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FORUM REVIEW ARTICLE

Coupling Heme and Iron Metabolism *via* Ferritin H Chain

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Abstract

Significance: Inflammation and immunity can be associated with varying degrees of heme release from hemoproteins, eventually leading to cellular and tissue iron (Fe) overload, oxidative stress, and tissue damage. Presumably, these deleterious effects contribute to the pathogenesis of systemic infections. ***Recent Advances*:** Heme release from hemoglobin sensitizes parenchyma cells to undergo programmed cell death in response to proinflammatory cytokines, such as tumor necrosis factor. This cytotoxic effect is driven by a mechanism involving intracellular accumulation of free radicals, which sustain the activation of the c-Jun N-terminal kinase (JNK) signaling transduction pathway. While heme catabolism by heme oxygenase-1 (HO-1) prevents programmed cell death, this cytoprotective effect requires the co-expression of ferritin H (heart/heavy) chain (FTH), which controls the pro-oxidant effect of labile Fe released from the protoporphyrin IX ring of heme. This antioxidant effect of FTH restrains JNK activation, whereas JNK activation inhibits FTH expression, a cross talk that controls metabolic adaptation to cellular Fe overload associated with systemic infections. ***Critical Issues and Future Directions:*** Identification and characterization of the mechanisms *via* which FTH provides metabolic adaptation to tissue Fe overload should provide valuable information to our current understanding of the pathogenesis of systemic infections as well as other immune-mediated inflammatory diseases. *Antioxid. Redox Signal.* 20, 1754–1769.

Introduction

IRON (FE) IS THE METAL most commonly used in biologic reduction–oxidation (redox) chemistry (42). Presumably, due to its intrinsic capacity to exchange electrons with a variety of donors/acceptors, Fe became, through evolution, a central component of "core biologic functions," such as the generation of metabolic energy or sensing and transport of gaseous molecules. However, this same property makes the participation of Fe in the production of free radicals *via* the Fenton chemistry potentially cytotoxic (50). One of the mechanisms limiting this cytotoxic effect relies on a highly evolutionary conserved multistep enzymatic reaction that incorporates Fe into a protoporphyrin IX carrier ring, giving rise to heme (4, 74). In this manner, the pro-oxidant and cytotoxic effect of Fe can be controlled within the protoporphyrin ring of heme by its incorporation into the "heme pockets" of hemoproteins (4). These play an essential role in a variety of vital cellular functions, based on the capacity of Fe-heme to exchange electrons (4). The heme pockets of hemoproteins are usually composed of aromatic amino acids, including phenylalanine (F), tryptophan (W), or tyrosine (Y) with few or no charged amino acids (108). This confers a relative hydrophobic environment, required for stable heme binding. Five conserved amino acids, that is, histidine (H), methionine (M), cysteine (C), tyrosine (Y), and lysine (K), can act as axial heme ligands (108) with hydrophobic amino acids, such as leucine, (L), isoleucine (I), and valine (V), creating further interactions with the porphyrin structure, whereas positively charged amino acids such as arginine (R) interact with the negatively charged propionate groups (101, 146). The evolutionary success of this strategy is probably best illustrated by the fact that most of the biologic available Fe in mammals exists in the form of heme instead of labile Fe (4). However, this strategy still poses some major biologic challenges in that the eventual release of heme from hemoproteins can be cytotoxic (16, 57, 66, 67, 102, 123, 148). The evolutionary conserved solution that emerged to overcome this problem was to couple heme release to its catabolism, such as afforded by heme oxygenases (HO) (165). These are evolutionary conserved and ubiquitously expressed enzymes that catalyze the degradation of the protoporphyrin ring of heme,

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producing equimolar amounts of labile Fe, biliverdin, and the gasotransmitter carbon monoxide (CO) (165). However, the salutary effect conferred by this strategy requires yet another regulatory mechanism that controls the redox activity of the labile Fe extracted from the protoporphyrin IX ring of heme. This is accomplished by coupling heme catabolism by HO to Fe extracellular export (17, 54) or to Fe incorporation into ferritin complexes (14, 72). As discussed in the following sections, coupling heme catabolism to Fe storage by ferritin plays an essential role in providing Fe metabolic adaptation in the context of inflammation and immunity, restraining the pathological outcome of systemic infections and possibly other immune-mediated inflammatory diseases.

