Heme Oxygenase-1 and Iron Metabolism Crosstalk in Macrophages

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Heme oxygenase-1 (HO-1) is an enzyme that catalyzes the degradation of heme, releasing equimolar amounts of carbon monoxide (CO), biliverdin (BV), and iron. The anti-inflammatory and antioxidant properties of HO-1 activity are conferred in part by the release of CO and BV and are extensively characterized. However, iron constitutes an important product of HO-1 activity involved in the regulation of several cellular biological processes. The macrophage-mediated recycling of heme molecules, in particular those contained in hemoglobin, constitutes the major mechanism through which living organisms acquire iron. This process is finely regulated by the activities of HO-1 and of the iron exporter protein ferroportin. The expression of both proteins can be induced or suppressed in response to pro- and anti-inflammatory stimuli in macrophages from different tissues, which alters the intracellular iron concentrations of these cells.

heme oxygenase-1 iron macrophages immunity inflammation

1. Introduction

Heme oxygenase-1 (HO-1) is an enzyme encoded by the Hmox1 gene and its main function is to degrade heme molecules into three sub products: carbon monoxide gas (CO), iron (Fe^{2+}), and biliverdin; the latter is converted into bilirubin by the action of biliverdin reductase [1][2]. Due to its activity in heme metabolism, HO-1 is constitutively expressed in macrophages from tissues involved in the recycling of erythrocytes and hemoglobin, such as bone marrow (BM), spleen, and liver [3]. In addition, HO-1 expression can be induced in response to a variety of stress signals in different cell populations, but specially in macrophages from different tissues of the organism [4].

Heme, the substrate of HO-1, is a tetrapyrrolic cofactor of extreme importance for living organisms due to its role as a major oxygen (O_2) transporter. Composed of a protoporphyrin IX ring complexed to an iron ion, heme participates in several functions in the body, such as cellular respiration, electron transport, modulation of reactive oxygen species, as well as regulation of transcription and gene translation [5][6]. Heme is synthesized in the mitochondria and cytosol of developing erythroid progenitor cells and is further conjugated to hemoglobin molecules, which are abundantly present in mature erythrocytes [7]. However, this molecule is also commonly found in macrophages that perform the physiological process of recycling senescent red blood cells [8].

The intracellular accumulation of heme is harmful to the organism and triggers cellular and tissue damage due to its highly pro-oxidant nature. Genotoxicity, induction of paraptosis in endothelial cells, and consequent dysfunction in the angiogenesis process are examples of detrimental effects caused by heme accumulation in different cells

and tissues [9][10]. HO-1 is widely known for its antioxidant properties and, in this sense, the catalysis of heme degradation by the enzyme activity alone can be considered as an important antioxidant function of HO-1 [11][12][13][14]. In humans, HO-1 deficiency induces high sensitivity to oxidative stress, intravascular hemolysis, perturbations of iron homeostasis, kidney, liver, and endothelial inflammation [15][16][17].

Particularly in macrophages, HO-1 and its products play important roles in the regulation of inflammatory and immune responses. In fact, HO-1 along with the products CO and biliverdin/bilirubin are classically associated with the promotion of antioxidant, anti-inflammatory, and immunosuppressive activities in macrophages [4].

2. Heme Acquisition by Macrophages

2.1. Erythrophagocytosis

Erythrocytes (red blood cells—RBCs) promote the transport of O_2 for cellular respiration. The average life span of these cells is approximately 120 days, after which they undergo structural changes and enter senescence [18]. Senescent RBCs express molecules in their membranes known as “eat me” signals, which will be recognized by receptors expressed by macrophages, in particular those from the splenic red pulp and liver (Kupffer cells—KCs) [19][20]. Some of these signals include: (a) The formation of Band 3 (RBC surface protein) clusters in senescent RBC membrane, which are bound by naturally occurring antibodies (Nabs) and activate complement, being further recognized by Fc or C3 receptors in macrophages [20][21][22]; (b) exposure of phosphatidylserine (PS) in the extracellular portion of the membrane, which can be directly bound by PS receptors in macrophages, such as Tim-1, Tim,4, CD300, and Stabilin-2, or can bind to GAS-6 or PROS1 that will be further recognized by TAM receptors in macrophages [20][23][24][25][26][27][28]; (c) expression of CD47 on the RBC surface and its interaction with thrombospondin-1 (TSP-1), which bind to the signal-regulatory receptor protein alpha (SIRPα) present in macrophages [29][30]. All of those signals trigger the phagocytic machinery of macrophages that result in phagocytosis of senescent RBC.

