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Heme Scavenging and the Other Facets of Hemopexin

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Abstract

Hemopexin is an acute-phase plasma glycoprotein, produced mainly by the liver and released into plasma, where it binds heme with high affinity. Other sites of hemopexin synthesis are the nervous system, skeletal muscle, retina, and kidney. The only known receptor for the heme–hemopexin complex is the scavenger receptor, LDL receptor–related protein (LRP)1, which is expressed in most cell types, thus indicating multiple sites of heme–hemopexin complex recovery. The better-characterized function of hemopexin is heme scavenging at the systemic level, consisting of the transport of heme to the liver, where it is catabolyzed or used for the synthesis of hemoproteins or exported to bile canaliculi. This is important both in physiologic heme management for heme-iron recycling and in pathologic conditions associated with intravascular hemolysis to prevent the prooxidant and proinflammatory effects of heme. Other than scavenging heme, the heme–hemopexin complex has been shown to be able to activate signaling pathways, thus promoting cell survival, and to modulate gene expression. In this review, the importance of heme scavenging by hemopexin, as well as the other emerging functions of this protein, are discussed. *Antioxid. Redox Signal.* 12, 305–320.

Introduction

Hemopexin (Hx) is a 60-kDa plasma glycoprotein composed of a single 439 amino acid–long peptide chain. It is encoded by an 11-Kb gene located on human chromosome 11 (chromosome 7 in mice) and is expressed mainly in liver. Other sites of Hx synthesis are the nervous system, skeletal muscle, retina, and kidney. The concentration of Hx in plasma is 0.5–1 mg/ml. This can, however, increase during inflammatory events, because Hx is an acute-phase protein (118), and its production is known to be regulated by the cytokines interleukin (IL)-6, IL-11, IL-1β, leukaemia inhibitory factor, oncostatin M, and tumor necrosis factor (TNF)-α (50).

Hx is the protein with the highest binding affinity to heme (K_d less than p*M*), and, subsequent to heme binding, the heme–Hx complex is internalized in liver cells through receptor-mediated endocytosis. The only known receptor for the heme–Hx complex is the scavenger receptor, LDL receptor–related protein (LRP)1 (18, 46, 71) (reviewed by S. K. Moestrup in this Forum). LRP1 is expressed in several cell types, including hepatocytes, macrophages, neurons, and syncytiotrophoblasts, thus indicating multiple sites of heme–Hx catabolism.

For a detailed analysis of Hx structure, see the recent review of Baker *et al*. (7). In this review, we focus on the functional properties of Hx. The better-characterized function of Hx is heme scavenging at the systemic level. This aspect is discussed, highlighting the double face of heme, essential for life but also highly toxic (see later). Then we describe the role of Hx in the nervous system, which, because of its isolation by the blood–brain barrier, cannot use plasma Hx and synthesizes the protein *in situ*. Furthermore, the importance of Hx in the immune system is discussed, considering that iron is one of the regulators of the immune response (see later). Finally, other Hx functions, not strictly related to its heme-scavenging properties, are taken into consideration (see later).

Heme as a Double-Faced Molecule

Heme is a tetrapyrrole containing a central iron ion (130). It is synthesized in all cells because of a series of reactions taking place partly in the mitochondrion and partly in the cytoplasm. The most rapid rates of heme synthesis occur in the erythroid cells of the bone marrow and in hepatocytes. In human erythroid cells, $\sim$300 mg of heme, which accounts for 75% of total body heme, is synthesized daily to support hemoglobin production, whereas in hepatocytes, $\sim$50 mg of heme per day is produced and incorporated into cytochrome P450, catalases, reticuloendoplasmic cytochrome B5, and mitochondrial cytochromes (127).

The importance of heme lies in its ability to act in a multitude of biologic functions, both as a prosthetic group in hemoproteins and as a crucial factor in the regulation of the expression of numerous proteins.

As far as its role as a prosthetic group is concerned, heme can interact with many different apo-hemoproteins to give

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rise, for example, to hemoglobin, myoglobin, cytochromes, catalases, peroxidases, and other enzyme systems.

Regarding the regulation of gene expression, heme is able to control the activation of several genes by binding the transcriptional repressor Bach1 (52, 57). Bach1 forms heterodimers with proteins of the Maf-related oncoprotein family (MafK, MafF, MafG). The Bach1–Maf heterodimers bind to the Maf recognition element (MARE) in the regulatory region of their target genes (47–49, 57, 132). MAREs are found in the regulatory regions of genes involved in heme metabolism, such as oxidative stress–response genes, globin genes, heme oxygenase (HO)-1, and heme biosynthetic enzymes (51, 57, 112). Under normal conditions, when expressed with small Maf proteins, Bach1 is located in the nucleus and represses transcription (132). However, under conditions with increased heme levels, Bach1 is exported from the nucleus to the cytoplasm, thus allowing gene expression (132).

Finally, some authors suggested that heme controls the rate of its own transport across the plasma membrane. Heme might regulate proteins like the heme and the folate importer heme carrier protein (HCP)1, whose redistribution from the cytoplasm to the plasma membrane is already known to be regulated by iron (99), or the heme exporter feline leukemia virus subgroup C receptor (FLVCR), whose expression is finely regulated during the differentiation of erythroid precursors according to the heme requirement, thus indicating its capability to sense heme (93).

Besides the important biologic functions, heme also bears toxic properties, mainly deriving from its hydrophobic nature and from the iron atom contained in the porphyrin ring. The lipophilic nature of heme allows it to intercalate into biologic membranes, perturbing lipid bilayers, promoting the conversion of low-density lipoprotein to cytotoxic oxidized products, and favoring iron (Fe^{2+}) to participate in Fenton reaction, a process during which reactive oxygen species (ROS) are produced (10, 11, 16, 53, 60, 97).

As heme overload usually occurs in the bloodstream under pathologic conditions, circulating and endothelial cells are the first cell populations exposed to heme-mediated damage. Heme entraps into red blood cell membranes and shortens the erythrocyte life span, thus enhancing hemolysis (60). Moreover, heme activates endothelial cells by increasing the surface expression of specific adhesion molecules, as intercellular adhesion molecule (ICAM)1, vascular cell adhesion molecule (VCAM)1, and selectins (13, 14, 125, 126), required to promote recruitment of circulating activated leukocytes at the site of inflammation. By enhancing adhesion molecule expression and generating oxidative stress known to damage cells, heme also acts as a proinflammatory molecule and starts the inflammatory cascades (38, 126). Finally, free heme is considered to be a trigger of vasopermeabilization, which results from the partial retraction of endothelial cells of venules in the vicinity of inflammation, leaving a small intercellular gap. Vascular leakage results in slower blood flow by allowing the passage of water, salts, and small proteins from the plasma into the damaged area (78). By binding heme with high affinity, Hx participates both in heme recycling, thus preventing iron loss, and in heme detoxification.

Hemopexin in Heme Recycling

To fulfil the *ex novo* synthesis of heme described earlier, life forms have evolved efficient mechanisms to support a constant availability of iron to cells. Iron circulating in the human body corresponds to ~4–5 g and is derived mainly from continuous recycling instead of from new intake from external sources. In humans, only ~1–2 mg of iron is absorbed daily by the intestine, and, at the same time, an approximately equal amount is lost by epithelial shedding in the gastrointestinal tract and skin and through blood loss in menstruating women (41).