Fe and Heme Homeostasis

Fe homeostasis is regulated by a series of integrated mechanisms acting at cellular and systemic levels (78). Disruption of Fe homeostasis is often associated with defective erythropoiesis and/or anemia as well as with tissue Fe overload, tissue damage, and disease. As such, mechanisms that control Fe homeostasis are critical to prevent these pathological outcomes (57, 66).

Cellular Fe homeostasis is controlled by several evolutionary conserved genes that act in a concerted manner to regulate intracellular Fe uptake, trafficking, and export (6, 78) (Fig. 1). Mammalian cells acquire Fe bound to transferrin (TF), a plasma homodimeric beta-globulin that binds two ferric Fe molecules with exceedingly high affinity ($Kd = 10^{-20}$ *M*) (3). Soluble TF-Fe complexes are recognized by transferrin receptor 1 (TFR1) and 2 (TFR2) (81), two transmembrane disulfide-linked glycoproteins encoded by distinct genes sharing 45% homology (90) (Fig. 1). The affinity of TFR2 for diferric TF is 30 times lower to that of TFR1 (90), suggesting that the contribution of TFR2 to intracellular Fe uptake is not as critical compared to TFR1. Cubilin is another TF-Fe receptor expressed in more restricted cellular subsets, including small intestine epithelial cells, renal proximal tubule cells, visceral yolk sac cells, and placenta cytotrophoblasts (Fig. 1) (38).

Fe is extracted from TFR1/TF-Fe or TFR2/TF-Fe complexes through acidification of the endolysosomal compartment, leading to conformational changes in the tertiary structure of TFR1/2. This decreases the affinity for TF-Fe and allows for Fe release from TF (81). Endosomal Fe^{3+} is reduced into Fe^{2+} by metalloreductases, including the six-transmembrane epithelial antigen of the prostate family member 3 (STEAP-3) (121) and transported into the cytoplasm by the Fe^{2+} regulated divalent metal transporter 1 (DMT1) (68). Cytoplasmic Fe^{2+} can be shuttled to the mitochondria, either bound to chaperone proteins or *via* interorganelle interactions (139), being internalized by the mitochondria *via* mitoferrins, of which mitoferrin 2 is ubiquitously expressed (124). In the mitochondria, Fe is used essentially for heme biosynthesis and Fe-sulfur cluster assembly (139) (Fig. 1). Alternatively, Fe can be stored in the mitochondria, by mitochondrial ferritin (105).

When intracellular labile Fe increases above a certain threshold level, its pro-oxidant activity must be controlled to avoid cytotoxicity. This is achieved, in large measure, *via* a mechanism regulated by iron regulatory proteins (IRPs) 1 and 2 (IRP1/2) (79). Both IRPs act as Fe sensors and bind to short conserved *cis*-regulatory stem-loop iron responsive elements

