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Haptoglobin and Hemopexin in Heme Detoxification and Iron Recycling

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1. Introduction

The acute phase reaction is an early response aimed at the defence of the organism and at the re-establishment of homeostasis in response to acute infection, inflammation and other pathological states (Kushner, 1982).
The putative mechanism responsible for this reaction is based on an initial signal derived from macrophages and other cells that synthesize and secrete several factors (probably cytokines) capable of inducing, in hepatocytes, a series of events. One of the most important mediators of the liver acute phase response is the monokine Interleukin (IL)-6 (Gauldie et al., 1987). The acute phase response consists in a change in the concentration of several plasma proteins, generally synthesized in the liver, including α_1-glycoprotein (AGP), complement factor C3, serum amyloid A, Haptoglobin and Hemopexin.

2. Haptoglobin

2.1 Gene structure

The Haptoglobin (Hp) locus is located on chromosome 16 in humans (chromosome 8 in mice) and shows an unusual polymorphism involving duplication, and, rarely, also triplication of parts of the coding region. Some Hp polymorphisms are linked to cardio-vascular and renal diseases.
A second gene exists, adjacent to the Hp gene and highly homologous to it, called Hpr (Haptoglobin related). Hpr arose by gene duplication and subsequent modification by a 7-kilobase retrovirus-like insertion into the first intron of the Hp gene (Marinkovic and Baumann, 1990). Both Hp and Hpr gene are transcribed in liver, but Hpr transcript level is only approximately 6% of that of Hp (Nielsen and Moestrup, 2009).
Although the primary site of Hp expression is the liver, it can also be detected in several other organs, including the nervous system, lung, spleen, thymus and heart (Nielsen and Moestrup, 2009).

2.2 Protein structure

Hp is a tetrachain ($\alpha_2\beta_2$) glycoprotein synthesized in the adult (but not fetal) liver and secreted into the plasma. The pro-Hp form is proteolytically processed into an α– and a β–chain. The two α-subunits and the two β-subunits of Hp protein are joined by inter-chain

disulfide bonds. In humans, the precise structure of Hp protein is different according to the different Hp alleles, that give rise to an (αβ)-dimer or to various (αβ)-multimers (Figure 1). Hp has a high binding affinity for hemoglobin that is bound to the β-chain (Adams and Weiss, 1969; Nielsen and Moestrup, 2009).

2.3 Gene, mRNA and protein regulation

Regulation of the expression of the Hp gene occurs at least at three levels: (i) developmental control, responsible for the lack of expression in fetal liver; (ii)tissue-specific control, responsible for the selectivity of the expression of the gene in the hepatocyte; (iii)modulation of its expression during the acute phase reaction (Oliviero et al., 1987).

A short DNA segment of the 5' flanking region of Hp gene contains sufficient information for tissue-specific expression and transcriptional activation by acute phase stimuli (Oliviero et al., 1987). Among these cis-acting elements in the Hp promoter, there are two IL-6 responsive elements, accounting for dramatic increase of Hp mRNA levels in the presence of this monokine (Oliviero and Cortese, 1989). Hp production is also regulated by glucocorticoids, but no information is available about a glucocorticoid-responsive element (GRE) in the human Hp gene (Marinkovic and Baumann, 1990). Transcription factor C/EBPβ and the nuclear matrix protein p55 were identified as the major proteins that bound the hormone-responsive cis-element of Hp gene during the acute phase response, at least in rat (Poznanovic et al., 1999).

The Hp gene is transcribed quite selectively in hepatocytes about fifty times more in adult than in fetal liver nuclei, compared to about a twenty-fold increase in the case of the hemopexin gene (Oliviero et al., 1987). As a consequence, Hp is found in very low concentrations in fetal plasma, whereas its levels in the adult are about 0.45-3 mg/ml.

Regarding cell lines, the Hp mRNA is present in some human hepatoma cell lines, such as HepG2, but it is completely absent in others, such as Hep3B.

Finally, ectopic production of Hp was reported in cases of inflammation and cancer. In fact, the expression of Hp mRNA was observed in a small number of pancreatic cancer cell lines. Moreover, some pancreatic cancer cells, thanks to secretion of IL-6, are able to induce the production of fucosylated Hp in hepatoma cell lines and, according to this, high levels of fucosylated Hp can be found in sera from patients with pancreatic cancer (Narisada et al., 2008). A similar condition is also common in cases of advanced ovarian cancer, mammary carcinomas and severe inflammation diseases, such as rheumatic arthritis and inflammatory bowel disease.

2.4 Conservation of gene, protein and regulation

New World primates and rats possess only a single Hp gene, while Old World monkeys carry two to three tightly clustered Hp genes. In humans, four structural alleles have been identified: HplS, HplF, Hp2, and Hp3. Hp2 and Hp3 differ from Hp1 by having seven rather than five exons resulting in an increase of the α-subunit amino acids content.

Hp1 and Hp2 are the two major allelic forms of human Hp and they can give rise to three major Hp genotypes: Hp1-1, Hp2-1 and Hp2-2 (Nielsen and Moestrup, 2009).

An extensive study has been made on rat Hp, demonstrating that rat Hp cDNA sequence shows a high degree of similarity to the human Hp1 allele and that no Hpr gene can be found in rat genome. The rat Hp shows 75% amino acid sequence homology for the α-subunit and 86% for the β-subunit when compared with the human Hp1 gene product. Rat β-subunit contains two potential N-glycosylation sites, in contrast to the human β-subunit,

which has four sites. Finally, rat Hp gene responsiveness to IL-6 is lower than in humans, and in rat cells the combination of IL-1, IL-6 and glucocorticoids (as dexamethasone) is required for maximal Hp expression (Marinkovic and Baumann, 1990).

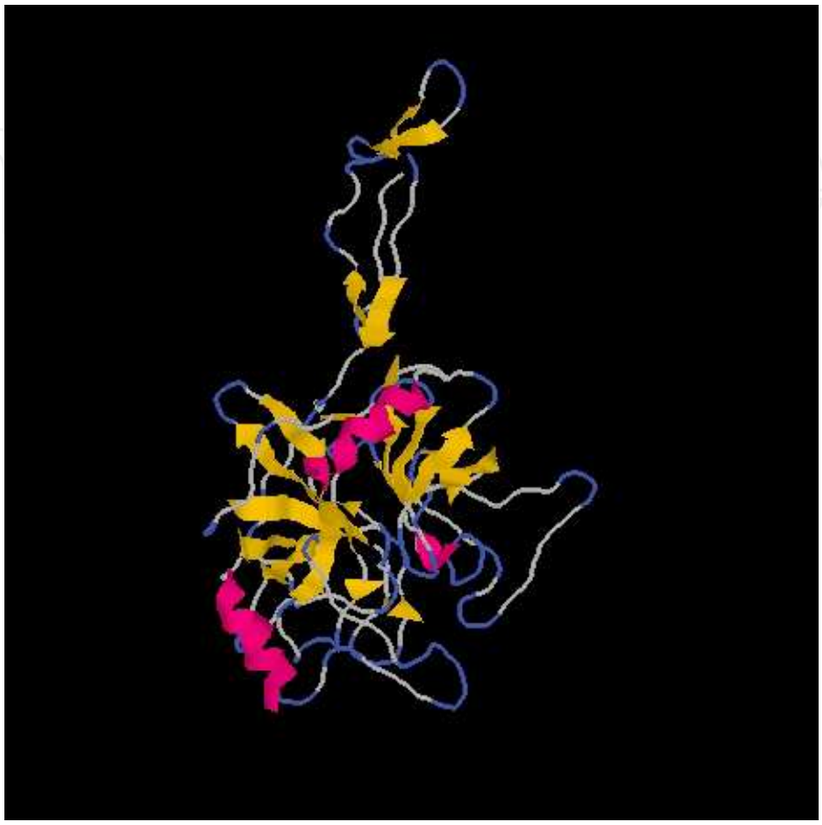


Fig. 1. Model of the human Haptoglobin isoform 1 monomer. α-Helices and β-strands are shown in pink and yellow, respectively. Loops are drawn in blue. The Hp model was generated using CPHmodels available at http://www.expasy.org/tools. The model was drawn with the Rasmol available at http://www.expasy.org/tools.