2.2. Haptoglobin and Hemopexin

Haptoglobin (Hp) and hemopexin (Hx) are plasma glycoproteins produced mainly by hepatic cells, which retain high binding affinity with free hemoglobin and heme, respectively [31][32][33]. Hp (alpha—2 glycoprotein) is composed of an alpha and a beta chain with approximately 328–388 amino acids each [34][35], while Hx has 439 amino acid residues divided into two homologous domains, N-domain and C-domain, in which both have four-bladed β-propeller fold helix [32][33].

Free hemoglobin in plasma can undergo structural changes caused by H_2O_2 that interfere with its internalization by cells, which causes its accumulation in the blood. The formation of the hemoglobin–Hp complex prevents H_2O_2 from modifying amino acids in the beta-globulin chain, thus limiting cross-link reactions that would occur with the alpha-globulin chain [36]. The Hb–Hp complex is recognized by the CD163 transporter in the surface of monocytes

and macrophages and is further endocytosed by these cells. Following CD163-mediated internalization, hemoglobin–Hp complexes will be degraded in the lysosome, resulting in the release of heme molecules [34][37].

Hx stably binds free heme at a pH greater than 5.0 and undergoes a conformational change that prevents additional binding of peptides to its structure, consequently protecting the complex from proteolysis [32][33]. The Hx–heme complexes are recognized by the low-density lipoprotein receptor-related protein (LRP)/CD91, which are present on the surface of macrophages but also in several other cell types, such as fibroblasts, hepatocytes, neurons, adipocytes, syncytiotrophoblasts, and columnar epithelial cells of the gastrointestinal tract [38]. Following LRP/CD91-mediated internalization of Hx–heme complexes by endocytosis, Hx is degraded by lysosomal enzymes releasing the heme molecules and the LRP/CD91 receptor is recycled to the cell surface [39].

2.3. Autophagy of Hemoproteins and Mitophagy

Hemoproteins/heme proteins comprise a group of more than 2300 proteins that have one or more heme groups in their structure [40][41]. They can be classified into different types and contain heme complexed to the amino acids in different forms as well, such as heme a, heme b, heme c, heme or heme o. Hemoproteins perform different functions within the cells, which range from transport, storage, and activation of O_2 molecules; electron transfer; and substrate for oxidation reactions [42][43]. Therefore, hemoproteins are normally found in cell cytoplasm and mitochondria.

In addition to the various forms of heme acquisition, the cell is also able to obtain heme via its synthesis in the inner membrane of the mitochondrial matrix. Once its production occurs, heme is routed to be incorporated among the hemoproteins present in the mitochondria and cellular cytoplasm [44]. Thus, it is implied that the process of autophagy or mitophagy caused by cellular or mitochondrial damage, inflammatory stimuli, or regular processes of organelle recycling by the cells [45], can also cause the release of free heme within the cell.

3. Heme Degradation by HO-1 and Iron Release

After heme is released from hemoglobin, Hx–heme complexes or from other hemoproteins in phagolysosomes or autophagolysosomes, and is transferred to the cytosol by the heme transporters heme-carrier protein 1 (HCP1) and heme responsive gene 1 protein (HRG1). Following their transport to the cytoplasm, heme molecules are then metabolized by HO-1, which is anchored to the membranes of the endoplasmic reticulum [8][19][46][47][48]. As mentioned previously, HO-1 is constitutively expressed in macrophages involved in the recycling of RBCs in the liver and the spleen [8]. However, the enzyme expression can also be induced in different cells in the organism in response to several cellular stressors, such as ultraviolet radiation, endotoxins, heavy metals, physical stress, heme-containing enzymes, and ROS [49]. The major signaling pathway responsible for the induction of HO-1 expression involves the action of the nuclear transcription factor erythroid 2p45-related factor 2 (Nrf2) [4]. Nrf2 is normally found in the cytosol in its inactive form, bound to the protein Kelch-like ECH-associated protein 1 (Keap1), which promotes the ubiquitination of Nrf2 and consequent proteasomal degradation of the transcription factor. However, under oxidative stress, Keap1 undergoes oxidation of its cysteine residues and releases Nrf2, which