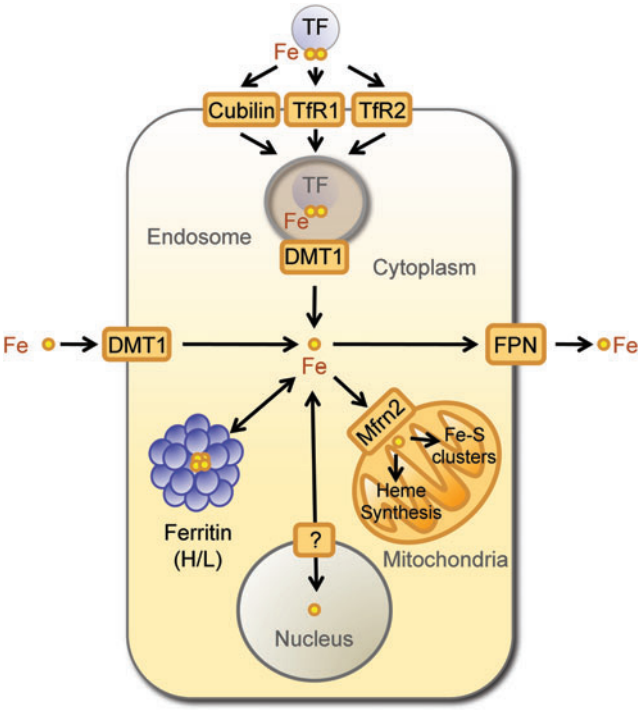

FIG. 1. Cellular Fe homeostasis. Fe metabolism is maintained at a cellular level by a series of evolutionary conserved mechanisms regulating intracellular Fe uptake, trafficking, and export. The scheme provides an overlook of these mechanisms without taking into account species and/or cell specificities. Extracellular Fe (*yellow circles*) exists in plasma mainly bound to transferrin (TF). Fe uptake occurs *via* a mechanism that involves the recognition of Fe-TF complexes by transferrin receptor 1 (TFR1), transferrin receptor 2 (TFR2), or Cubilin. Upon internalization of Fe-TF complexes by endocytosis, the acidification of the endolysosomal compartment allows for Fe release. Labile Fe is reduced and transported into the cytoplasm by divalent metal transporter 1 (DMT1). In the specific context of Fe absorption by enterocytes, DMT1 is used to absorb Fe from the intestinal lumen. Intracellular labile Fe is directed mainly to the mitochondria, being imported by Fe transporters, which include the mitochondrial Fe importer mitoferrin-2 (Mfrn2). In the mitochondria, Fe is used for heme biosynthesis and Fe-sulfur cluster assembly or stored by mitochondrial ferritin (not depicted in the scheme). When intracellular labile Fe accumulates above a certain threshold level, Fe can be stored and neutralized intracellularly by multimeric ferritin complexes composed of FTH and FTL chains or exported by the Fe transporters ferroportin (FPN). Fe, iron; FTH, ferritin H (heart/heavy) chain; FTL, ferritin liver/light chain. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

(IRE) in the untranslated mRNA regions (UTR) of genes, where expression is regulated by IRPs (12, 76, 77). Binding of IRPs to IRE located in the 3′UTR increases mRNA stability and promotes translation (71), whereas translation is repressed upon binding of IRPs to IRE located in the 5′UTR (44, 72, 181).

Ferritin H (heart/heavy) chain (FTH) is a prototypical gene regulated by IRPs, encoding a 21 kDa protein that catalyzes

the conversion of Fe^{2+} into ferric Fe^{3+} and allowing for intracellular storage of inert Fe^{3+} (72) (Fig. 1). The *FTH* gene contains one 5′UTR IRE, recognized under low cellular Fe content by IRP1/2, repressing *FTH* translation (71). Although *TFR1* is also a gene regulated by IRPs, it contains several IRE in its 3′UTR that are recognized under low Fe by IRP1/2, promoting its translation (71). This is not the case for *TFR2*, where expression is not regulated by Fe (56), consistent with the lack of IRE in *TFR2* mRNA (56). Another gene regulated by IRPs is ferroportin (*FPN*), which encodes a 62 kDa transmembrane Fe transporter that exports Fe^{2+} from cells (45) (Fig. 1). The *FPN* gene has a 5′UTR IRE regulated by IRPs that represses its translation under low cellular Fe (79). The regulation of FTH, TFR1, and FPN by Fe, sensed by IRPs, allows for rapid adaptation to changes in intracellular Fe content. Regulated expression of *FTH* appears to be of particular importance for cellular adaptation to Fe overload in the context of inflammation and immunity, as discussed in the next sections (66, 141, 167, 168, 182).