3. Hemopexin

3.1 Gene structure

The Hemopexin (Hx) gene is an 11Kb long gene located on human chromosome 11 (chromosome 7 in mice), the same location as the β-globin gene cluster (Law et al., 1988).
It is mainly expressed in the liver and, to a lesser extent, in neurons and astrocytes of the central nervous system, ganglionic and photoreceptor cells of the retina, Schwann and fibroblast-like cells of the peripheral nervous system, kidney mesangial cells and skeletal muscle (Tolosano et al., 1996).

3.2 Protein structure

Hx is a plasma 60-kD β-1B-glycoprotein composed of a single 439 amino acids long peptide chain, which forms two domains resembling two thick disks that lock together at a 90° angle and are joined by an interdomain linker peptide.

It contains about 20% carbohydrate, including sialic acid, mannose, galactose, and glucosamine and it does not present free sulfhydryl groups (Takahashi et al., 1984). Twelve cysteine residues were found in the protein sequence, probably accounting for six disulfide bridges.

The structure of human Hx is characterized by its unique clustering of histidine and tryptophan residues. The histidine residues are present in His-Gly sequences presumably exposed at the surface, while tryptophan mostly occurs in four clusters (Takahashi et al., 1984) (Figure 2).



Fig. 2. Model of the human Hemopexin precursor. α-Helices and β-strands are shown in pink and yellow, respectively. Loops are drawn in blue. The Hx model was generated using CPH models available at http://www.expasy.org/tools. The model was drawn with the Rasmol available at http://www.expasy.org/tools.

Hx has the highest known heme affinity (Kd<1 pM) of any characterized heme-binding protein. It binds heme in an equimolar ratio, but there is no evidence that heme is covalently bound to the protein (Takahashi et al., 1984). The heme ligand is bound between the two domains of Hx in a pocket formed by the interdomain linker peptide. Heme binding and release results from opening and closing of the heme binding pocket, through movement of the two domains and/or interdomain linker peptide. The heme affinity decreases on lowering pH, on reduction of the heme iron atom, on nitric oxide (NO) binding to the ferrous heme iron atom, and in the presence of the chloride anion and of divalent metal ions, while the sodium cation increases the heme affinity for Hx (Tolosano et al., 2010).

Other than heme, Hx can also interact with a wide variety of natural and synthetic metalloporphyrins. As in cytochrome b5 (with which Hx shares several chemical and physical properties), two histidines in the N-terminal domain are proposed to be the ligands to heme iron, while tryptophan residues seem to reinforce the interaction of Hx with heme (Takahashi et al., 1984).
Regarding the C-terminal domain, its structure is common to that found in other proteins such as metalloproteinases which, for this reason, are indicated as "hemopexin-like domain" containing proteins (Bode, 1995).

3.3 Gene, mRNA and protein regulation

In murine development, Hx mRNA expression appears in the fetal life and the hepatic production of the protein and its serum concentration increase considerably during postnatal development, reaching the maximum level in the adult (Takahashi et al., 1984).
Similarly, Nikkilä et al. showed that hepatic Hx mRNA in rat is first detected on day 14 after gestation. Hx gene expression is not present in yolk sac, placenta, decidua, uterus or early embryonic tissues (Nikkila et al., 1991).
Apart from liver, other sites of Hx synthesis are the nervous system, skeletal muscle, retina and kidney, while Hx mRNA is not detectable in lung, heart, gastrointestinal tract and spleen (Poli et al., 1986).
Among human hepatoma cell lines, Hep3B cells have been shown to produce the highest amount of Hx mRNA (Poli et al., 1986).
After its synthesis, Hx is released in plasma where it can reach a concentration of about 0.5-1mg/ml. Its level can, however, increase during hemolyses or inflammatory events (Tolosano and Altruda, 2002; Tolosano et al., 1996).
Hx production is known to be regulated in large part at the transcriptional level. The tissue specific and the temporal expression of the Hx gene is directed by a 500bp fragment located upstream of the transcription start point in the Hx promoter. This region contains a specific cis-acting element, called Hpx A site, which, apart from being important for the cell-specific transcription of Hx, is also responsible for its regulation during the acute phase response (Poli et al., 1989).
Among inducers of Hx expression there are the cytokines IL-6, IL-11, leukaemia inhibitory factor (LIF), oncostatin M, IL-1β and Tumor Necrosis Factor (TNF)α (Immenschuh et al., 1995), while unlike most acute-phase proteins the serum amount of Hx is only slightly affected by dexamethasone. The regulation of Hx expression in response to IL-6 is mediated by a liver specific nuclear protein, IL6DBP (a member of the C/EBP family), which binds to the Hpx A site, and by the IL6RE-BP, an inducible nuclear factor which binds to another similar, but functionally distinct, IL6-responsive element in the Hx promoter (Tolosano et al., 1996).
Besides being regulated during inflammation, Hx production increases in response to extracorpuscular heme, while the levels of other acute-phase proteins remain unchanged after this kind of stimulus. Interestingly, rat Hx expression is also promoted by hyperoxia (Nikkila et al., 1991).

3.4 Conservation of gene, protein and regulation

The physiological importance of Hx is suggested by the extensive homologies in the sequence of this protein in different species and by the fact that its structure is very similar in all vertebrates.

As an example, the N-terminal domain of Hx has been identified as the heme-binding domain in human, rabbit and pig. Moreover, human and rat Hx share a high degree of homology at the amino acid level (76%) and a comparison of the interdomain disulfide bond formation reveals a similarity in their N-terminal and C-terminal domain structure.
Finally, the perfect conservation of the cysteine residues of rat and human Hx indicates that the same disulfide configuration is present in both proteins (Nikkila et al., 1991).
Beside protein structure conservation, gene expression regulation was maintained during evolution. Indeed, at least in human, rabbit, rat and chicken Hx gene expression is quite entirely confined to the liver and follows a peculiar temporal pattern during development, increasing several folds from fetal to adult life.

4. Haptoglobin and Hemopexin function into the bloodstream

4.1 Antioxidant and cytoprotective function of both Haptoglobin and Hemopexin

Hp and Hx belong to the acute-phase proteins whose expression can be induced by various cytokines in a context of inflammatory processes and act as soluble scavengers of free hemoglobin and heme, respectively.
Before starting to discuss in detail the role of Hp and Hx in heme metabolism, we want to open a short parenthesis on why heme scavenging from circulation is crucial.

4.1.1 Heme

Heme (protoporphyrin IX and iron) plays critical roles in several biological processes as it is the prosthetic group of a lot of essential proteins, such as hemoglobin, myoglobin, catalases, peroxidases and cytochromes (Tsiftsoglou et al., 2006).
On the other hand, free heme is highly toxic as it is a source of redox-active iron. In the cytoplasm, iron can participate in the Fenton reaction to produce the highly toxic reactive oxygen species (ROS) that damage lipid membranes, proteins and nucleic acids (Papanikolaou and Pantopoulos, 2005). Heme toxicity is further exacerbated by its ability to intercalate into lipid membranes. Heme-iron may initially lodge within the hydrophobic interstices of the phospholipid bilayer. Within this highly oxidizable matrix, iron catalyzes the oxidation of cell membrane constituents and assists in the formation of cytotoxic lipid peroxide, which enhances permeability and membrane disorder. Oxidation of membrane components may promote cell lysis and death.
Free heme is also a potent hemolytic agent. It affects erythrocyte membrane stability as a result of ROS formation and oxidative membrane damage thus shortening erythrocyte life span. Finally, free heme is an important source of iron for pathogenic microorganisms, predisposing to infections (Kumar and Bandyopadhyay, 2005).
Release of hemoglobin into the bloodstream is a physiologic process due to intravascular hemolysis that occurs during enucleation of erythroblasts and destruction of senescent erythrocytes. It has been calculated that, even if senescent red blood cells are mostly phagocytosed by macrophages, intravascular hemolysis accounts for at least 10% of red cell breakdown in normal individuals. However intravascular hemolysis becomes a severe pathological complication when it is accelerated in various disorders, such as hemorrhage, hemolytic anemia and hemoglobinopathies, polycitemia vera, malaria, ischemia reperfusion and muscle injury (Ascenzi et al., 2005; Stuart and Nagel, 2004). Under physiologic conditions, released hemoglobin is bound by Hp and transported to macrophages and hepatocytes. After massive hemolysis, when the buffering capacity of plasma Hp is