migrates to the nucleus and binds, in conjunction with small Maf proteins, to stress-responsive DNA sequence elements (StREs) containing Maf recognition element sequences (MARE). StREs/MARE are present upstream of the HO-1 gene, and therefore, binding of Nrf2 to these regions, results in induction of enzyme expression [49][50][51][52][53]. In homeostatic conditions, the MARE sequences in HO-1 gene promoter are found complexed to the transcription repressor Bach1, which prevents the induction of HO-1 expression. However, concurrently to Nrf2 activation, the accumulation of free heme favors the binding of these molecules to Bach1, which results in the release of this repressor from the MARE regions, therefore promoting expression of HO-1 [54][55][56].

Iron, the third product of heme degradation by HO-1, is an essential ion for the organism. Free $Fe2^{+}$ in the cytoplasm, also known as labile iron, is involved in several vital processes in the cell, such as cellular respiration, oxygen sensing and metabolism, cell signaling, energy metabolism, as well as DNA synthesis and repair. However, free Fe^{2+} is highly reactive and can promote the production of ROS by the Fenton reaction, which can consequently cause oxidative damage to cellular components [8][57]. Because of that, the cell prevents the cytotoxic effects of iron by promoting the conversion of Fe^{2+} ions to the ferric Fe^{3+} form, which is further stored intracellularly, or by exporting the Fe^{2+} to the extracellular environment. Iron storage occurs through a multimeric protein complex called ferritin (FT), which is composed of heavy (H—ferritin heavy/heart chain—FTH) and light (L—ferritin light/liver chain—FTL) chains [58][59]. FTH is responsible for catalyzing Fe^{2+} into Fe^{3+} by ferroxidase, forming ferrihydrite aggregates, which are inert and incapable of generating free radicals. FTH chains provide stability to the ferritin structure but also assist in the formation of inorganic ferrihydrite aggregates. It is estimated that one ferritin molecule can store as much as 4500 iron atoms [59]. Alternatively, if Fe^{2+} is not used by the cell or stored in ferritin molecules, this ion is directed to be exported out of the cell through the transmembrane transporter ferroportin (FPN1), encoded by the gene SLC40A1 (Solute Carrier Family 40 Member 1) [60][61].

Macrophages can also acquire iron through other ways besides heme metabolization by HO-1. In the serum, iron is oxidized by ceruplasmin and majorly converted to the Fe^{3+} form, which will then be complexed to transferrin (each transferrin molecule can accommodate two iron ions) [62]. Iron-loaded transferrin is recognized by the transferrin receptor (TFR) on the surface of macrophages and endocytosed. Inside the endosomal compartment, Fe^{3+} is reduced to ferrous iron (Fe^{2+}) by the six-transmembrane epithelial antigen of prostate (STEAP3) enzyme and further transported into the cytosol through the divalent metal transporter 1 (DMT1), which is a transmembrane glycoprotein that can only transport iron in its ferrous (Fe^{2+}) form. Following this, TFR is subsequently recycled back to the cell surface [63][64][65]. DMT1 is also commonly found in the plasma membrane, where it promotes the internalization of extracellular free iron ions [66]. In the cell surface, ferric iron is reduced to its ferrous form by cytochrome B DCYTB and subsequently internalized though DMT1 [58]. Macrophages can further mobilize iron by nuclear receptor coactivator 4 (NCOA4)-induced autophagy of iron loaded ferritin molecules (ferritinophagy). Ferritin is then degraded and iron ions are transported to the cytosol through the same mechanisms described earlier [64].