Among the wide range of events that account for heme recovery, erythrophagocytosis plays the major role (Fig. 1A). The term erythrophagocytosis indicates the process through which spleen macrophages, as well as bone marrow macrophages and liver Kupffer cells, normally eliminate 90% of senescent erythrocytes. Senescent red blood cells are recognized by macrophages because of changes in the erythrocyte shape and because of a series of biochemical modifications that accumulate at the red blood cell membrane during aging. After recognition, the erythrocytes undergo a process of phagocytosis and subsequent degradation of their constituents [for review, see Beaumont and Canonne-Hergaux, 2005

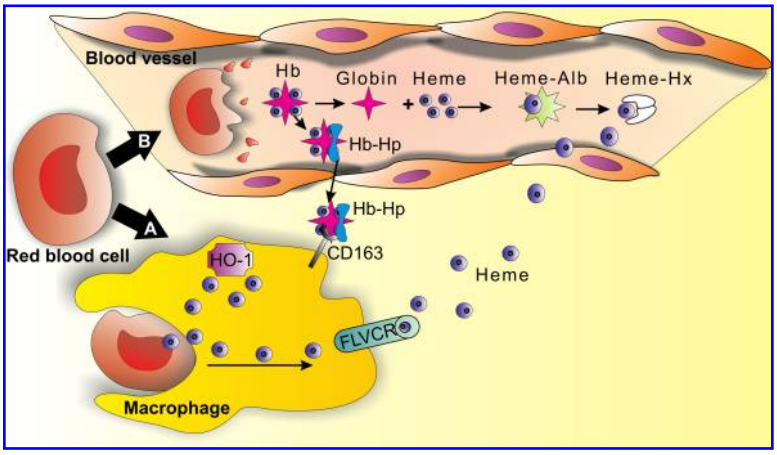

FIG. 1. Heme recycling. Most heme is recycled by macrophages through erythrophagocytosis of senescent erythrocytes (**A**). Heme released during this process either is degraded by HO-1 or is exported out of the cell through the heme exporter, FLVCR. Ten percent to 20% of normal erythrocyte destruction occurs intravascularly, resulting in the release of hemoglobin, which is bound by haptoglobin (**B**). The haptoglobin–hemoglobin complexes are subsequently delivered to hepatocytes and macrophages of the reticuloendothelial system and internalized through CD163 receptor–mediated endocytosis. When the buffering capacity of haptoglobin is exceeded, hemoglobin undergoes a rapid conversion to methemoglobin, liberating heme. Ferriheme then binds to albumin to form methemalbumin and is subsequently transferred to Hx. Hb, hemoglobin; Hp, haptoglobin; Alb, albumin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

(12)]. During these events, a large amount of heme is released in the macrophage, where it is catabolyzed by the enzyme heme oxygenase (HO) [see the review "Heme degradation and vascular injury" by Belcher *et al*., in this Forum (12a)], anchored to the endoplasmic reticulum membrane. Moreover, it was recently reported that not all heme derived from erythrophagocytosis is degraded by HO, but is in part exported to plasma (55).

Furthermore, circulating heme can be generated by different processes, such as enucleation of mature erythroblasts, intravascular oxidation of free haemoglobin, and dietary heme absorption. Besides these physiological events, many pathologic conditions can account for the liberation of heme in the organism, including trauma, hemoglobinopathies, hemorrhages, malaria, bacterial infections, and other diseases, all associated with intravascular hemolysis.

In intravascular hemolysis (Fig. 1B), erythrocytes release a high amount of hemoglobin, which first stimulates the formation of stable complexes with the acute-phase protein haptoglobin (5). The haptoglobin–hemoglobin complexes are subsequently delivered to hepatocytes (42, 56, 129) and macrophages of the reticuloendothelial system and internalized through CD163 receptor–mediated endocytosis (59). This function carried out by haptoglobin is fundamental, as demonstrated by studies on haptoglobin-null mice exposed to various hemolytic stimuli. Haptoglobin limits the loss of hemoglobin through renal filtration, thus preventing renal iron loading (36, 72, 76).

When the buffering capacity of haptoglobin is overwhelmed, hemoglobin undergoes a rapid conversion to methemoglobin, liberating heme. Ferriheme then binds to albumin ($K_d \sim 10$ n*M*) to form methemalbumin and is subsequently transferred to Hx (83, 84). Heme is initially associated with albumin because the molar concentration of albumin is considerably greater than that of Hx (albumin, 650 μM; Hx, 10–20 μM). However, methemalbumin is an abnormal component of plasma and has been found only in diseases associated with massive hemolysis, when Hx-binding capacity is exceeded.

A role for Hx in heme catabolism was originally proposed on the basis of two observations: (a) injection of hematin increased the catabolic rate of Hx and the plasma clearance of the protein (64, 65, 70, 131); and (b) in human hemolytic disorders, Hx concentrations decreased (32, 86), and its catabolism increased (131). Subsequently, several lines of evidence supported the conclusion that heme is delivered to the liver by Hx. *In vivo* studies showed that the liver is the major site of heme uptake after intravenous injection of ^{55}Fe-heme–^{125}I-Hx, as nearly 90% of the administered heme is transported to the liver within 2 h (K_d, 700 n*M*) without significant urinary excretion of either isotope (105, 108). Conversely, the heme–albumin complex appears to act only as a transient heme deposit before the transport of heme–Hx complexes to the liver. No experimental evidence exists about albumin-transport functions *in vivo* (107). Furthermore, after injection of doubly labeled hemoglobin-haptoglobin or methemalbumin, it was demonstrated that heme dissociates from albumin before its hepatic uptake and catabolism (17). Smith and Morgan (106) demonstrated that plasma membranes isolated from rabbit liver retained the ability to interact specifically with heme–Hx and to remove heme from the complex. The membranes bound heme-Hx with high affinity (K_d, 680 n*M*) and with an apparent capacity of 2.3 pmol/mg of membrane protein (106). Finally, specific binding of heme–Hx has been demonstrated in freshly isolated hepatocytes (K_d, 50 n*M*) (107) and in a murine hepatoma cell line, Hepa (K_d, 17 n*M*) (104, 107).

As mentioned earlier, Hx-mediated heme uptake by the liver has been shown *in vivo* and *in vitro* to be a saturable, tissue-specific process, dependent on time, temperature, and energy. Occurring within minutes, the association is on the same time scale as the receptor-mediated uptake of asialoglycoproteins (63) and of iron–transferrin complexes (37).

Conversely, some *in vitro* studies suggested that Hx delayed heme uptake by hepatocytes and failed to demonstrate that Hx is the main carrier involved in heme delivery to the liver. Taketani *et al*. (115) observed that, in the presence of Hx, only a small amount of heme associates with hepatocytes, unless the molar ratio of heme to Hx exceeded 1:1 (115). The authors concluded that Hx plays a limited role in heme uptake by cultured hepatocytes and hepatoma cells, and that heme exceeding the Hx-binding capacity is taken up directly from heme–albumin (102, 115). The discrepancies between the *in vivo* and some *in vitro* data can be explained by the fact that hepatocyte cultures fail to reproduce faithfully the complex architecture of the liver and the correct polarity and differentiation state of hepatic parenchymal cells. Furthermore, heme–Hx binding sites on hepatocytes may be lost during the preparation of primary cell cultures or may be downregulated in hepatoma cell lines. Thus, it is difficult to compare different experimental systems.

The heme–Hx complex is thought to be taken up by hepatic parenchymal cells by receptor-mediated endocytosis. The only known Hx receptor is LRP1, which mediates heme–Hx internalization, resulting in cellular heme uptake (46). Once inside the cell, the heme–Hx complex is dissociated by lysosomal activity. LRP1 is known to recycle to the plasma membrane, whereas data on Hx turnover are controversial. Some studies suggested that Hx can be recycled as an intact molecule to the extracellular milieu (105), whereas others proposed that following hepatic uptake of heme from heme–Hx, a certain amount of Hx returned to the circulation, and the rest is degraded in the liver (92). Recently, Hvidberg *et al*. (46) reported that most Hx is degraded in lysosomes. Accordingly, the plasma Hx level decreases in several disorders associated with heme overload in both humans and mice (32, 87, 121, 124).