overwhelmed, hemoglobin is quickly oxidised to ferrihemoglobin, which releases free heme (Tolosano et al., 2010). Ferriheme then binds to albumin [Kd~10nM] and is subsequently transferred to Hx [Kd<1pM]. Heme is initially associated with albumin, presumably because the molar concentration of albumin in plasma is considerably greater than that of Hx (300 μM vs. 20 μM). After heme binding, Hx specifically delivers heme to the liver (Figure 3).

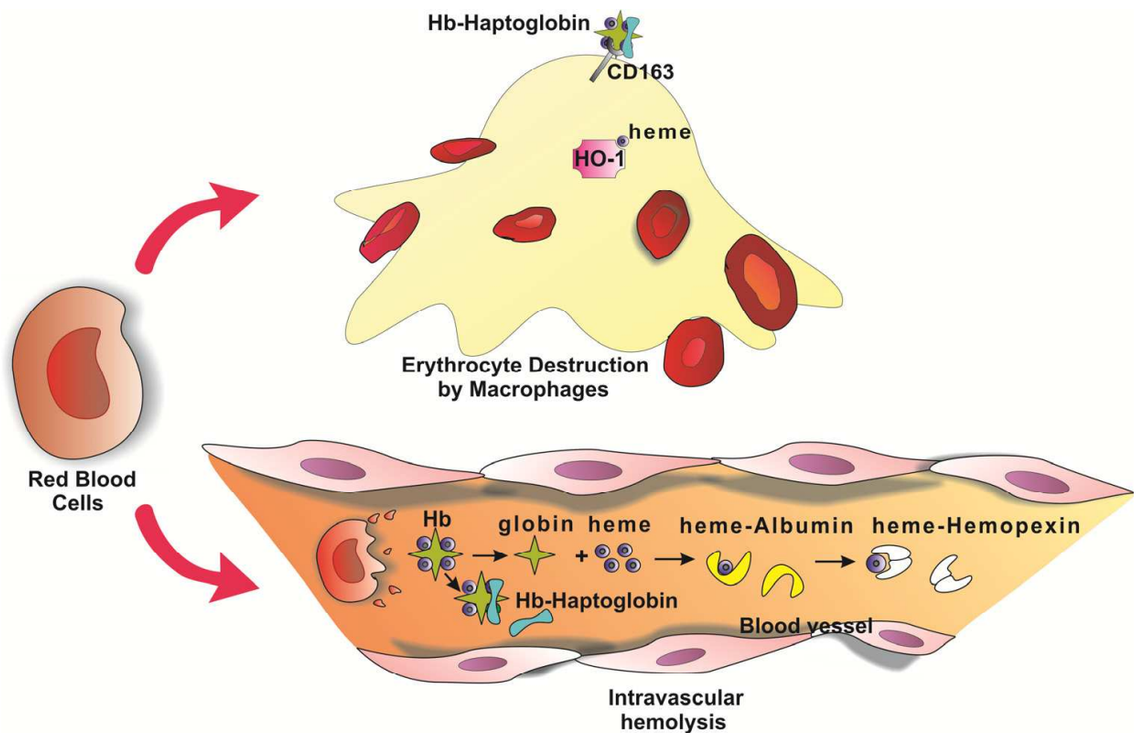


Fig. 3. Hemoglobin catabolism. Heme contained in red blood cells is mostly recycled by macrophages through erythrophagocytosis. During this process heme is degraded by HO-1 and iron recycled. A minor part of erythrocytes undergoes intravascular destruction, releasing hemoglobin which is bound by Hp and the complexes are subsequently delivered to hepatocytes and macrophages of the reticuloendothelial system, where they are internalized through CD163 receptor–mediated endocytosis. When the buffering capacity of Hp is exceeded, hemoglobin liberates heme, which binds to albumin and is subsequently transferred to Hx. (Hb: hemoglobin).

Under these conditions, the physiological mechanisms of removing free hemoglobin and heme from the circulation collapses, allowing nonspecific hemoglobin and heme uptake and heme catalyzed oxidation reactions (Kumar and Bandyopadhyay, 2005; Wagener et al., 2003b).

The vasculature is one of the most susceptible tissue to heme-mediated oxidative injury as it is continuously exposed to circulating erythrocytes, exogenous hemoglobin and heme released by damaged cells, (Balla et al., 2000; Jeney et al., 2002; Ogita and Liao, 2004; Wagener et al., 2001a). Heme can threaten vascular endothelial cell integrity directly by promoting intracellular ROS formation (Balla et al., 2000; Stocker and Keaney, 2004; Wagener et al., 2001a; Wagener et al., 2003a) and indirectly by its ability to oxidize low density lipoproteins (LDLs) (Grinshtein et al., 2003). The initial step of heme-mediated LDL

oxidation involves the spontaneous insertion of heme into LDL particles. The inserted heme directly promotes extensive oxidative modification of LDL. Accordingly, when endothelial cells are exposed to LDL from plasma containing hemoglobin or free heme, oxidative endothelial damage ensures (Grinshtein et al., 2003).
Under physiological condition, the endothelial layer is non-adhesive for leukocytes. However, when exposed to free heme, activated endothelial cells increase the surface expression of adhesion molecules, as Intercellular Cell Adhesion Molecule (ICAM)-1, Vascular Cell Adhesion Molecule (VCAM)-1 and selectins (Belcher et al., 2003; Wagener et al., 2001a) which may subsequently promote the recruitment of leukocytes at the site of inflammation. By enhancing adhesion molecule expression and generating oxidative stress known to damage cells, heme also acts as a pro-inflammatory molecule and starts the inflammatory cascades (Wagener et al., 2001b). Finally free heme is considered a trigger of vasopermeabilization, which results from the partial retraction of endothelial cells of venules in the vicinity of inflammation, leaving small intercellular gaps. Vascular leakage results in slower blood flow by allowing the passage of water, salts and small proteins from the plasma into the damaged area (Mehta and Malik, 2006).
Other than for the vessels, free heme is also highly toxic for other tissues and organs causing oxidative stress and damage.

4.1.2 Haptoglobin

Following intravascular hemolysis, stable hemoglobin-Hp complexes are formed in plasma and are delivered to the reticuloendothelial system by CD163 receptor-mediated endocytosis and to liver parenchymal cells through a yet unidentified receptor. CD163 is a member of the cysteine-rich scavenger receptor family and is exclusively expressed by cells of monocyte/macrophages lineage (Kristiansen et al., 2001; Nielsen and Moestrup, 2009). The existence of another receptor for hemoglobin-Hp complexes in hepatocytes has been hypothesized as it has been demonstrated that after injection of labeled hemoglobin-Hp complexes in rats, most of labeled hemoglobin is taken up by liver parenchymal cells (Higa et al., 1981; Kino et al., 1982; Ship et al., 2005; Weinstein and Segal, 1984). In macrophages, upon endocytosis, the receptor–ligand complex enters early endosomes where hemoglobin-Hp complexes are released from CD163. The receptor then recycles to the cell surface while hemoglobin-Hp complexes continue through the endocytic pathway to end up in lysosomes where the protein moieties and the ligand are degraded (Nielsen and Moestrup, 2009).
In this manner, Hp reduces the loss of hemoglobin through the renal glomeruli hence protecting against peroxidative kidney injury and allows heme-iron recovery.
This has been extensively confirmed by studies in Hp-null mice which have shown that the loss of Hp did not affect hemoglobin clearance (Fagoonee et al., 2005; Lim et al., 1998) but influences the pattern of hemoglobin distribution. Following the injection of low doses of labeled hemoglobin, hemoglobin-Hp complexes are mainly delivered to hepatocytes and Kupffer cells in the liver and to macrophages in the spleen of wild-type animals; in the absence of Hp, hemoglobin is mainly recovered by the kidney instead of the liver and spleen suggesting that Hp is important for the delivery of hemoglobin complexes to the liver and spleen. In a similar way, when high doses of labeled hemoglobin were injected into wild-type mice, causing the saturation of Hp binding capacity, in addition to the liver and spleen, hemoglobin is also delivered to the kidney thus mimicking what occurs during pathological conditions such us chronic hemolysis.