HO-1 and FPN1 expression in macrophages play a pivotal role in the systemic iron homeostasis. The genetic deletion of HO-1 profoundly affects iron levels in the body, causing anemia and iron accumulation inside cells in several tissues [67]. The deficiency of ferroportin gene in macrophages was also shown to result in the development

of anemia and iron accumulation in the spleen, liver, and BM [68]. Ferroportin expression can be regulated transcriptionally or through a post-translational mechanism by the action of hepcidin, a peptide hormone secreted by liver cells in response to increases in serum iron concentration or inflammation [69]. Hepcidin binds to ferroportin in the surface of cells and induces its internalization and further degradation. As a consequence, the export of iron ions to the extracellular environment ceases and the metal accumulates inside the cells. Accordingly, overproduction of hepcidin leads to tissue iron overload and hypoferremia [70].

4. Cross Regulation of Iron Homeostasis, Inflammation, and Immunity

Serum iron levels can regulate the production of hepcidin and consequently, the expression of ferroportin in cell membranes. High serum iron levels induce the production of hepcidin, which promotes the degradation of ferroportin and ceases further export of iron to serum, while in situations of low iron levels, hepcidin expression is suppressed, favoring ferroportin expression and promoting iron export to the circulation [69]. However, hepcidin production can also be induced in macrophages in response to inflammatory stimuli, the most studied of which is IL-6 biding to its receptor and subsequent activation of signal transducer and activator of transcription 3 (STAT3) signaling pathway [19].

Hepcidin itself has antibacterial properties, however, its main role in the immune response to infectious diseases has been associated to the induction of nutritional immunity or “hypoferremia of inflammation” [71][72]. The production of hepcidin by macrophages and other cells in response to infection-derived stimuli is intended to decrease ferroportin expression and consequently limit iron bioavailability to pathogens [19]. Armitage et al. demonstrated that pathogen-derived Toll-like receptor 5 agonists stimulate hepcidin production by leukocytes and hepatoma cells in an IL-6-dependent manner, while IL-22, an important cytokine produced in response to extracellular infections, also induces phosphorylation of STAT3 and upregulation of hepcidin production. The scholars additionally found that following in vivo infection with *C. albicans* or Influenza A/PR/8/34 virus (H1N1), hepcidin expression is upregulated causing a decrease in serum iron levels in mice [73]. Intraperitoneal challenge with *Pseudomonas aeruginosa* was also shown to induce TLR4-dependent hepcidin expression and consequent iron deposition in splenic macrophages [74].

Some of the pro-inflammatory signals that trigger hepcidin production and/or ferroportin downregulation, also induce the expression of HO-1. Such scenario promotes increased iron release by HO-1-mediated degradation of heme molecules along with reduced iron export by ferroportin, favoring intracellular iron accumulation. Although these mechanisms can restrict nutrient iron for extracellular pathogens, they may have the opposite effect in infections with intracellular microorganisms [4]. In addition, the pro-inflammatory signals that modulate the expression of HO-1, hepcidin, and ferroportin are also produced in several sterile inflammatory conditions, such as autoimmune diseases, ischemia-reperfusion injuries and tumors, and, therefore, in all of those conditions, intracellular iron accumulation can also occur.

As discussed in the following sections, the fluctuations in intracellular iron levels in response to the mechanisms discussed above can regulate several intracellular signaling pathways that play important roles in the modulation of inflammatory and immune responses. Therefore, the crosstalk between iron homeostasis and inflammatory/immune responses holds promise as an important target for new immunomodulatory therapies.

4.1. Iron Regulation of HIF1α Expression

Hypoxia inducible factors (HIFs) are alpha/beta heterodimeric transcription factors that play critical roles in the adaptive transcriptional responses to O_2 deprivation (hypoxia). Under normoxia, the prolyl hydroxylase (PHDs) and asparaginyl hydroxylase (factor inhibiting HIF—FIH) enzymes use O_2 as a cofactor in order to catalyze a hydroxylation reaction in the HIF-α chains that will culminate in their ubiquitination and degradation by the proteasome [75][76]. These enzymes can also use Fe^{2+} ions as cofactors, and therefore, under hypoxia or iron depletion, the hydroxylation of HIF-α subunits is inhibited and the expression of the transcription factors as a whole is stabilized, culminating in the increased expression of genes induced by them [75][77].