Ferritins are multimeric complexes (~450 kDa) made of FTH and FTL (liver/light) chains (72). These form heteropolymeric nanocage-like structures comprising of 24 subunits, which assemble as a hollow shell providing a central 80-Å diameter storage cavity that can incorporate up to 4500 Fe atoms (72), in the form of inorganic ferrihydrite aggregates (94). The proportion of the two FTH and FTL subunits composing ferritin multimeric complexes is regulated in a tissue-specific manner with FTL-rich nanocages being produced mainly by hepatocytes and FTH-rich nanocages by the brain and muscle cells (72). Proportion of FTH and FTL subunits is also regulated in response to inflammation, with proinflammatory cytokines inducing FTH expression and hence enriching its content in ferritin nanocages (141, 168, 182). This creates several isoferritins with different Fe storage capacity as well as other physiological properties (72).

FTH has ferroxidase activity (28, 80), which catalyzes the reaction: $4Fe^{2+} + 4\ H^+ + O_2 = 4Fe^{3+} + 2H_2O$, converting reactive Fe^{2+} into Fe^{3+} and forming inert ferrihydrite aggregates that do not partake in the production of free radicals *via* the Fenton chemistry (9, 50). The ferroxidase activity of FTH is provided by a core of evolutionary conserved amino acids, that is, Glu-27, Glu-62, and His-65 (80, 103). These are absent from FTL, which does not have ferroxidase activity (8). FTL, however, is essential to promote Fe nucleation by ferritin nanocages, a process leading to the formation of inorganic ferrihydrite aggregates (107). FTL also confers stability to multimeric ferritin, protecting the nanocage from elevated temperatures and denaturants (145). While FTL can form homopolymers (106), this is not the case for FTH, which promote aggregation and degradation of ferritin nanocage (65).

As mentioned above, FTH and FTL are prototypical Fe-dependent genes, which expression is regulated essentially at post-transcriptional level by the dissociation of IRP1/2 from mRNA 3′UTR IREs (71). In response to high cellular Fe content, IRP1/2 are degraded *via* the 26S proteasome pathway (71, 86) promoting mRNA stability and hence *FTH* and *FTL* translation. Physiological modulators of FTH translation, *via* the IRP/IRE system, include nitric oxide (NO) as well as reactive oxygen species and hypoxia (77). Expression of FTH is also regulated at the transcriptional level *via* the activation of the transcription factors nuclear factor kappa B (NF-κB) (100, 127) and NF-E2-related factor 2 (NRF2) (129). More recently, FTH expression was show to be post-transcriptionally regulated by miR-200b, which reduces its expression (151).

Presumably, when the levels of cellular Fe are reduced, redistribution of the Fe sequestered inside the ferritin nanocages occurs through a mechanism regulated at least partially by FTH and FTL proteolytic degradation (10). The mechanisms involved in Fe release from ferritins are not well defined, but are thought to involve cysteine and/or serine proteases acting in acidic or autophagic lysosomes (11, 99, 166). Additional mechanisms involved in ferritin disassembly and Fe release from ferritin include FTH ubiquitin-dependent 26S proteasomal degradation (117, 143), a notion in keeping with our own observation that pharmacological inhibition of the 26s proteasome increases FTH expression in cells exposed *in vitro* to heme plus tumor necrosis factor (TNF) (Raffaella Gozzelino, unpublished).

More than 80% of bioavailable Fe in mammals is contained within the hydrophobic methane-bridged tetrapyrrole ring of heme, where it is used within the prosthetic groups of hemoproteins (Fig. 2). The most abundant hemoproteins in mammals are hemoglobin (Hb) and myoglobin, expressed in red blood cells (RBCs) and muscle cells, respectively (67) (Fig. 2). Another significant pool of hemoproteins is formed by ubiquitously expressed cytochromes (67) (Fig. 2).