The role of Hp in preventing hemoglobin filtration through the glomerular barrier is further supported by the observation that Hp-null mice develops kidney iron overload with ageing (Fagoonee et al., 2005). Particularly, hemoglobin derived iron accumulate mainly in the proximal tubular cells of the kidney. Similarly, HO-1 knockout mice which completely lack macrophages expressing the hemoglobin-Hp receptor CD163 also develop kidney iron loading (Kovtunovych et al., 2010).

Moreover, excessive hemolysis or transfusion of hemoglobin solution have been shown to result in Hp depletion and subsequent renal failure, particularly acute tubular necrosis (Tam and Wong, 1988).

In the absence of Hp, hemoglobin is filtered through the glomerular barrier and is reabsorbed by proximal tubular cells through the endocytic receptors megalin and cubilin. Megalin and cubilin are multiligand endocytic receptors expressed at the apical membrane of proximal tubules. Their primary function is to reabsorb small molecules that pass the glomerular filtration barrier. It has been previously demonstrated that hemoglobin is one of their ligands (Christensen and Birn, 2001; Gburek et al., 2002). Once in tubular cells, hemoglobin is degraded in the endosomal compartment and heme is catabolized by heme oxygenase (HO) (Figure 4).

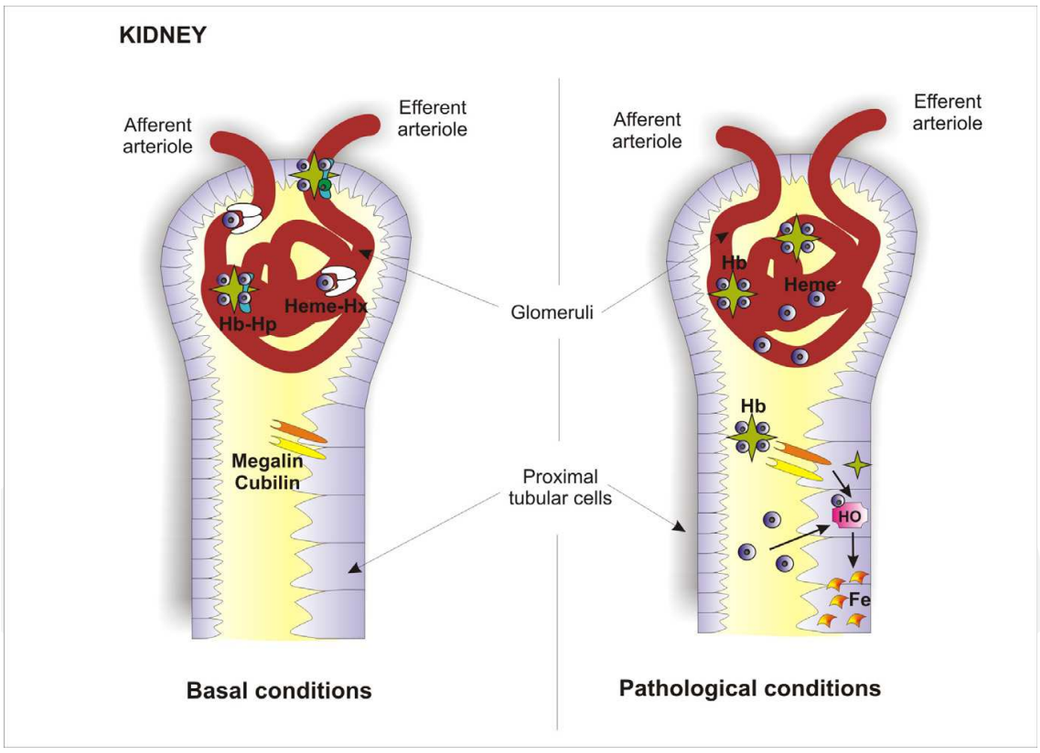


Fig. 4. Haptoglobin and Hemopexin prevent kidney iron loading. Under basal conditions hemoglobin and heme are targeted to macrophages and hepatocytes by Hp and Hx, respectively. Under pathologic conditions when Hp and Hx binding capacity is overwhelmed, hemoglobin and heme are filtered through the glomerular barrier and are re-absorbed by proximal tubular cells. Hemoglobin is recognized by the endocytic receptors megalin and cubilin while heme enters into the cells through a yet unidentified mechanism. Into the cells, heme is degraded by HO and iron stored bound to ferritin (Ft).

In agreement with renal iron loading, Hp-null mice show higher basal level of renal lipid peroxidation and suffered greater tissue damage, as evidenced by the induction of the hepatic acute phase response resulting in increased AGP levels (Lim et al., 1998). Moreover, these mice showed increased susceptibility to acute hemolysis induced by phenylhydrazine treatment and are more sensitive to kidney injury than wild-type animals. Accordingly, an increased susceptibility to hemoglobin driven lipid peroxydation has been observed in human patients with hypo- or anhaptoglobinemia.

4.1.3 Hemopexin

Hx represents the primary line of defence against heme toxicity thanks to its ability to bind heme with high affinity and to function as a heme specific carrier from the bloodstream to the liver (Tolosano et al., 2010). The formation of heme-Hx complexes has been demonstrated to promote heme delivery to the parenchymal cells of the liver (Smith and Morgan, 1978, 1979). On the contrary, the heme-albumin complex appears to act only as a heme depository, before transport to the liver as heme-Hx, and there is no experimental evidence that albumin has a transport function in vivo (Smith and Morgan, 1981). Nowadays, several lines of evidence support the fact that the liver is the main target tissue for heme-Hx complex internalization and heme-derived iron recycling. In vivo studies showed that the liver is the major site of radioactive heme uptake after intravenous injection of ^{55}Fe-heme-^{125}I-Hx: nearly 90% of the administered heme is transported to the liver within 2 hours (K_D700nM) without significant urinary excretion of either isotope (Smith and Ledford, 1988; Smith and Morgan, 1978, 1979, 1981, 1984). Hx-mediated heme uptake by the liver has been shown in vivo and in vitro to be a saturable process: saturation is indicative of an interaction with a rate-limiting step and a finite number of binding sites and is characteristic of receptor-mediated uptake. Furthermore, heme-Hx internalization has been demonstrated to be a highly tissue-specific process, time-, temperature- and energy-dependent (Smith and Morgan, 1978, 1979). Occurring within minutes, the association is on the same time scale as the receptor-mediated uptake of asialoglycoproteins (LaBadie et al., 1975) and of iron-transferrin complexes (Gardiner and Morgan, 1974). Nowadays, the only known Hx receptor on hepatocytes is represented by the LDL receptor-related protein 1 (LRP1), a multi-ligand scavenger receptor, involved in the metabolism of lipoprotein and expressed in several cell types including macrophages, hepatocytes and neurons (Boucher et al., 2003; Lillis et al., 2005). LRP1 has been shown to mediate heme-Hx internalization, resulting in cellular heme uptake (Hvidberg et al., 2005). Once entered the cell, the heme-Hx complex is dissociated by lysosomal activity: LRP1 is then recycled to the plasma membrane, whereas Hx destiny, after complex internalization, is somewhat controversial. Some studies have suggested that Hx can be recycled as an intact molecule to the extracellular milieu (Smith and Morgan, 1979). However, it has also been proposed that following hepatic uptake of heme from heme-Hx, varying proportion of the protein are either returned to the circulation or degraded in the liver (Potter et al., 1993). Recently, Hvidberg et al. have shown that most Hx is degraded in lysosomes (Hvidberg et al., 2005). Accordingly, in a model of heme overload, plasma Hx level has been found to decrease, thus indicating that Hx is actively involved in heme scavenging and subjected to degradation (Vinchi et al., 2008). Furthermore, a decrease in plasma Hx concentration reflects a recent release of heme compounds in the extracellular compartment. Invariably, high concentrations of heme are associated with low concentration of Hx (Muller-Eberhard et al., 1968). Hx is in fact found to decrease in plasma after hemolytic stress associated to

pathologies like hemolytic anemias, acute intermittent porphyria and chronic neuromuscular diseases.