The expression of HIF-1α, which is widely characterized to play major roles in glycolytic metabolism, apoptosis, angiogenesis, cellular stress, and inflammation, among other biological processes [75], is partcularly highly susceptible to changes in intracellular iron concentration. The chelation of iron by bacterial siderophores, for example, was demonstrated to be able to induce HIF-1α stabilization and expression independently of hypoxia, while increases in iron levels induce its degradation by PHDs [78].

4.2. Iron Regulation of IRPs/IRE Interactions

The iron-regulatory proteins (IRPs) 1 and 2 (IRP1 and IRP2) are mRNA-binding proteins that recognize and interact with non-coding sequences, known as iron responsive elements (IREs) present at the 3′ or 5′ untranslated region (UTR) of mRNA transcripts of particular genes, forming conserved RNA stem loop structures. The binding of IRPs to IREs located at 3′ regions protects the mRNA molecule from degradation and promotes its translation, while binding of IRPs to IREs at the 5′ regions blocks the translation of mRNA molecules into proteins [79][80]. Much of the cellular iron uptake, transport, storage, utilization, and release processes are controlled by the IRP/IRE system [19]. When intracellular iron is abundant, it binds to IRP1 and alters its conformation, making it incapable of binding to IREs, while high iron concentrations promote the degradation of IRP2. In situations of low iron tension, IRP1 is not bound to iron and assumes a conformation with high affinity for the IREs, while expression of IRP2 is stabilized. Therefore, in cases of intracellular iron accumulation, mRNAs from genes that have IREs at 3′ of UTRs will be degraded, while those that have IREs at 5′ of UTRs will be translated, due to the absence of IRP binding to the IREs. When iron tension is low, IRPs are able to bind to IREs, and the opposite effect is observed [81]. The mRNAs of the iron importer proteins transferrin receptor 1 (TFR1) and DMT1 (SLC11A2) have IREs at 3′ of UTR, and therefore, their translation is increased when intracellular iron concentration is low and repressed when iron levels in the cytosol are high. On the other hand, the mRNAs for ferroportin (SLC40A1), as well as the heavy (FTH) and light (FTL) chains of ferritin have IREs at 5′ of the UTR, which results in induction of translation when intracellular iron concentration is high and repression at low iron levels [82][83]. The mRNA of other genes that are

not involved or at least not exclusively involved in iron homeostasis also have IREs, and therefore, their translation can be regulated by the IRP/IRE system.

4.3. Iron Induction of ROS Generation by Fenton Reaction

Macrophages produce inflammation-related proteins, such as myeloperoxidase, NADPH oxidase, indoleamine 2,3-dioxygenase, nitric oxide synthases, or lipoxygenases, all of which contain iron [84]. Moreover, iron induces the generation of ROS by the Fenton reaction and is also involved in the production of such radicals by the phagocyte oxidase. NOX2, a NOX family member that is part of the phagocyte oxidase system, is a transmembrane hemoprotein that uses heme iron to transport electrons across membranes to catalyze the generation of superoxide ($\bullet O_2$), via the following reaction: $O_2 + \bullet O_2$. When NOX2 accumulates in macrophages, $\bullet O_2$ can give rise to other ROS, such as hydrogen peroxide (H_2O_2), which reacts with iron to generate hydroxide ions (OH^-) and hydroxyl radicals (•OH), leading to the production of hydrogen peroxide radicals (HOO•). This latter step occurs via two iron-catalyzed reactions, first $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + \bullet OH$ and second ($Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HOO\bullet + H^+$). These ROS play a critical role in the destruction of pathogens in phagolysosomes but also support other macrophage functions, such as disassembling of dying cells internalized by phagocytosis [8][85]. Accordingly, it was demonstrated that Fe chelation dramatically exacerbates murine infection with *S. typhimurium* via inhibition of the host phagocyte oxidase-dependent respiratory burst and the production of nitrogen radical catalyzed by the inducible nitric oxide synthase [86].