Once delivered into the hepatocyte, heme is released into the cytoplasm and used to build new hemoproteins or is catabolyzed by HO. HO is the enzyme responsible for heme catabolism, as it breaks down the porphyrin ring to yield equimolar amounts of biliverdin, free iron (Fe^{2+}), and carbon monoxide (CO). In mammals, biliverdin is then rapidly converted into bilirubin by biliverdin reductase. HO proteins are anchored to the endoplasmic reticulum, and, to date, three isoforms of HO have been identified (HO-1, HO-2, and HO-3), encoded by three different genes, the expression, distribution, and regulation of which differ among cell types and tissues. Although the regulation of HO-3 is poorly characterized, and it seems to have low heme-degrading capacity, HO-1 levels have been demonstrated to be low under normal physiologic conditions but highly inducible by several stimuli, including heme and other oxidant agents, and HO-2 has been described as a constitutively expressed enzyme (1, 75, 81). The activity of

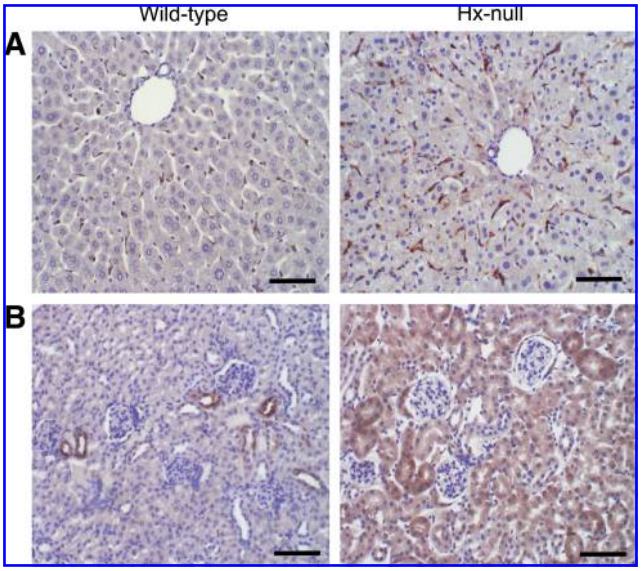

FIG. 2. Hemopexin prevents heme-induced HO-1 expression. Immunohistochemistry with an anti-HO-1 antibody, on liver (**A**) and kidney (**B**) sections from heme-overloaded wild-type and Hx-null mice. HO-1 induction was stronger in Kupffer cells and proximal tubules of Hx-null mice than in wild-type controls. Bar, 100 μm. For details, see ref. 63. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

HO *per se* could apparently seem a paradox as, other than its involvement in heme detoxification, it may represent a source of Fe^{2+}, a noxious agent. This problem is overcome because of the iron-sequestering protein, ferritin, which is generally regulated in response to iron-level changes and thus, indirectly, through to HO activity. Ferritin is composed of two different kinds of subunits: H-ferritin (endowed with a ferrioxidase activity and essential for iron incorporation in L-ferritin) and L-ferritin (4). An increase in Fe^{2+} is generally accompanied by an increase in H-ferritin, the role of which is to convert Fe^{2+} into Fe^{3+}, a less toxic element, and to promote its incorporation into L-ferritin. Conversely, L-ferritin in most cells is only partially saturated, and its upregulation does not occur every time iron enters the cell (4).

Several authors reported that the heme–Hx complex was able to induce HO-1 expression in hepatoma cells and other cell types (2, 3, 33, 45, 46). Davies *et al*. (30) showed that iron derived from heme transported to hepatocyte by Hx is rapidly stored in ferritin. Nevertheless, in our experience and in that of others (46), heme alone or bound to albumin is a stronger HO-1 inducer than the heme–Hx complex for most cell lines in culture. Accordingly, HO-1 induction after heme overload or intravascular hemolysis was stronger in Hx-null mice than in wild-type controls (120, 124) (Fig. 2). Based on these observations and considering that HO-1 is a stress-activated enzyme, not expressed under physiologic conditions, we think that normal Hx-mediated heme recycling occurs through HO-2 activity in hepatocytes (Fig. 3). HO-1 induction represents an attempt to counteract a nonphysiologic event when the standard system devoted to heme recycling is overwhelmed.

Hemopexin in Counteracting Heme Toxicity

As discussed in the previous section, the formation of heme–Hx or heme–albumin complexes allows heme to be transported in the body in a nontoxic form. After heme binding, Hx promotes heme detoxification in the liver through receptor-mediated endocytosis of the complex, followed by heme degradation or reutilization. Conversely, no receptor for the heme–albumin complex has been identified. Albumin-bound heme is thought to be transferred to Hx because of its

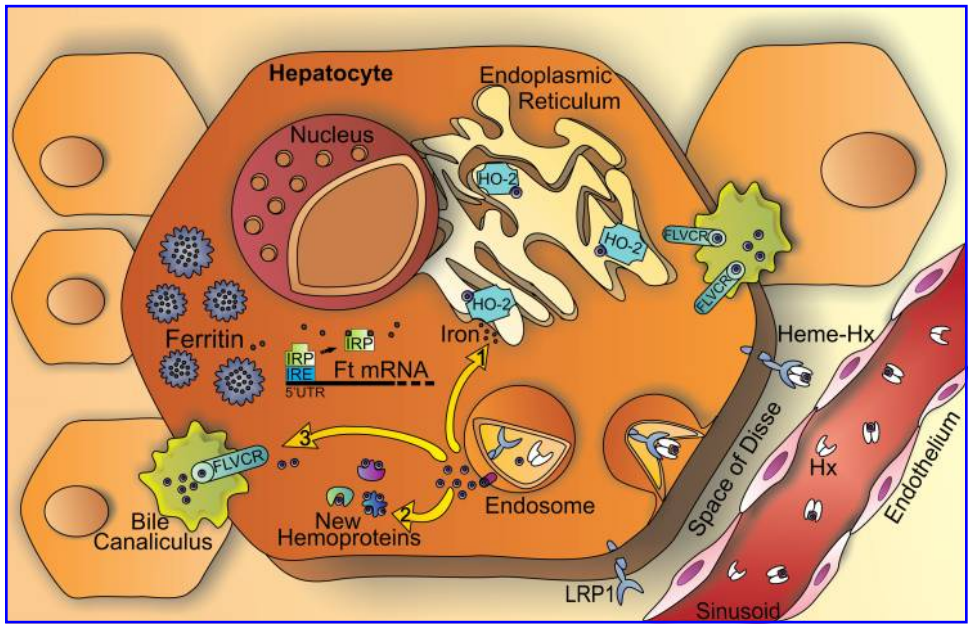

FIG. 3. Heme–hemopexin recovery by hepatocytes. The heme–Hx complex is taken up by hepatic parenchymal cells by LRP1 through receptor-mediated endocytosis. In the endosome, the heme–Hx complex is dissociated, and heme is released into the cytoplasm, where it (1) can be catabolyzed by HO-2 anchored to endoplasmic reticulum membranes, (2) can be used to build new hemoproteins, or (3) can be exported to bile canaliculi by FLVCR. Iron derived from heme catabolism is stored into ferritin, whose expression is controlled by iron itself through IRPs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

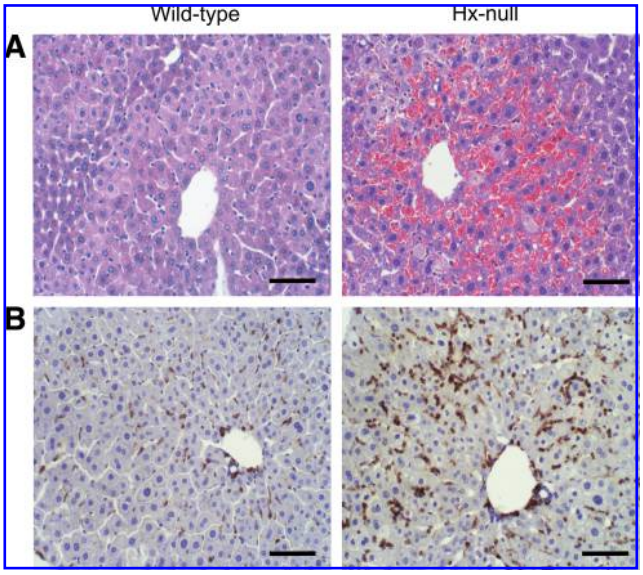

FIG. 4. Hemopexin prevents liver damage after heme overload. Liver sections of heme-overloaded wild-type and Hx-null mice, stained with hematoxylin and eosin (**A**) or, with immunohistochemistry (**B**), with an antibody to CD18 antigen. Note congestion around the centrolobular vein and massive leukocyte infiltrates in the Hx-null sample. Bar, 100 μm. For details, see ref. 63. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

higher affinity or, when Hx is saturated, to pass the lipophilic plasma membrane. Alternatively, heme could be taken up by cells through a heme importer (99).

