206). Unlike free heme, heme-HpX complexes are not deleterious (207), delivering free heme to the HO-1 system (208). Hpx-deficient ($Hpx^{-/-}$) mice as well as Hp/Hpx-deficient ($Hp^{-/-}/HpX^{-/-}$) mice develop severe renal damage (209) in response to hemolysis, suggesting that Hpx plays a central role in preventing the deleterious effects of extracellular free heme produced by hemolysis.

Albumin (molecular weight: ~66 kDa; human; P02768) can bind free heme in plasma with an affinity that is 10^4 times lower to that of Hpx ($K_d = 10^{-8}$ M in humans) (210) (**Figure 6**). However, given that albumin is the most abundant plasma protein (30–50 g/L), its low affinity

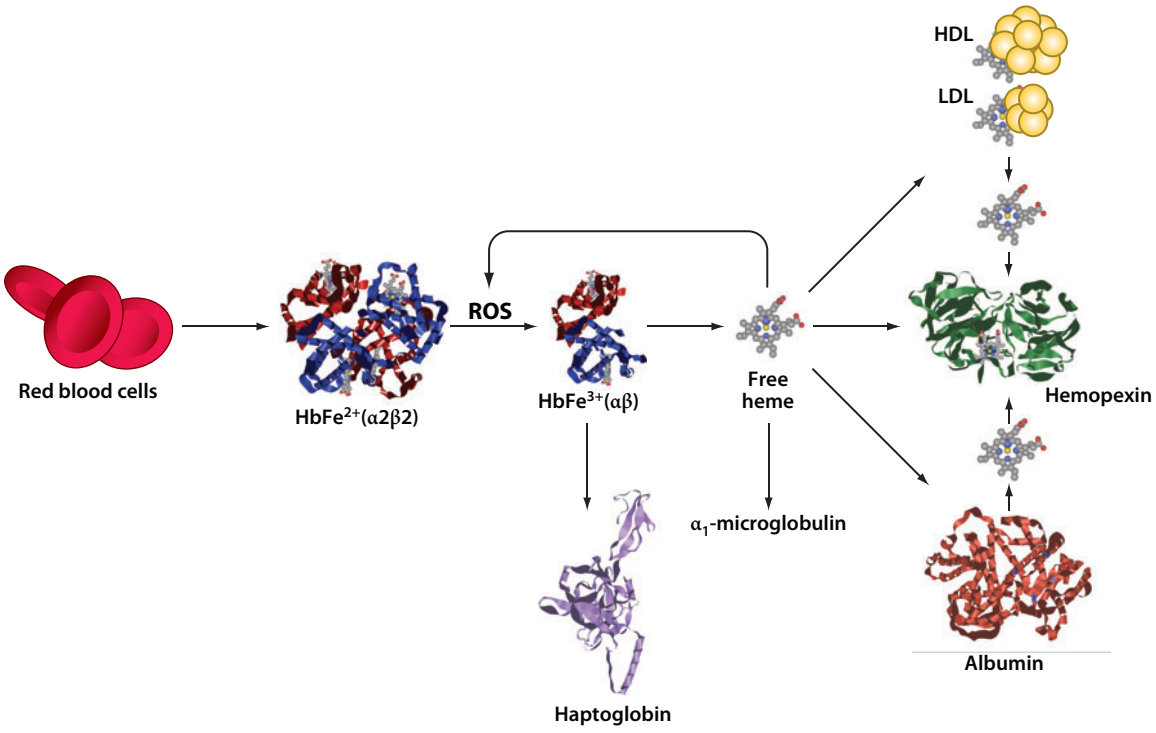

Figure 6

Systemic regulation of free heme. Under homeostasis, cell-free Hb released from red blood cells is scavenged by haptoglobin. Under pathologic conditions associated with hemolysis, the protective effect of Hp can be overwhelmed, and cell-free Hb accumulates in plasma and becomes oxidized, releasing its prosthetic heme groups. Extracellular free heme can be scavenged by several plasma proteins including hemopexin (Hpx) ($K_d \sim 10^{-12}$ M), albumin ($K_d \sim 10^{-8}$ M), α_1-microglobulin ($K_d \sim 10^{-6}$ M), and lipoproteins ($K_d \sim 10^{-11}$–10^{-12} M) such as low-density lipoproteins (LDL) or high-density lipoproteins (HDL). Hemopexin can remove heme from lipoproteins as well as from albumin, thus acting as the central scavenging system for free heme in plasma.

for free heme might be compensated by its high plasma concentration, in particular when Hpx has been depleted by severe hemolysis. Whether heme-albumin complexes can be recognized by specific cellular receptors, allowing for heme degradation by the HO-1 system, has not been established. However, because albumin inhibits the pro-oxidant activity of free heme, it is possible that its binding to free heme might provide some level of protection against extracellular free heme. This might help explain the salutary effects of albumin infusion into individuals developing cerebral malaria (211, 212), the pathogenesis of which is driven by free heme (21, 22).

Other proteins can bind free heme in plasma, including α_1-microglobulin (molecular weight: ~26 kDa; human; P02760), a member of the lipocalin protein superfamily with which it shares the ability to carry small lipophilic ligands in hydrophobic pockets (213) (**Figure 6**). Hemolysis cleaves α_1-microglobulin, producing a truncated protein that binds (Kd ~10^{-6} M) and degrades free heme, forming a yellow-brown protein-bound chromophore (204) that can be cleared by glomerular filtration. The mechanism via which α_1-microglobulin degrades heme is not well understood. Binding of α_1-microglobulin to free heme negates its pro-oxidant effects, suggesting that α_1-microglobulin might function as an extracellular heme scavenger (204).

Plasma LDL and HDL can bind free heme with high affinity (Kd $\sim 10^{-11}$–10^{-12} M) and with kinetics that are faster than those of Hpx or albumin (205), presumably acting as a major sink for

plasma free heme (205) (**Figure 6**). When bound to LDL or HDL, heme can be subsequently removed by Hpx (214) and presumably by albumin as well (**Figure 6**) (205). Heme binding to LDL or HDL can catalyze oxidative modification in these lipoproteins (215), precipitating their clearance by Mø (205, 216). However, based on the cytotoxic effects of oxidized LDL or HDL, in particular to endothelial cells (217), it is possible that heme-assisted LDL or HDL oxidation might promote the pathogenesis of some vascular diseases, such as lipid-mediated atherosclerosis, an effect that would explain the protective effect of HO-1 against these diseases (26; reviewed in 218).

CONCLUSION

Free heme might be a ubiquitous cytotoxic molecule involved in the pathogenesis of a broad spectrum of diseases. Various mechanisms afford protection against free heme. In a whole organism, however, these must be coupled to heme catabolism via the HO system. HO enzymes afford the only known mechanism that allows the efficient extraction of the reactive Fe atom from heme, providing the means for subsequent neutralization and eventually reutilization of that Fe atom. Presumably for this reason, expression of HO-1 appears to be essential, despite the existence of other mechanisms, to afford protection against free heme and thus exert salutary effects against the pathogenesis of a variety of immune-mediated inflammatory diseases.

DISCLOSURE STATEMENT

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