4.4. Iron and Polarization of M1 and M2 Macrophages

M1 macrophage polarization is induced in response to Th1 cytokines, such as TNF and IFN-γ, or by bacterial LPS recognition. These macrophages produce majorly pro-inflammatory cytokines, such as TNF, IL-1α, IL-1β, IL-6, IL-12, IL-23 and low levels of IL-10. M1 macrophages display potent anti-microbial activity mainly through the generation of NADPH-oxidase-dependent ROS and iNOS-induced NO production [87].

M2 macrophages display an anti-inflammatory profile and are polarized in response to Th2 and suppressor cytokines, such as IL-4, IL-13, and IL-10. IL-33 is another cytokine involved in M2 polarization, through the amplification of IL-13-induced effects. M2 macrophages are characterized by the high expression of arginase-1 (Arg-1) and production of both IL-10 and TGF-β, while the production of pro-inflammatory cytokines is very low or absent. M2 macrophages play an important role in the scavenging of cellular debris and apoptotic cells, as well in the promotion of tissue repair and wound healing, besides displaying pro-angiogenic and pro-fibrotic properties [87].

Iron overload, induced by treatment with ferric citrate, has been shown to induce M1 polarization in RAW 264.7 macrophages, which was associated with the induction of ROS production in iron-treated cells [88]. In an experimental model of chronic venous ulcers, the induction of iron overload in macrophages was shown to potently induce M1 polarization, which was characterized by elevated TNF production and to promote poor wound healing properties in these cells. In addition, the pro-inflammatory activity of these macrophages promoted DNA damage and senescence of skin-resident fibroblasts [89].

On the contrary, other studies found that the expression of Arg1 and IL-10 along with a series of genes associated with M2 polarized macrophages, such as Ym1, IL-10, and Stat6, were all upregulated in mice fed with a diet containing high levels of iron, while mice that were fed a diet poor in iron displayed increased production of pro-inflammatory cytokines and expression of M1 macrophage markers [90]. Along these same lines, studies employing experimental in vitro and in vivo models of *Salmonella* infection have demonstrated that a major mechanism used by macrophages to restrict infection is to enhance the expression of ferroportin, therefore decreasing the intracellular iron pool, which also enhances the levels of iNOS expression and NO production in response to the reduction of intracellular iron concentration [91][92][93].

These studies demonstrate that it is hard to draw a definite conclusion on whether different iron levels can be is associated with M1 or M2 macrophage phenotypes. Regardless, they clearly demonstrate that the levels of cytosolic iron can play important roles in the regulation of pro- and anti-inflammatory programs of macrophages.

5. Conclusions

HO-1 plays a key role in maintaining cellular homeostasis, particularly through its anti-inflammatory and antioxidant properties, which were proven to display several cytoprotective functions throughout the organism [1][94]. However, HO-1 activity also results in the release of pro-oxidant ferrous iron (Fe^{2+}). In fact, the recycling of iron from heme molecules in macrophages by the action of HO-1 is the major mechanism used by the organism to acquire the metal. The coordinated action of HO-1 and the hepcidin/ferroportin axis controls the release of iron to the serum and/or its retention inside cells to efficiently maintain optimal systemic iron levels [69].

Alterations in iron homeostasis can have profound impacts on the regulation of inflammation and immune responses. In particular, the changes in macrophage intracellular iron levels resulting from modulation of HO-1 expression and activity as well as from the transcriptionally or hepcidin production-induced regulation of ferroportin expression, can impact the activation of microbicidal effector functions as well as cytokine production by these cells. Given the important role played by macrophages in the pathogenesis of several autoimmune and auto-inflammatory disorders, as well as in the host response to infectious diseases with different pathogens [84][95], the iron metabolism of macrophages represents a potential target for novel immunomodulatory therapeutic strategies in these areas. In recent years, several advances have been achieved in the identification of novel inhibitors and inducers of HO-1 activity as well as in modulators of the hepcidin/ferroportin axis [96][97][98].

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