Several studies demonstrated the potent antioxidant role of Hx. Heme binding to Hx has been demonstrated to reduce heme-mediated free radical production from organic peroxides (117). Furthermore, *in vitro* studies by Grinberg *et al*. (39) demonstrated that Hx strongly decreased the peroxidative and catalytic activity of heme by forming inactive heme–protein complexes. Interestingly, heme activities were found to be inhibited by 80–90% with Hx but only by 50–60% with either human or bovine albumin. Moreover, binding to Hx was shown to inhibit heme-catalyzed lipid peroxidation in artificial liposomes (40), rat liver microsomes (123), and plasma low-density lipoprotein (80). Finally, Eskew and co-authors (33) demonstrated that Hx has an essential role in the prevention of heme-induced oxidative damage and cell death in Hepa cells.

Much experimental evidence also supports the antioxidant function of Hx *in vivo*. Hx-null mice have been demonstrated to be particularly sensitive to heme overload and more prone to heme-induced oxidative damage and inflammation during hemolytic processes. Furthermore, *in vivo* studies showed that the most-damaged tissues with heme overload conditions are the vasculature, the liver, and the kidney (121, 124).

After heme overload, Hx-null mice have shown an increased induction of the adhesion molecules ICAM1 and VCAM1 in the endothelium and an increased vascular permeability compared with wild-type mice, thus demonstrating that Hx activity is required to prevent heme-induced vasopermeabilization and endothelial activation. Furthermore, heme-overloaded Hx-null mice showed a higher expression of HO-1 in the endothelium than did wild-type animals, and the induction of HO-1 before heme overload preserved endothelial integrity, thus indicating that Hx and HO-1 work in the same pathway to counteract the toxic effect of heme (124).

Besides the vasculature, other tissues have been described as particularly sensitive to heme-mediated damage when Hx is lacking. Hx-null mice recovered more slowly after phenylhydrazine-induced hemolysis and had severe renal damage compared with control mice. These animals had prolonged hemoglobinuria, higher iron loading, and lipid peroxidation in the kidney than did wild-type mice (121). Increased oxidative damage in the Hx-null kidney was also evident after heme injection (124).

Finally, when Hx is lacking, heme overload resulted in a marked liver congestion accompanied by red blood cell stasis and sinusoidal dilation around the centrolobular vein (Fig. 4). Hepatic congestion was found to be associated with abnormal iron deposits, increased lipid peroxidation, and massive leukocyte infiltrates (124). Liver damage was also evident in compound mutant mice, lacking both Hx and haptoglobin, after intravascular hemolysis (120).

Hemopexin-Independent Heme-Detoxifying Systems

We conclude the first part of this review by considering a novel emerging player in the field of heme handling, that is, heme export, which may have important implications for Hx. Until now, all free heme was thought to be captured and catabolyzed by cells (mainly hepatocytes and macrophages) because of its toxicity. Nevertheless, increasing evidences suggest that a certain amount of intact heme can be exchanged among cells or between different tissues and compartments in the organism without being catabolyzed.

This new approach to heme metabolism was revealed mainly by studies on FLVCR. FLVCR is a single-chain cell-surface receptor composed of 12 membrane-spanning domains and intracellular amino and carboxyl termini (114). It is a member of the major-facilitator superfamily of secondary transporters, capable of transporting small solutes in response to chemiosmotic ion gradients, and much evidence suggests that it functions as an organic anion-Na^+ (or H^+) symporter (89).

Recent *in vitro* and *in vivo* studies have highlighted its role as a heme exporter (93), and its expression has been detected in all hematopoietic tissues (including erythroid precursors, granulocytes, monocytes, peripheral blood lymphocytes and fetal liver) and in most nonhematopoietic ones. Moreover, FLVCR is expressed in a broad range of cell lines and primary cells (55, 93).

The most important function described for FLVCR is the export of heme in colony- forming units-erythroid cells (CFU-E) to protect them from heme toxicity in a stage in which hemoglobinization of erythroid cells would be premature (93). Lack of FLVCR at this stage of erythroid maturation results in cell death (93). Accordingly, FLVCR-null mice die *in utero* because of the failure of fetal erythropoiesis (55).

Nevertheless, the expression of FLVCR in numerous tissues, other than erythroid cells, suggests the existence of further functions for this carrier. Keel and co-workers (55) demonstrated a role for FLVCR in the export of heme from

macrophages, thus suggesting that not all heme in these cells is broken down by HO, but rather, a certain amount is exchanged with other cells (Fig. 1). Furthermore, they hypothesized that FLVCR is important in hepatocytes to guarantee the export of intact heme from liver into bile canaliculi, providing a way through which heme–iron exits the body (Fig. 3).

A further indication that not all heme entering the organism is immediately catabolyzed comes from studies on the duodenum. The presence of a heme importer, HCP1, at the apical membrane of enterocytes, and the expression of the heme exporters FLVCR and ATP-binding cassette, subfamily G, member 2 (ABCG2) strongly suggest the possibility that, at the duodenal level, intact heme absorption could take place (66). This hypothesis is supported by the fact that heme absorption from dietary sources is favored *versus* iron uptake (27). Therefore, we can speculate that, besides the system of inorganic iron absorption, based on the divalent metal transporter (DMT)1–ferroportin–transferrin axis (in which DMT1 is responsible for iron import in the enterocytes, ferroportin for its export to the bloodstream, and transferrin for its transport in the circulation) (41), a similar mechanism for heme–iron uptake exists, based on a putative HCP1–FLVCR/ABCG2–Hx axis. Preliminary data from the group of Abkowitz (133) indicate that heme export through FLVCR is facilitated by the presence of Hx in the extracellular medium compared with albumin.

From this point of view, the relevance of Hx function under basal conditions is highlighted, as this protein might play an important role in the regulation of heme transport among different tissues in the organism, and perhaps in other physiologic processes.

Hemopexin as a Signaling Molecule

Several studies have demonstrated that the heme–Hx complex, other than mediating heme uptake, may activate intracellular signaling pathways, important for cell proliferation and survival. The heme–Hx complex supports and stimulates proliferation of human acute T-lymphoblastic cells (MOLT-3) by its ability to replace iron transferrin (103). Besides this function, heme–Hx has been demonstrated to increase serum-stimulated MOLT-3 cell growth, suggesting that the heme–Hx receptor activation triggers signaling pathways involved in the regulation of cell growth. Interestingly, the cobalt protoporphyrin (CoPP)–Hx complex, which binds to the receptor but is not internalized into the cell, also stimulated cell growth in presence of serum, suggesting once again that Hx receptor occupancy generates mitogenic signals in cells with sufficient nutrients to support growth (103). The heme-Hx–stimulated signaling pathway has been demonstrated to involve protein kinase C (PKC) activation (103). These findings supported the existence of a heme-Hx–activated signaling pathway, and this hypothesis was further strengthened by work on Hepa cells, which demonstrated that heme-Hx, but not free heme, activates the N-terminal c-Jun kinase (JNK) (33). JNK, also known as stress-activated protein kinase (SAPK), a member of the mitogen-activated protein (MAP) kinase family, is usually activated by exposure of cells to several dangerous stimuli, including DNA damage, heat shock, or inflammatory cytokines, and leads to the appropriate response (62). Thus, JNK activation is needed for the proper response to toxic heme. A similar activation has been seen after CoPP-Hx treatment but not with heme alone, thus indicating the involvement of the heme–Hx receptor (33).

Moreover, the heme–Hx complex has been shown to induce the nuclear translocation of nuclear factor-kappa B (NF-κB), a key transcription factor activated by stress and involved in the cellular responses to inflammation (33). Interestingly, both JNK/SAPK and PKC have been implicated in NF-κB activation. Finally, NF-κB–binding sites were found in the promoter of the gene coding for HO-1 (67).

In Hepa cells, it was demonstrated that the heme–Hx complex increased the expression of p53 and p21, thus allowing long-term survival (33). Furthermore, the cell-cycle inhibitor p21, by binding JNK/SAPK, may prevent apoptosis. This may explain why, despite sustained elevated levels of phospho-c-Jun, Hepa cells exposed to heme–Hx do not undergo apoptosis. These findings represent additional evidence for the pleiotropic protective effects of heme–Hx and may explain why Hx-null mice showed extensive cellular damage and cell death after heme overload (120, 124).