As a consequence of Hx-mediated heme delivery to the liver, heme deleterious effects are efficiently counteracted as demonstrated by several experimental data. First, heme binding to Hx has been demonstrated to reduce the heme-mediated free radical formation from organic peroxides (Timmins et al., 1995). Furthermore, in vitro studies demonstrated that Hx strongly decreases the peroxidative and catalatic activity of heme by forming inactive heme-protein complexes. Interestingly, these hemin activities were found to be inhibited by 80-90% with Hx but only by 50-60% with either human or bovine albumin (Grinberg et al., 1999). The marked effectiveness of Hx at inhibiting heme toxicity was most probably the result of its very high affinity to heme with a dissociation constant K_D of 10^{-13}M. Moreover, binding to Hx was shown to inhibit heme-catalyzed lipid peroxidation in artificial liposomes (Gutteridge and Smith, 1988), rat liver microsomes (Vincent et al., 1988) and plasma LDL (Miller et al., 1996). Thus, Hx has an essential role in the prevention of heme-induced oxidative damage and cell death (Eskew et al., 1999).

Many experimental evidences also support 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 (Tolosano et al., 1999; Vinchi et al., 2008). Furthermore, in vivo studies showed that the most damaged tissues upon heme overload conditions are the vasculature, the liver and the kidney.

It has been demonstrated that Hx has a crucial role in the protection of the endothelial wall against heme toxicity. It has been observed an increased induction of the adhesion molecules ICAM-1 and VCAM-1 in the endothelium and increased vascular permeability in Hx-null mice compared to wild-type mice, after intravenous heme injection (Vinchi et al., 2008), thus demonstrating that Hx activity is required to prevent heme-induced vasopermeabilization and endothelial activation.

Oxidative stress has already been shown to induce vascular HO-1 expression in rats, mice, and humans. Even if HO-1 induction is significantly higher in the vascular endothelium of Hx-null mice compared to controls, it cannot prevent endothelial damage (Vinchi et al., 2008). On the other hand, the induction of HO-1 before intravenous heme injection preserved endothelial integrity in Hx-null mice, thus indicating that the lack of Hx may be tolerated if the cells are already equipped to metabolize an excess of heme and suggesting that Hx and HO-1 work in sequence to counteract the toxic effect of heme, Hx being the first line of defence.

Besides the vasculature, other tissues have been described as particularly sensitive to heme-mediated damage. Studies on Hx-null mice have demonstrated that these animals are particularly sensitive to acute hemolysis. These mice recover more slowly after phenylhydrazine-induced hemolysis and suffer from more severe renal damage compared to wild-type mice. In fact, after hemolytic stimulus, Hx-null mice present prolonged hemoglobinuria, higher kidney iron loading and lipid peroxidation than wild-type mice (Tolosano et al., 1999). These findings emphasize the protective role of Hx in hemolytic processes. Moreover, Hx-null kidneys exhibit increased lipid peroxidation not only after phenylhydrazine treatment but also after intravenous injection of hemin (Vinchi et al., 2008). Therefore Hx, together with Hp, plays a fundamental role in the kidney during hemolysis: Hp has a major function in the protection of renal tubules from hemoglobin-mediated oxidative damage; then, once Hp disappears from the circulation, the delayed presence of Hx in the plasma takes on a relevant role in the protection against heme derived from hemoglobin oxidation.

Interestingly Hx and Hp compound mutant mice subjected to phenylhydrazine-induced hemolysis presented, other than kidney damage, a more severe injury in the liver characterized by inflammation, necrosis and fibrosis (Tolosano et al., 2002). The liver is also the most sensitive organ to heme overload in Hx-null mice. Indeed the liver of heme-overloaded Hx-null mice developed a marked congestion characterized by red blood cell stasis and sinusoidal dilation around the centrolobular area (Figure 5). Hepatic congestion was found to be associated with abnormal iron deposits, increased lipid peroxidation and massive leukocyte infiltrates (Vinchi et al., 2008).

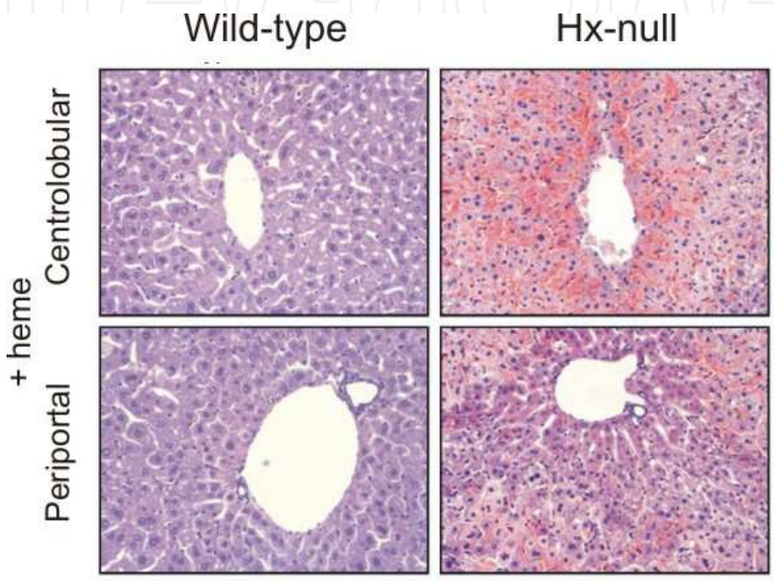


Fig. 5. Liver congestion in heme-overloaded Hemopexin-null mice. Liver sections of a wild-type and a Hx-null mouse injected with heme into the tail vein and sacrificed 6 hours later. Note the marked congestion around the centrolobular vein in Hx-null animal. Hematoxylin and eosin staining; X200.

This phenotype underlines the increased susceptibility of Hx-null mice to acute hepatic damage in condition of heme overload and highlights a role for Hx in the protection from liver injury. Liver damage in Hx-null mice may be prevented by induction of HO-1 before heme overload, thus confirming once again that Hx and HO-1 work together to ensure tissue protection against heme toxicity.

The congestion observed in the liver of heme-overloaded Hx-null mice resembles the hepatic phenotype of patients suffering from vaso-occlusive diseases like veno-occlusive disease (VOD) (Bayraktar et al., 2007; Senzolo et al., 2007) and Budd-Chiari syndrome (BCS) (Aydinli and Bayraktar, 2007; Hillmen et al., 1995) or experienced painful vaso-occlusive crises associated with hemoglobinopathies (Ahn et al., 2005; Dampier et al., 2004). Particularly, in sickle cell disease, vascular occlusions are the major causes of the pain, morbidity, and mortality (Stuart and Nagel, 2004).