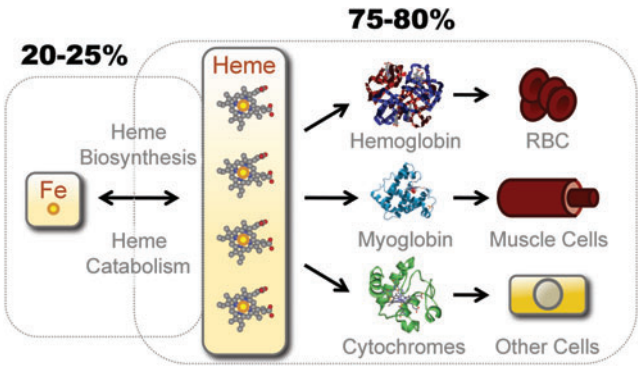

FIG. 2. Bioavailable Fe. Only an estimated 20%–25% of the bioavailable Fe in mammals exists in the form of nonheme (labile) Fe, either bound to TF, stored by ferritin, as part of a Fe-Sulfur cluster or transiently bound to Fe chaperones and transporters. The remaining 75%–80% of the bioavailable Fe is contained inside the protoporphyn IX ring of heme as prosthetic groups of hemoproteins. The major hemoproteins compartments in mammals are hemoglobin (Hb) in red blood cells (RBCs), myoglobin in muscle cells, and cytochromes in virtual all cell types. Hb contains an estimated 70% of the total pool of heme, myoglobin 5%–10%, and cytochromes 25%–20%, respectively. Fe can transit from the "nonheme" to the "heme" compartment through *de novo* heme synthesis, a process driven by a sequence of eight enzymatic steps, the last being catalyzed by ferrochelatase, which inserts Fe inside the proptoporphyrin IX ring giving rise to heme. Fe can also transit from the heme to the nonheme pool *via* the catabolism of heme by HO enzymes or *via* heme oxidation, which releases Fe from the proptoporphyrin IX ring of heme. HO, heme oxygenases. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Under conditions of oxidative stress, noncovalently bound heme can be released from hemoproteins, as demonstrated for Hb (32, 123). The redox activity of nonhemoprotein-bound heme, referred hereby as "free heme," is no longer controlled by the heme pockets of hemoproteins, and thus, free heme becomes pro-oxidant and cytotoxic (66, 67, 102, 148). This deleterious effect is restrained by two proteins expressed at high levels in human plasma, namely haptoglobin (36–195 mg/dL) and hemopexin (40–150 mg/dL). Binding of haptoglobin to cell-free Hb ($K_d \approx 10^{-15}$) prevents heme release from Hb, with the resulting heterodimeric complex being recognized by the macrophage (Mø) transmembrane receptor CD163 (98). Hemopexin scavenges free heme ($K_d < 10^{-12}$ *M* in humans), forming a heterodimeric complex recognized by the macrophage transmembrane low-density lipoprotein (LDL) receptor-related protein 1 (LRP1/CD91) (82) (Fig. 3). Cellular Hb uptake can also occur irrespectively of haptoglobin or hemopexin *via* engulfment of senescent RBC by hemophagocytic Mø in the spleen (88).

In a similar manner to TF-Fe, cellular uptake of haptoglobin/Hb and hemopexin/heme complexes converges at the endolysosomal compartment, where heme is extracted (82, 98) (Fig. 3). Subsequent transport of heme into the cytoplasm is mediated by the heme transporter heme responsive gene-1 (HRG-1) (137), as demonstrated in hemophagocytic Mø (184) (Fig. 3). *Hrg-1* gene deletion impairs erythroid development, as demonstrated in zebrafish (137) and attenuates heme transport from the phagolysosomal compartment of mouse erythrophagocytic Mø (184), illustrating the nonredundant role of these heme transporter in heme/Fe homeostasis (184).

Presumably, most of the cytoplasmic heme transits to the mitochondria *via* a mechanism involving specific heme chaperones and mitochondrial transporters (189). Several other genes are known to control the fate of cytoplasmic heme, including HO, which catabolize heme degradation (165). There are two HO isoforms, namely HO-1 (≈32 kDa) and HO-2 (≈36 kDa), encoded by the *HMOX1* and *HMOX2* genes respectively (25, 113, 171). HO-2 is constitutively expressed in most cells (171), whereas excess intracellular heme induces the expression of the stress-responsive HO-1 isoform, which is constitutively expressed by hemophagocytic Mø as well as by natural regulatory T cells (29, 190).