The data reported (summarized in Fig. 5) were published before the discovery that LRP1 is the heme–Hx receptor (46). LRP1 is a scavenger receptor for an array of ligands, and some of these may trigger receptor-activation and -signaling pathways (71). For instance, tissue-type plasminogen activator has been shown to act as a cytokine by its ability to bind and activate LRP1, thus triggering phosphorylation of the intracellular mediators Mek1 and Erk-1/2, and inducing matrix-metalloproteinase-9 gene expression (43). Moreover, platelet-derived growth factor (PDGF) induced LRP1 phosphorylation and Shc recruitment, which might constitute a link to downstream PDGF-dependent signaling events such as MAP kinase activation (73, 88). In addition to Shc, the neuronal adaptors Disabled and FE65 were also shown to bind to LRP1 (122). Thus, it is possible to speculate that the heme–Hx complex acts in a similar manner by activating LRP1 and recruiting different adaptor molecules, thus triggering appropriate intracellular signals. Hence, cells expressing the heme–Hx receptor may catabolyze heme and activate pathways controlling cell proliferation, survival, and apoptosis.

Heme–Hemopexin Complex as a Regulator of Gene Expression

As reported earlier, incubation of Hepa cells with heme–Hx induces HO-1 and ferritin expression (3, 30). It also downregulates the transferrin receptor (TfR) mRNA level (3, 30). These effects were observed not only in hepatic cells but also in promyelocytic HL-60 cells, in leukemic U937 cells, and in HeLa cells (3, 116). Regulation of HO-1 expression is thought to occur through heme binding to the transcriptional inhibitor Bach1 (79). Conversely, the effect on TfR and ferritin expression is likely mediated by heme-derived iron affecting iron regulatory protein (IRP)1/2 activity (41) (Fig. 6). Moreover, other than an effect on TfR mRNA level, heme–Hx treatment also was shown rapidly to affect receptor redistribution between the plasma membrane and the cytosolic vesicle pool (128). Conversely, T lymphocytes isolated from Hx-null mice exhibited decreased expression of TfR compared with cells from wild-type animals (34). Again, it is difficult to reconcile these data with results obtained from *in vitro* experiments. However, the rapid kinetics of heme–Hx internalization by hepatocytes and the much higher affinity of heme for Hx than

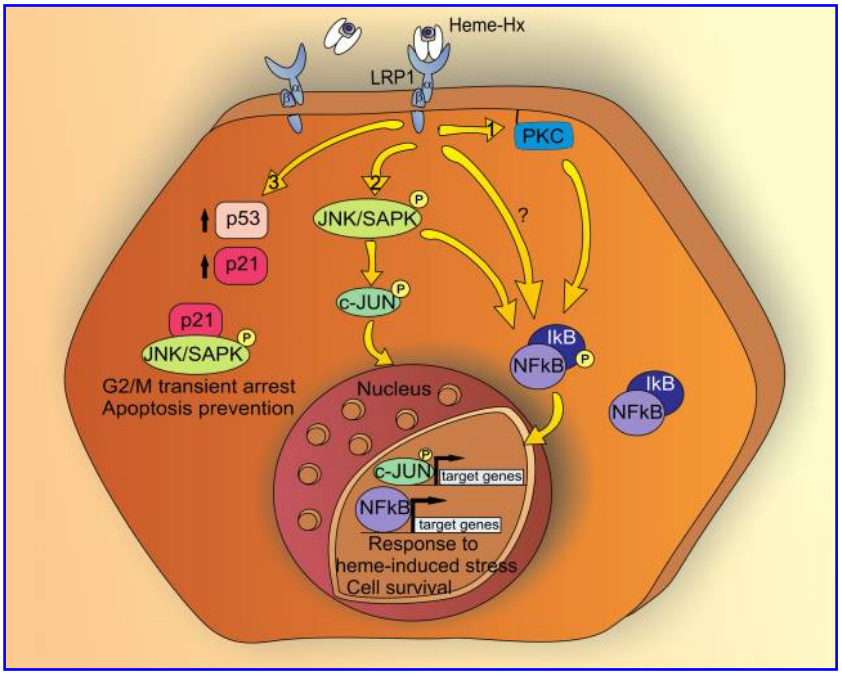

FIG. 5. Signaling pathways activated by heme–hemopexin. The heme–Hx complex has been shown to mediate: (1) PKC activation, which can lead to IkB phosphorylation and NF-κB nuclear translocation; (2) JNK/SAPK activation that may in turn phosphorylate c-jun, which translocates to the nucleus and activates its target genes; and (3) induction of p53 and p21 expression. p21 might sequester JNK/SAPK, thus inducing G_2/M arrest. NF-κB activation might also be induced by JNK/SAPK or by other mechanisms. All together, these events prevent apoptosis and promote cell survival. No experimental evidence has demonstrated that these effects are triggered by LRP1 activation (see text for details). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

for other plasma proteins may explain why heme is not taken up by other cell types under physiologic conditions. Thus, the reduced expression of TfR in T lymphocytes of Hx-null mice is probably the consequence of an "aspecific" heme uptake, which occurs when Hx is lacking, resulting in an increased intracellular iron pool that destabilizes TfR mRNA (see later and Fig. 10).

Finally, binding of heme–Hx to the plasma membrane stimulates gene expression of metallothionein (MT)1 (2, 96). Metallothioneins are cysteine-rich metal-binding proteins, thought to play a role in heavy metal detoxification, zinc, and copper homeostasis, and cellular adaptation to stress (28). On incubation with heme–Hx, MT1 mRNA steady-state levels rapidly increase in both mouse hepatoma and human HL-60

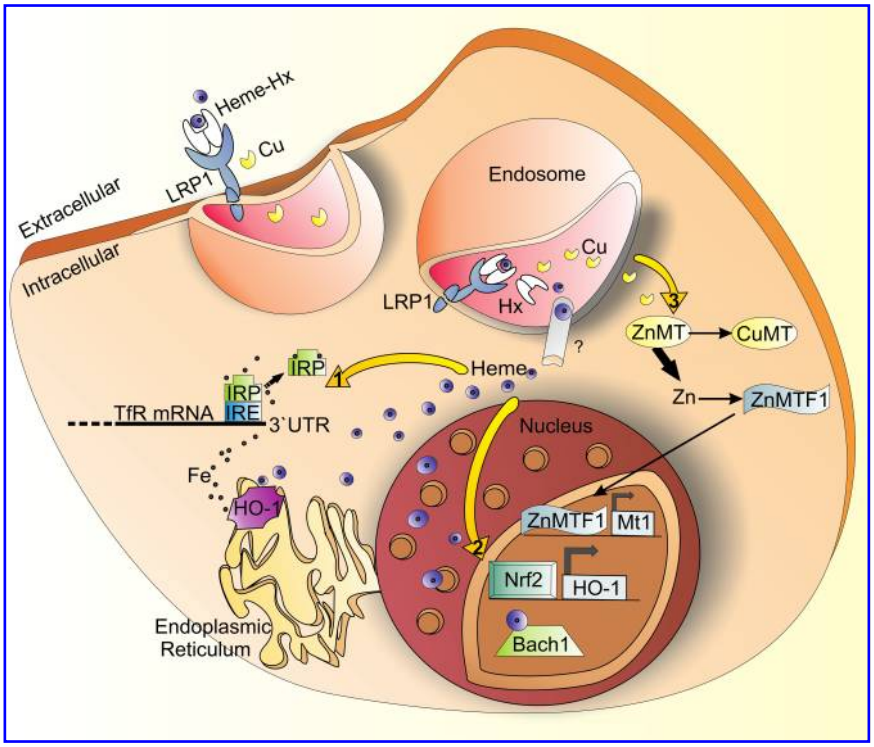

FIG. 6. Regulation of gene expression by heme–hemopexin. The heme–Hx complex is internalized, together with copper. Once released from the endosome, heme is degraded by HO, and iron derived from heme catabolism regulates IRPs activity, thus destabilizing TfR mRNA (1). In a similar way, iron may regulate ferritin mRNA translation (see Fig. 3). Alternatively, heme can translocate to the nucleus and remove Bach1 inhibition, thus inducing the expression of *HO-1* and other MAREs-bearing genes (2). Copper, released from the endosome, can bind to metallothioneins, displacing zinc from its storage sites. Free zinc then binds to the MTF1, stimulating its nuclear translocation, needed for *MT1* gene stimulation (3). The mechanisms of copper internalization and release from the endosome, as well as the mechanism of heme delivery from the endosome to the cytosol, are unknown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