Since all the disorders mentioned above are usually related to pathological conditions wherein extracellular hemoglobin and free heme are released in massive amounts, it could be speculated that heme represent a predisposing factor for vaso-occlusion and that Hx is important to counteract its pro-occlusive effects. This hypothesis is also in agreement with

the mentioned role of Hx as a detoxification mechanism that prevents endothelial damage by removing free heme from circulation.
In conclusion in vivo studies highlight the critical importance of Hx in preventing firstly vascular inflammation and acute liver injury and secondly renal damage, thanks to its ability to limit heme-induced oxidative stress. Interestingly all the toxic effects of heme are exacerbated in Hx-null mice, indicating not only that Hx has an important protective role in plasma but also that none of the plasma proteins able to bind heme (ie, albumin, α1-microglobulin, high- and low-density lipoproteins) may substitute for Hx after heme overload.

4.2 Role of Haptoglobin and Hemopexin in iron recycling

4.2.1 Haptoglobin- and Hemopexin-mediated heme recovery

Besides their function as hemoglobin and heme scavengers respectively, Hp and Hx are essential in the re-utilisation of heme-bound iron and represent a fundamental part of the iron-conservation mechanisms of the body (Hershko,1975; Davies,1979).
As reported above, the hemoglobin-Hp complexes are mainly taken up by macrophages through the specific receptor CD163, whereas the heme-Hx complexes enter into hepatocytes through LRP1. Once in macrophages or hepatocytes, heme is degraded by HO-1 to iron, biliverdin and CO (see next section). Iron is then stored in cells bound to ferritin or exported to the plasma and transported throughout the body. The contribution of Hp to iron recovery is further highlighted by the observation that the Hp phenotype modify iron loading in hemochromatosis both in humans and in mice (Delanghe and Langlois, 2002; Langlois et al., 2000; Tolosano et al., 2005; Van Vlierberghe et al., 2004; Van Vlierberghe et al., 2001). In addition, deletion of the Hx gene in mice results in abnormal extrahepatic iron deposits (Morello et al., 2008), thus suggesting that also in humans mutations in the Hx gene might modify iron distribution and accumulation in the body.
Other than by Hp and Hx other mechanisms have been reported to mediate hemoglobin or heme delivery to cells. Recent data suggest that in macrophages CD163 is also able to mediate the entrance of free hemoglobin through a low affinity binding. Particularly, (a)hemoglobin uptake has been observed in the absence of Hp in human macrophages and in CD163 transduced HEK293 cells but not in CD163-negative cells; (b)highly purified hemoglobin inhibits CD163 mediated uptake of labeled hemoglobin-Hp complexes or free hemoglobin, implying a common receptor binding site; (c)free hemoglobin induces transcriptional induction of HO-1, an indirect measure of hemoprotein internalization and degradation, in CD163 expressing cells in a dose dependent manner; (d)disruption of the hemoglobin interaction with Hp by chemical cross-linking of hemoglobin between its alpha chains or, alternatively, by proteolytic cleavage does not significantly affect the CD163-hemoglobin interaction.
Moreover, other than free hemoglobin, macrophages may also take up free heme, or, in other words, heme not bound to Hx. Treatment of primary macrophages or macrophage cell lines with heme resulted in the induction of HO-1 and ferritin indicating that heme enters in these cells and is degraded (Hvidberg et al., 2005; Liang et al., 2009). Moreover, Hx-deficient mice showed a prolonged HO-1 induction in Kupffer cells after acute hemolysis (Tolosano et al., 2002) and intravenous heme injection (Vinchi et al., 2008), thus suggesting that Hx limits heme delivery and thus heme-mediated HO-1 induction in these cells. Moreover, several other cell types, other than macrophages, may take up free hemoglobin and heme.

Nevertheless these alternative mechanisms do not ensure an adequate protection against oxidative damage nor an efficient iron recovery as demonstrated by the observation that, under conditions of massive hemolysis, free hemoglobin and heme accumulate in proximal tubular cells of the kidney. As mentioned in section 4.1.2,, Hp-null mice accumulated heme-derived iron in proximal tubular cells during ageing and after phenylhydrazine-induced hemolysis. This is true also for Hx-null that, after phenylhydrazine treatment show renal iron loading. Moreover, heme overloaded-Hx-null mice upregulate HO-1 and ferritins in the kidney. These data indicate that excess of free heme is recovered by the kidney, during hemolytic stress, when the buffering capacity of Hp and Hx is overwhelmed (Figure 4). (Lim et al., 1998; Tolosano et al., 1999).

In conclusion as shown in Figure 6, Hp plays a major role in mediating haemoglobin recovery in macrophages through CD163, whereas Hx promotes heme uptake by

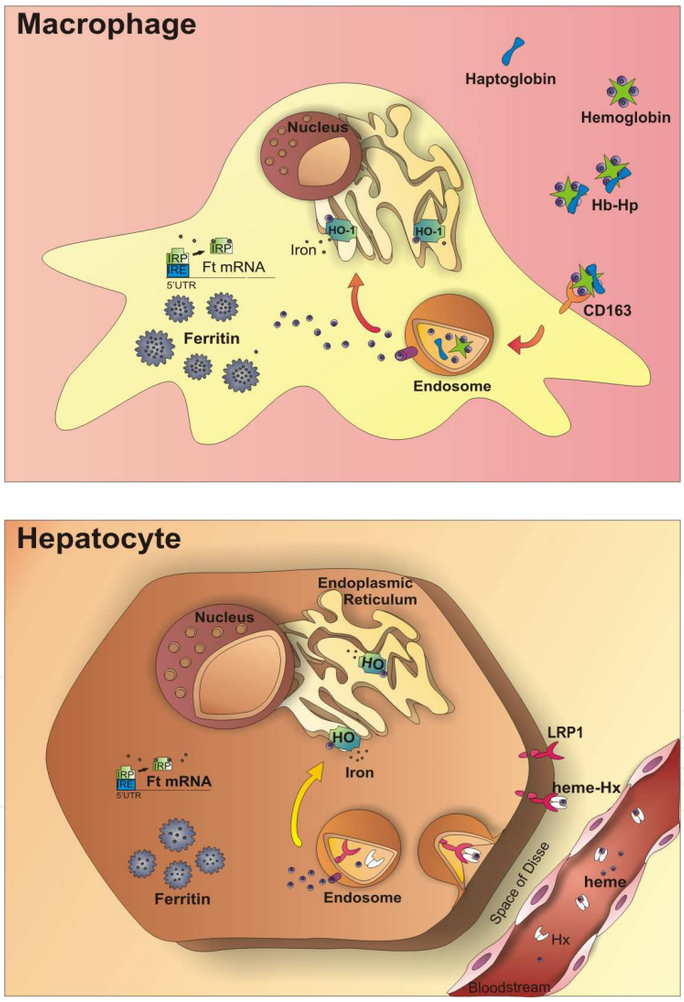


Fig. 6. Role of macrophage and hepatocyte in hemoglobin and heme recovery respectively. Macrophage takes up the hemoglobin-Hp complexes through CD163, whereas the hepatocyte recovers the heme-Hx complexes through LRP1. Once into the cell heme is degraded by HO to iron, which is bound to ferritin, CO and biliverdin (see section 4.2.2 for details). As depicted in the figure iron by itself may control the expression of ferritin (see section 4.3.2).

hepatocytes through LRP1. These mechanisms ensure an adequate protection against heme-mediated oxidative stress and mediate heme-iron reutilization. Under conditions of massive hemolysis when Hp and Hx are saturated free hemoglobin and heme may be taken up by macrophage through not well-characterized mechanisms. However, under these conditions heme pro-oxidant potential is not adequately inactivated and the vasculature and tissues are damaged.

4.2.2 Role of HO and Ferritin in heme iron recovery

Once that the hemoglobin-Hp or the heme-Hx complexes are respectively taken up by macrophages and hepatocytes, heme is released in the cytoplasm and presumably used to build new hemoproteins or catabolized by HO.