cells. Free heme, although an effective inducer of HO gene transcription, was found to be a poor inducer of MT1 (2, 96). Moreover, it has been reported that endocytosis of the heme–Hx complex increases intracellular copper, and that copper is needed for the heme-Hx–mediated induction of HO-1 and MT1 (109). Based on these observations, Smith and co-authors (109) proposed a model to explain the concerted induction of HO-1 and MT1 in hepatoma cells. After receptor-mediated endocytosis *via* coated pits, heme–Hx complexes were shown to enter endosomes. During this process, a certain amount of copper also is internalized through an unknown mechanism. Within endosomes, the acid pH and the presence of copper favor the release of heme from Hx and its subsequent export to the endoplasmic reticulum, where heme can be catabolyzed by HO-1, or to the nucleus, where heme can regulate a series of genes, among which the transcription of HO-1 gene *via* Bach-1 de-repression. Besides the release of heme, endosomes liberate copper into the cytosol. The copper coming both from endosomes and plasma membrane can bind to metallothioneins, displacing zinc from its storage sites. Free zinc then binds to the metal transcription factor (MTF)1, stimulating its nuclear translocation, needed for *MT1* gene stimulation (Fig. 6).

Hemopexin in the Nervous System

Heme is an essential cofactor for many proteins involved in the normal function of neuronal tissue, such as enzymes required for neurotransmitter synthesis and myelination of axons (26). Conversely, an excess of heme is usually associated with such pathologic conditions as intracerebral or subarachnoid hemorrhages and ischemia/reperfusion injury. In addition, some neurodegenerative disorders, like Alzheimer's and Parkinson's diseases, are associated with iron accumulation in specific brain regions (15, 134). As the central nervous system is separated from the body by the blood–brain barrier, it has evolved mechanisms of local heme and iron management.

The expression of Hx in central nervous system (CNS) has been gradually established. The first analysis in rats failed to detect Hx in the CNS (113). Conversely, one work on the human brain showed immunoreactivity for Hx in neurons and a few scattered glial cells, whereas oligodendrocytes and epithelial cells of choroid plexi were negative (85). Human Hx promoter activity has been reported in several brain regions in mice, including cortex, hippocampus, thalamus, cerebellum, and brainstem nuclei (24, 119). Finally, Western blotting analysis of brain homogenates confirmed Hx expression (82), and a proteomic study identified the protein in human cerebrospinal fluid (CSF) (29). Detection of β-galactosidase activity on brain sections from Hx-null mice, carrying the lacZ gene into the Hx genomic locus, demonstrated that Hx was expressed primarily by ependymal cells lining the ventricular system and hippocampal neurons (82). These data demonstrate that Hx is expressed at different levels by ependymal cells and cell populations within the brain.

Little is known about the function of Hx in the CNS, the only data coming from the analysis of the brain of Hx-null mice. Hx-null mice have shown a strong increase in the number of iron-loaded oligodendrocytes in the basal ganglia and thalamus compared with wild-type controls (Fig. 7), not associated with an increase in H- and L-ferritin expression in these regions. Consistent with increased iron deposits and inadequate ferritin expression, oxidative stress markers were higher in the brains of Hx-null mice than in those of wild-type controls (82). These data suggest a role for Hx in controlling heme–iron recovery within the brain. Considering its ependymal expression, Hx may be released both in CSF, at the apical side of ependymal cells, and in brain parenchyma, at their basolateral side. In this manner, Hx may exert a double function by scavenging heme through the ventricular system, and by protecting brain parenchyma from heme-mediated damage. Previous studies demonstrated that LRP1 is expressed in brain and, in particular, in epithelial cells of choroid plexi, endothelial cells of microvessels, neurons, perivascular astrocytes, and microglial cells (31, 100, 135), thus supporting the possibility that Hx acts at multiple sites, as presented in Fig. 8.

Other than in the CNS, Hx expression has been demonstrated in the peripheral nervous system (PNS). Under basal condition, Hx is synthesized at low levels in the sciatic nerve (113). During postlesion degeneration, Hx strongly accumulates in the extracellular matrix near the injury site of most types of peripheral nerves, irrespective of their nature and origin. Otherwise, during nerve regeneration, Hx expression decreases progressively toward normal levels (20, 74). Hx-expressing cells in degenerated nerves are Schwann cells, fibroblast-like cells, and invading blood macrophages (74). Based on its expression after nerve lesion, Hx may be considered a molecule involved in wallerian degeneration, a process that occurs after a nerve trauma, as all these proteins are overexpressed during degeneration and disappear during nerve regeneration (111). Supporting this hypothesis is the fact that Hx does not increase in mice that display severely impaired wallerian degeneration (21). At sites of nerve lesions, Hx might merely act as a heme scavenger to remove heme released by injured vessels, or, alternatively, it might have a different role, not closely related to heme-binding capacity. Based on its protease activity (see later), it is possible to

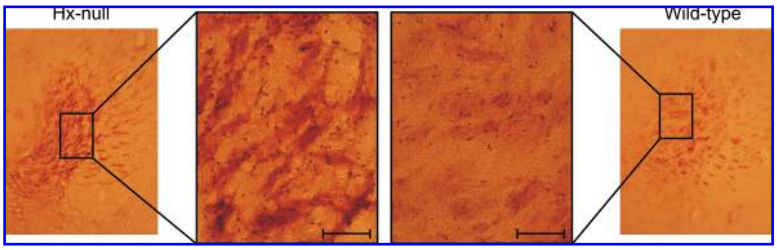

FIG. 7. Increased numbers of iron-loaded cells in Hx-null brain. Brain sections, at the level of the basal ganglia region, of an Hx-null and a wild-type mouse at 6 months of age stained with the Perl reaction to detect iron deposits. The images show a strong increase in the number of iron-loaded cells in Hx-null brain compared with that of the wild-type animal. Bar, 100 μm. For details, see ref. 105. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

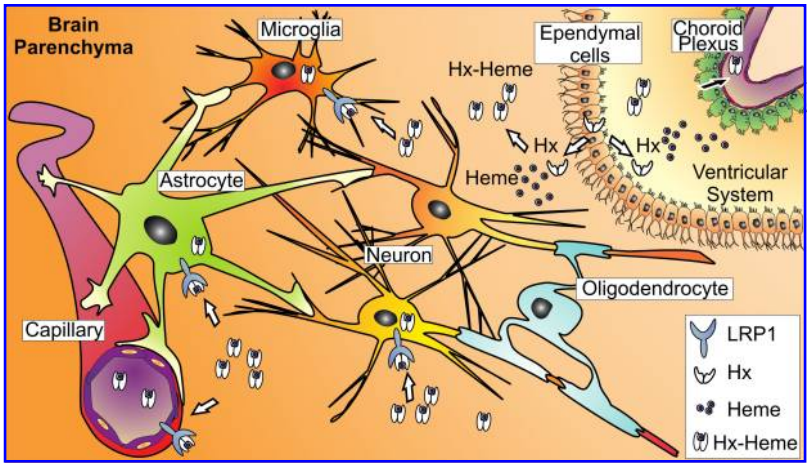

FIG. 8. A hypothetical model for Hx function in the brain. Hx is produced by ependymal cells and may be released both in the ventricular system and in brain parenchyma, where it binds free heme. In brain parenchyma, the heme–Hx complexes may be taken up by LRP1-expressing cells (*i.e.*, neurons, astrocytes, and microglial cells) and degraded. Oligodendrocytes do not express LRP1 and probably accumulate heme-derived iron released by other LRP1-expressing cells (for details, see ref. 105). In the ventricular system, the heme–Hx complexes may be removed by endothelial cells of choroids plexi. In this way, Hx might contribute to maintain heme homeostasis in the brain. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

speculate that Hx might be involved in the process of matrix remodelling at injured sites and in axonal regrowth.

Finally, Hx expression has been demonstrated also in the retina, which is separated from the circulation by the blood–retinal barrier. Chen and co-workers (22) showed that Hx is expressed in neural retina by photoreceptors and ganglion cells. Moreover, retinal pigment epithelial cells are able to bind the heme–Hx complex and, after its internalization, to induce the expression of HO-1, MT1, and ferritin (44, 45). At this level, Hx-mediated heme scavenging might be important in preventing oxidative damage after traumas and hemorrhages.

Hemopexin and the Immune System

As reported, several *in vitro* works described the effects of the heme–Hx complex on cells of the immune system (103). Conversely, it is known that iron can affect the immune response. Several studies indicate a decrease in circulating T lymphocytes in response to iron deficiency in humans and mice (19, 61, 98, 110). However, many controversies relate to iron overload and T lymphocytes, generated by the heterogeneous nature of iron overload. For instance, hereditary hemochromatosis, an autosomal recessive disorder of iron metabolism, has been associated with several T-cell abnormalities (91). The effects of iron on autoimmunity are just beginning to emerge. Iron supplementation in the MRL/MPJ-lpr/lpr mouse model of systemic lupus erythematosus leads to more severe renal disease and increased mortality (68). Moreover, the iron-transporter NRAMP1 has been associated with autoimmune diseases like rheumatoid arthritis, juvenile rheumatoid arthritis, type I diabetes, and multiple sclerosis (19, 58). The increase in HO-1 activation in lymphocytes has been previously reported to suppress the autoimmune response and to limit T-cell activation (25). Iron also regulates T-lymphocytes sensitivity to interferon (IFN)γ. In particular, iron uptake mediated by TfR delivers a signal that leads to IFN-γ receptor (R)2 internalization, thus attenuating activation of the IFN-γ/STAT1 pathway. Conversely, the iron-chelating agent desferroxamine upregulates IFN-γR2 surface expression and reinstates IFN-γ/STAT1 activation (94). The interplay between TfR and IFN-γR2 during $CD4^+$ T-cell activation is not yet clear, but it has been shown that TfR1 can physically associate with the T-cell receptor (TCR) in the immunologic synapse and that TCR engagement also induces a rapid co-polarization of IFN-γR with TCR (94, 95). Thus, it is possible to speculate that agents able to affect TfR expression may regulate T-lymphocyte activation. Supporting this conclusion is the observation that, in a model of mercury-induced autoimmunity, $CD4^+$ T cells from Hx-null mice, which had reduced the amount of TfR, had a blunted response to IFN-γ (34). Moreover, Hx-null mice produced significantly fewer

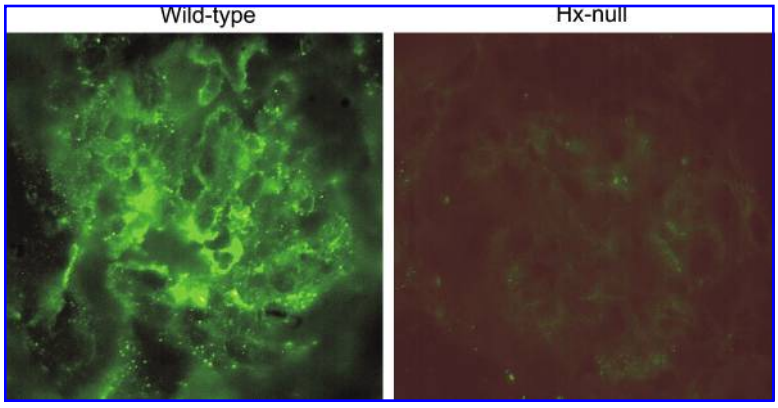

FIG. 9. Hx-null mice show a dampened autoimmune response. Kidney sections of mercury-treated wild-type and Hx-null mice were stained with FITC-conjugated goat anti-mouse IgG. Wild-type kidneys show granular deposits of immune complexes in the glomerular capillary wall and mesangium, whereas Hx-null kidney has no such deposits. For details, see ref. 94. Magnification, x400. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

autoantibodies and had fewer immune-complex deposits than did their wild-type counterparts (34) (Fig. 9). Based on these results, we conclude that Hx mediates heme uptake by lymphocytes in a controlled manner. In the absence of Hx, free heme contributes significantly to the intracellular iron pool, thus impinging on the immune response (Fig. 10). This might have important implications in the control of the immune response in patients with hemolytic disorders.

Other Functions

Hemopexin as a serine protease

Several studies have provided data demonstrating a protease activity for plasma Hx. Hx purified from human plasma and the recombinant protein were both able to induce transient protein leakage after contact with the rat kidney *ex vivo* and showed protease activity that could be inhibited with various serine protease inhibitors or ATP *in vitro* (8, 23). Hx was able to modify the permeability of the glomerular microvasculature *in vivo*, inducing the same histologic changes seen in minimal-change disease (MCD) *i.e.*, diminished expression of glomerular ecto-ATPase and retraction of foot processes (23). Accordingly, Bakker and co-authors (9) demonstrated that patients with MCD in relapse showed enhanced protease activity of their plasma Hx, whereas decreased Hx plasma titers were measured. Conversely, Hx could barely be detected in the urine of MCD in patients in relapse compared with patients with proteinuria due to other