Microsomal HO is the rate-limiting enzyme in the degradation of heme and plays a key role in the protection of cells from heme-induced oxidative stress (Ferris et al., 1999). It breaks down the pro-oxidant heme into the antioxidant biliverdin, the vasodilator carbon monoxide (CO) and iron. Biliverdin is then reduced to bilirubin by the enzyme biliverdin reductase. Hitherto, three isoforms of HO have been identified: HO-1, HO-2, and HO-3.

HO-1 is highly inducible by a variety of stimuli including oxidative stress, heat shock, hypoxia, heavy metals, ischemia-reperfusion, cytokines and its substrate heme. The constitutively expressed HO-2 participates in the normal heme capturing and metabolism, while the function of HO-3 is still under investigation (Wagener et al., 2003b). HO-1 plays a crucial function in regulating heme degradation and protects against heme-mediated oxidative injury.

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 by producing CO, a final product of heme degradation. Once bound to the heme groups of hemoproteins, CO inhibits heme-iron oxidation, thus limiting the oxidation of hemoproteins and preventing heme release. It has been recently demonstrated that by this mechanism HO-1 inhibits the accumulation of free heme in plasma following Plasmodium infection, thus preventing the onset of severe malaria in mice (Ferreira et al., 2008; Pamplona et al., 2007; Pamplona et al., 2009; Seixas et al., 2009).

Analysis of HO-1-null mice has shown that these animals accumulated, with age, hepatic and renal iron that contributed to oxidative damage, tissue injury and chronic inflammation. On the other hand, HO-1-null mice presented low serum iron concentration and developed anemia (Koizumi, 2007; Yachie et al., 1999). These data demonstrated that, although HO-1 is a stress-induced protein, it is important under basal conditions to protect liver and kidney from oxidative damage and that it is an essential regulator of iron metabolism and homeostasis.

Overexpression of HO-1 is associated to the resolution of inflammation through the generation of beneficial molecules like CO, bilirubin, and ferritin resulting from catabolism of toxic heme (Kapturczak et al., 2004; Wagener et al., 2001b). Some of the end products of heme catabolism by HO-1 might 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 biliverdin reductase into the potent lipid-soluble antioxidant bilirubin. Owing to its lipophilic nature, free heme might act as a pro-oxidant primarily within cellular membranes. This deleterious effect may be inhibited by lipophilic bilirubin, that efficiently

scavenges peroxyl radicals, thereby inhibiting lipid peroxidation and attenuating heme-induced endothelial activation. This mechanism would explain the ability of HO-1 to inhibit, via the production of bilirubin, lipid peroxidation in cells exposed to free heme and TNF. CO controls the activity of several heme proteins and causes vasodilation. It also exerts anti-inflammatory effects by inhibiting the expression of pro-inflammatory cytokines through a pathway involving the mitogen-activated protein kinases (Ndisang et al., 2002). In the last years several studies have shown the therapeutic potentialities of HO-1 and its products in counteracting the toxic effect of heme associated to pathologic conditions (Farombi and Surh, 2006; Lindenblatt et al., 2004).

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 ferritin (Ft) expression to avoid the pro-oxidant effects of labile iron produced via heme catabolism. This notion is consistent with the observation that overexpression of Ft can mimic the cytoprotective effects of HO-1.

Ft is the major intracellular depot of non-metabolic iron and acts as a heme-detoxification system by scavenging free iron and protecting cells from its adverse effects. Ft is a multimeric protein composed of 24 subunits of two types, the heavy chain (H-Ft) and the light chain (L-Ft) and has a very high capacity for storing iron (up to 4500 mol of iron per mol of Ft). In the Ft shell, the proportion of heavy and light subunits depends on the iron status of the cell or tissue and varies among organs and species. H-Ft manifests ferroxidase activity that catalyses the oxidation of ferrous iron to ferric iron to allow intracellular iron storage in L-Ft, which acts as intracellular iron deposit (Arosio and Levi, 2002). Iron released during heme catabolism has been demonstrated to be rapidly stored in Ft (Davies et al., 1979).

Together, HO and Ft allow rapid iron shifting from heme into Ft core where iron is less available to catalyze deleterious reactions. Hence their potent antioxidant role. By increasing the expression of HO-1 and Ft, cells can survive lethal heme-induced oxidative stress (Balla et al., 2005).

Interestingly, in vivo work showed that Hx-null mice failed to up-regulate Ft in the liver after heme overload, thus demonstrating that the lack of Hx decreases the ability of the liver to recover heme-iron, under heme overload condition. Conversely, up-regulation of Ft in wild-type liver indicates a strong iron detoxifying capacity and an active iron storage and demonstrates, once again, that Hx is crucial to mediate heme delivery to hepatocytes.

4.3 Regulation of gene expression by hemoglobin-Haptoglobin and heme-Hemopexin complexes.

4.3.1 Haptoglobin mediated regulation of Ferroportin expression

Recent studies suggest an important role of Hp in modulating iron export from the duodenum. Hp-null mice showed increased iron export from the duodenum compared to wild-type mice, while iron uptake was normal (Marro et al., 2007). Iron export out of the duodenum was due to the increased expression of the iron exporter Ferroportin.

Following the injection of a low dose of hemoglobin into wild-type and Hp-null mice, a little amount of hemoglobin is delivered to the duodenum, suggesting the existence of a yet unknown mechanism for hemoglobin uptake into duodenal cells (Fagoonee et al., 2005). So, it has been proposed that hemoglobin taken up into duodenal cells could regulate Ferroportin transcription.

In vitro data on macrophages, showed that hemoglobin and heme directly activate the transcription of Ferroportin through the transcription factors Bach1 and Nrf2 (Marro et al., 2010). Thus, Hp, by controlling plasma levels of hemoglobin, participates in the regulation of ferroportin expression, thus contributing to the regulation of iron export. In the same way it is possible to speculate that Hx by controlling heme uptake by the cells may contribute to the control of ferroportin expression.

4.3.2 Hemopexin-mediated regulation of genes involved in iron recycling and cell survival

By its ability to mediate heme uptake into the liver, Hx promotes an increase in intracellular concentrations of heme, that directly affects the surface expression of transferrin receptor (TfR) and the expression level of HO-1 and ferritin. Heme has been shown to regulate the expression of several genes, including HO-1, by inhibiting the transcriptional repressor Bach1. Moreover, when intracellular heme increases, a rapid downregulation of TfR on the plasma membrane and concomitant induction of ferritin synthesis occur. It has been demonstrated that incubation of mouse Hepa cells with heme-Hx causes a rapid dose- and time-dependent decrease in the level of TfR mRNA. These regulatory effects have been observed not only in hepatic cells but also in human promyelocitic HL-60 cells (Alam and Smith, 1989), in human leukemic U937 cells and in HeLa cells (Taketani et al., 1990). Down-regulation of TfR on the plasma membrane was the result of multiple steps: a rapid redistribution of the protein between the plasma and intracellular membrane compartments and a decrease in the biosynthesis of the receptor. The latter is due to iron released from heme, that affects the stability of iron regulatory proteins (IRP), which regulate TfR mRNA stability and ferritin mRNA translation by binding to the iron responsive elements (IRE) in their 3′ and 5′ UTRs, respectively (Hentze et al., 2004). In this manner heme-derived iron enhance the expression of the iron storage protein ferritin and down-regulates the uptake of inorganic iron.