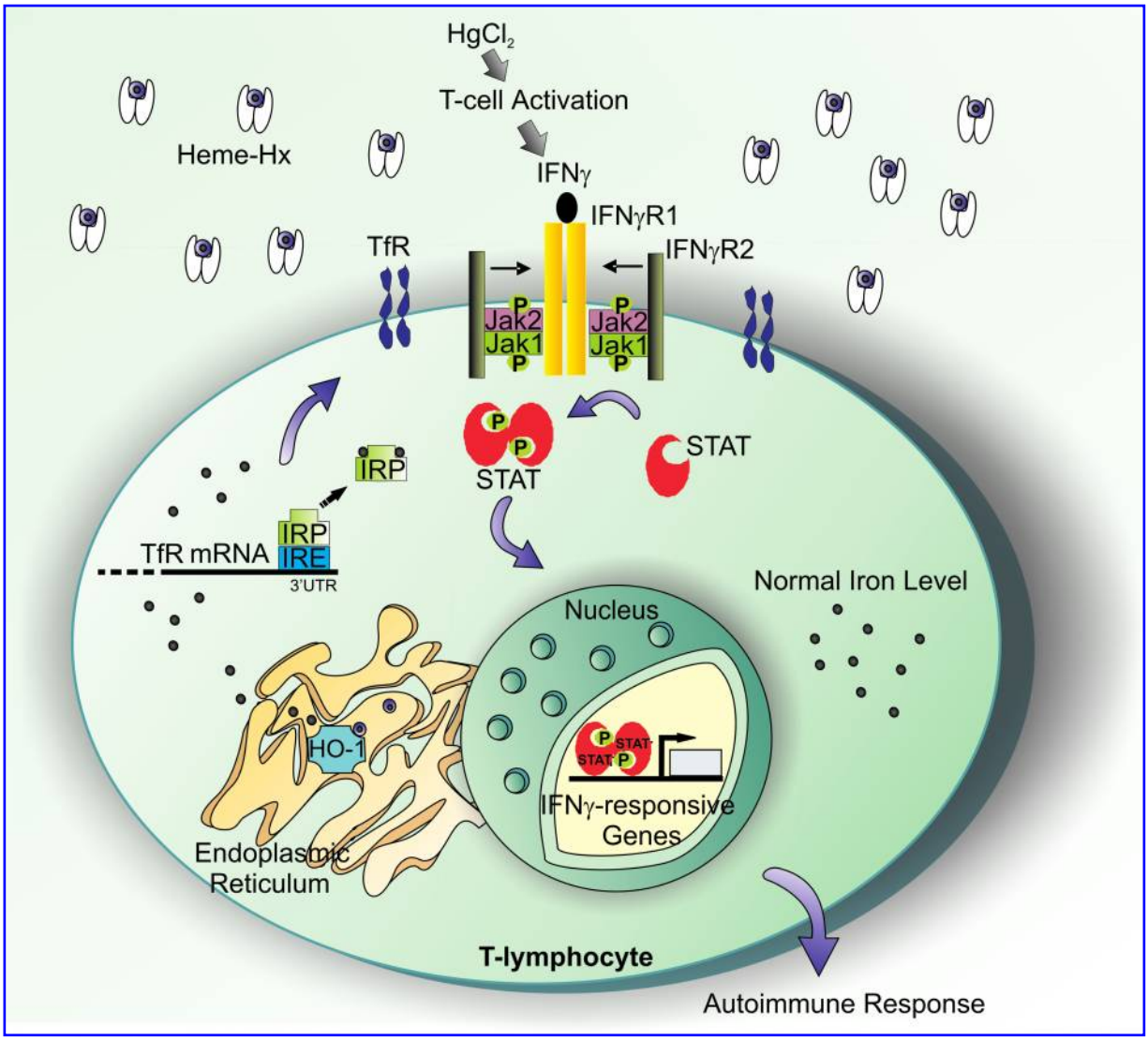

FIG. 10. A model to explain how hemopexin may contribute to control of CD4$^+$ T-lymphocyte responsiveness to IFN-γ. After the appropriate stimulus (in this case, $HgCl_2$ treatment),. IFN-γ, released by activated CD4$^+$ T cells, binds to IFN-γR1 (binding chain), and the latter recruits IFN-γR2 (transducing chain) to the complex. JAK1 and JAK2 constitutively bound to IFN-γR1 and IFN-γR2, respectively, phosphorylate each other, as well as IFN-γR1. This creates a docking site for STAT1, which is phosphorylated, dimerizes, and translocates to the nucleus, where it activates IFN-γ–regulated genes. The expression of IFN-γR2 at the plasma membrane is controlled by the TfR level through an unknown mechanism. As TfR expression is regulated by the intracellular iron pool through IRPs, Hx, by controlling heme–iron delivery to CD4$^+$ T cells, may affect their responsiveness to IFN-γ. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

forms of primary glomerulopathy (9). Taking these data together, the authors suggested that during disease, an active form of Hx with protease activity, which is responsible for glomerular alterations and proteinuria, emerged. Further supporting this possibility is the recent report that Hx induces nephrin-dependent reorganization of the actin cytoskeleton in podocytes and affects the permeability of the glomerular filtration barrier by degrading the glycocalyx (69).

Finally, Hx synthesis could be induced in human mesangial cells *in vitro* by proinflammatory cytokines like TNF-α (54). Local production of Hx might serve a protective function by limiting the proinflammatory and prooxidant effects of heme and, conversely, might alter glomerular permeability through its protease activity. This might be relevant both for the pathogenesis of renal diseases and for other pathologic conditions, such as those reported previously concerning peripheral nerve lesions. In this case, Hx protease activity might be important during wallerian degeneration, when axonal–Schwann cell contacts are disrupted (111).

We can speculate that, under basal conditions, Hx is associated with cofactors like serine protease inhibitors in plasma and hence, is inactive. During disease processes, the inhibitors are released, and Hx becomes an active and potentially toxic protease. This is supported by our data generated with a bioinformatic approach to identify genes coexpressed with *Hx*. Genes that were potentially functionally related to *Hx* and obtained by this procedure were analyzed for their gene-ontology annotation, and interestingly, the annotation term "serine protease inhibitor" was obtained with the mouse *Hx* list (35). Thus, this suggests that Hx may be associated with serine protease inhibitors that help keep the protein in its inactive form. Disease processes may lead to removal of the inhibitors, hence rendering Hx active.

Hemopexin as a binding protein for ligands different from heme

In this section, we discuss the binding properties of apo–Hx and heme–Hx complex. Porath and Olin in 1983 (90) described the ability of Hx to bind bivalent metal ions. They discovered that immobilized metal-affinity chromatography composed of Ni^{2+}-tris (carboxymethyl) ethylenediamine-Sepharose 4B could be used to separate several metal-binding plasma proteins, including Hx. By developing an advanced purification method, Mauk *et al*. (77) showed that apo–Hx binds bivalent metal ions in the following order: Ni^{2+}> Cu^{2+}>Co^{2+}>Zn^{2+}>Mn^{2+}. The heme–Hx complex exhibited similar behaviour, except that the order of retention of the complex on Zn^{2+} and Co^{2+} was reversed (77). Apo–Hx was found to have at least two metal ion–binding sites, and the capacity or affinity or both for metal ion binding decreased after heme binding. Thus, presumably, apo–Hx may have a role in the transport of metal ions in the blood or in exchange of metal ions between proteins. This might become relevant during injury or various disease processes, thus limiting the potential toxicity of metal ions (77).

Finally, it was observed that the geometry of the heme–Hx complex is reminiscent of hemoproteins endowed with ligand binding and (pseudo)-enzymatic properties. Accordingly, the heme–Hx complex binds reversibly two key regulatory molecules, CO and nitric oxide (NO), other than oxygen (6). CO is a by-product of heme degradation by HO and has vasodilatory and immunomodulatory properties. NO can both stimulate and inhibit superoxide-mediated tissue injury. It can act as an antioxidant or, in the presence of sufficient superoxide, as a strong oxidant to generate peroxynitrite, which produces potentially toxic NO-containing oxidized lipid species. By binding NO at sites of injury, heme–Hx may abrogate NO-mediated toxicity and protect endothelial cells lining blood vessels or neurons (101). Thus, the heme–Hx complex may have a key role in NO homeostasis, especially in conditions of trauma, inflammation, and hemolysis, as well as in stroke and reperfusion after ischemia. Accordingly, endothelial damage is strongly enhanced in Hx-null mice after heme overload (124).

Conclusions

It is clear that Hx is not only the systemic scavenger of heme but also acts as a multifunctional agent in important processes such as iron homeostasis, antioxidant protection, signaling pathways to promote cell survival, and gene expression. This has important implications in several pathologic conditions, not only associated to the release of heme, but also in nerve regeneration, in the control of the immune response, and in the pathogenesis of renal diseases.

It has emerged that, like heme, Hx has a double face, protective in many conditions, but detrimental in others. Thus, the protease activity of Hx might be important in promoting regeneration at sites of nerve injury, but also contributes to glomerular alterations in minimal-change disease. Similarly, the ability of Hx to control the intracellular heme and iron pools, thus affecting gene expression, is fundamental in cells deputed to manage high amounts of heme–iron as hepatocytes and macrophages, but may alter the response of T lymphocytes, thus causing the loss of immune tolerance.

Therefore, the potential manipulation of Hx function or expression or both in therapeutic interventions requires the future development of selective pharmacologic tools targeting Hx activity in a cell type–specific manner.

Acknowledgments

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Abbreviations Used

ABCG2 = ATP-binding cassette, subfamily G
Alb = albumin
ATP = adenosine triphosphate
ATPase = adenosine triphosphate phosphatase
CFU-E = colony-forming units-erythroid
CNS = central nervous system
CO = carbon monoxide
CoPP = cobalt protoporphyrin
CSF = cerebrospinal fluid
DMT1 = divalent metal transporter 1
FLVCR = feline leukemia virus subgroup C receptor

Abbreviations Used (cont'd)

Hb = hemoglobin
HCP1 = heme carrier protein 1
HO = heme oxygenase
Hp = haptoglobin
Hx = hemopexin
ICAM1 = intercellular adhesion molecule 1
IFN-γ = interferon γ
IFN-γR1 = interferon γ receptor 1
IFN-γR2 = interferon γ receptor 2
IL = interleukin
IRP = iron regulatory protein
JAK = Janus kinase
JNK = c-Jun N-terminal kinase
LDL = low-density lipoprotein
LRP1 = LDL receptor–related protein 1
MAP = mitogen-activated protein
MARE = Maf recognition element
MCD = minimal-change disease
MT-1 = metallothionein-1
MTF-1 = metal transcription factor 1
NF-κB = nuclear factor kappa B
NO = nitric oxide
NRAMP1 = NRAMP metal ion transporter 1
PDGF = platelet-derived growth factor
PKC = protein kinase C
PNS = peripheral nervous system
ROS = reactive oxygen species
SAPK = stress-activated protein kinase
STAT = signal transducer and activator of transcription
TCR = T-cell receptor
TfR = transferrin receptor
TNF-α = tumor necrosis factor α
VCAM1 = vascular cell adhesion molecule 1

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