Furthermore, binding of heme–Hx to the plasma membrane Hx receptor stimulates the expression of metallothionein (MT)-1 (Alam and Smith, 1992; Ren and Smith, 1995) . Metallothioneins are cysteine-rich proteins thought to play a role in heavy metal detoxification, zinc and copper homeostasis, and cellular adaptation to stress. Upon incubation with heme-Hx, MT-1 mRNA steady state levels rapidly increase in both mouse hepatoma and human HL-60 cells. Regulation is controlled primarily at the level of MT-1 gene transcription in Hepa cells. Non protein-bound heme, although an effective inducer of HO gene transcription, was found to be a poor inducer of MT-1. This indicated that occupation of the Hx receptor itself by the heme-Hx complex is necessary for efficient accumulation of MT-1 transcripts. Activation of MT-1 gene transcription as a consequence of Hx-mediated heme transport may occur during endocytosis or via an indirect mechanism triggered by the interaction of heme-Hx with the Hx receptor on the cell surface. Recently Smith et al. demonstrated that the correct hypothesis was the first one: mainly copper, than the heme-Hx complex has been found to have an essential role in MT-1 induction (Smith et al., 2008). Copper endocytosis together with that of heme-Hx provides a mean to facilitate heme release from Hx in the maturing endosomes, by preventing the rebinding of heme to Hx. In this manner copper promotes heme export from endosomes and renders it available for HO-1 degradation. On the other hand, MT-1 induction is proposed to take place in response to a rise in cytosolic copper that directly contribute to MT-1 gene transcription. Therefore, cytosolic copper provide a link for the simultaneous regulation of HO-1 and MT-1 by heme-Hx.

5. Haptoglobin and Hemopexin function 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 (Connor and Menzies, 1996). On the other hand, excess of heme is usually associated to 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 (Berg and Youdim, 2006; Zecca et al., 2004). 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.

5.1 Haptoglobin and Hemopexin expression in the central nervous system

Both Hp and Hx were found in the human cerebrospinal fluid and their expression increases in several pathologic conditions including Parkinson's disease, Alzheimer's disease and Guillain-Barré syndrome (Arguelles et al.; Roher et al., 2009; Yang et al., 2008).

Hp was found to be expressed in human gliobastoma cell lines, in reactive astrocytes after transient forebrain ischemia in rats and in oligodendroglia in mice (Lee et al., 2002). Hx expression was demonstrated in cortical neurons and astrocytes (Morris et al., 1993). Moreover, detection of beta-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 (Morello et al., 2008). Finally, both Hp and Hx are expressed in the neural retina (Chen et al., 1998).

5.2 Neuroprotective roles of both Haptoglobin and Hemopexin

In humans Hp haplotypes were found to be correlated with the extent of cerebral deep white matter lesions in hypertensive patients and with cerebrovascular disease, thus suggesting that the efficiency of hemoglobin scavenging may be crucial for the resolution of neuronal injury.

Moreover, by using a mouse model of intracerebral hemorrhage, Zhao and co-authors demonstrated that Hp plays an important role in defending neurons from damage induced by hemolysis (Zhao et al., 2009). *In vitro* studies demonstrated that oligodendroglia-released Hp protects neurons and oligodendrocytes against hemoglobin-mediated toxicity (Zhao et al., 2009).

A protective role against intracerebral hemorrhage has also been reported for Hx by Chen and co-authors that demonstrated increased striatal injury and behavioral deficits in Hx-null mice subjected to intracerebral hemorrhage (Chen et al.). Moreover, it has recently been reported that, in a mouse model of transient ischemia, Hx is protective as neurologic deficits and infarct volumes were significantly greater in Hx-null than in wild-type mice (Li et al., 2009). Exogenous free heme was shown to decrease cell survival in primary mouse cortical neuron cultures, whereas the heme bound to Hx was not toxic and protection was achieved through heme-Hx-mediated induction of HO-1 (Li et al., 2009).

6. Other functions

Recent works highlighted a role for Hp and Hx in the control of the immune response, mainly achieved through their ability to control inflammation. Hp modulates both innate

and adaptive immune responses. Hp has been demonstrated to bind activated neutrophils, to inhibit several of their functions and to suppress secretion of TNF-α, IL-10, and IL-12p70 by macrophages upon LPS triggering (Arredouani et al., 2005; Rossbacher et al., 1999). CD11b has been identified as a macrophage receptor for Hp (El Ghmati et al., 1996). The binding of hemoglobin-Hp complex to the CD163 molecule on macrophages leads to anti-inflammatory cytokine secretion (Nielsen and Moestrup, 2009). Hp acts on Langerhans cells of the skin, preventing their differentiation and function during in vitro culture and affects proliferation and cytokine production by stimulated T cells and B cells (Huntoon et al., 2008; Xie et al., 2000). Recently, Galicia et al. demonstrated that, in a model of experimental autoimmune encephalomyelitis, Hp-null mice suffered from a more severe disease that was associated with increased expression of IL-17A, IL-6, and interferon (IFN)-γ mRNA in the CNS and with a denser cellular infiltrate in the spinal cord. During the recovery phase, a significantly higher number of myeloid DC, $CD8^+$ cells, IL-17^+ $CD4^+$ and IFN-γ^+ $CD4^+$ cells persisted in the CNS of Hp-null mice. Absence of Hp affected the priming and differentiation of T cells after induced encephalomyelitis (Galicia et al., 2009).

On the other hand, Hx-null mice produced significantly less autoantibodies and had less immune complex deposits than their wild-type counterpart in a model of mercury-induced autoimmunity and this response has been correlated to a blunted response of $CD4^+$ T cells from Hx-null mice to IFNγ. Some data suggested that Hx, by controlling heme-iron availability to T lymphocytes may control the expression of IFNγR at the cell membrane thus regulating IFNγ responsiveness (Fagoonee et al., 2008). However, other data demonstrated that Hx, like Hp, down-regulates LPS-induced proinflammatory cytokines from macrophages and suppresses neutrophil adhesion and phagocytosis by a mechanism unrelated to heme-binding (Liang et al., 2009). Furthermore, Spiller et al. have recently reported that Hx by inhibiting neutrophil migration leads to increased mortality in septic mice (Spiller et al., 2010).

All these results suggest that Hp and Hx play a modulatory role on the immune response likely by controlling cytokine production.

7. Conclusion

As discussed in the previous sections, Hp and Hx, by acting as plasma scavengers of hemoglobin and heme respectively, play a major role in the protection against heme-mediated oxidative stress and in preventing heme-iron loss during the acute phase response associated to massive intravascular hemolysis. In addition, they play a "local" role in the nervous system by limiting the pro-oxidant effect of heme after ischemia or intracerebral hemorrhage. Finally, they have a modulatory role in the immune system by regulating the inflammatory response.

Most of the work in the past decades has been focused on the definition of the mechanisms underlying the Hp- and Hx-mediated protection against heme toxicity. Nevertheless, recently, Schaer and co-authors investigated the potential of Hp supplementation as a strategy to counteract the intrinsic hypertensive and oxidative toxicities of free hemoglobin and demonstrated that the induction of Hp synthesis in dogs by glucocorticoid treatment prevented free hemoglobin-mediated hypertension. In a similar way, the co-infusion of exogenous Hp and hemoglobin in guinea pig prevents hemoglobin peroxidative activity and oxidative tissue damage (Boretti et al., 2009).

Thus, it is time to speculate that therapeutics that could increase Hp and/or Hx levels or act as Hp/Hx agonists might help to limit heme toxic effects in pathologic conditions associated to massive hemolysis as hemolytic anemia, sickle cell disease, ischemia-reperfusion injury.

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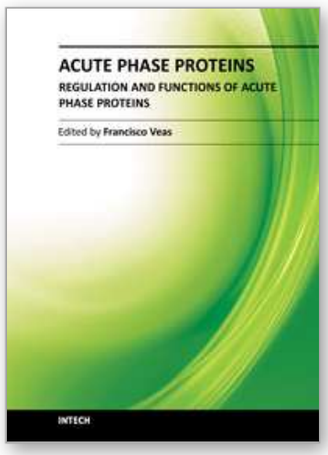
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The two volumes of Acute Phase Proteins book consist of chapters that give a large panel of fundamental and applied knowledge on one of the major elements of the inflammatory process during the acute phase response, i.e., the acute phase proteins expression and functions that regulate homeostasis. We have organized this book in two volumes - the first volume, mainly containing chapters on structure, biology and functions of APP, the second volume discussing different uses of APP as diagnostic tools in human and veterinary medicine.

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