Intracellular heme is exported from cells *via* the feline leukemia virus C receptor 1 (FLVCR1) (91) (Fig. 3). There are two FLVCR1 isoforms, namely FLCVR1α (≈60 kDa) and FLVCR1β (≈28 kDa), generated *via* alternative splicing of the same *FLCVR* gene (35, 91). FLCVR1α drives heme extracellular transport, whereas FLVCR1β mediates mitochondria heme export (35). At least two other ABC transporters can regulate intracellular heme trafficking, namely ABCG2 (≈72 kDa), which promotes heme extracellular transport (96) and ABCB6 (≈94 kDa), which is involved in intracellular heme import (92, 97) (Fig. 3). The heme carrier protein 1 (HCP1) is another putative heme transporter, probably involved in intracellular heme import (149).

A functional system ensuring heme transport and catabolism is essential to sustain systemic Fe homeostasis, as demonstrated unequivocally for HO-1 deficiency in mice (131, 132) and humans (188). In both cases, the lack of HO-1 expression is associated with impaired Fe recycling, anemia, vascular damage, and depletion of hemophagocytic Mø (95), presumably driven by heme cytotoxicity (59). Moreover, HO-1 deficiency is also associated with increased expression of FLVCR in the kidneys (160), suggesting that mechanisms regulating heme catabolism and transport cross-regulate each other to maintain Fe homeostasis. *Flvcr* gene deficiency is lethal in mice, due to defective erythropoiesis and severe macrocytic anemia (91). This is probably owed to the lack of

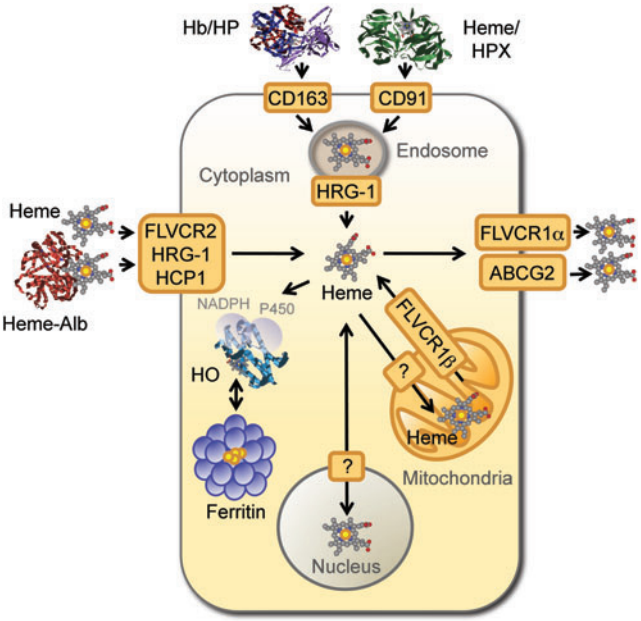

FIG. 3. Cellular heme homeostasis. When released from Hb, nonprotein bound heme, that is, free heme, is captured in plasma by hemopexin (HPX) or by albumin (Alb), forming heme-hemopexin and heme-Alb complexes, respectively. Cell-free Hb can also be captured in plasma by haptoglobin (HP), forming Hb-haptoglobin (HP) complexes, from which heme release is inhibited. Plasma Hb-haptoglobin and heme-hemopexin complexes are recognized by the macrophage transmembrane CD163 and the low-density lipoprotein receptor-related protein 1 (CD91) receptors, respectively, and internalized into endolysosomes. Possibly, extracellular heme-Alb or eventually "free heme" can also be internalized *via* the heme transporters feline leukemia virus C receptor 2 (FLVCR2), the heme responsive gene-1 (HRG-1), and the heme carrier protein 1 (HCP1), although this remains to be formally established. Intracellular heme transits from endolysosomes to the cytoplasm *via* a mechanism involving HRG-1, giving rise to intracytoplasmic heme, which can be transported into the mitochondria *via* a heme transporter that remains to be identified (?). A possible involvement of ATP-binding cassette subfamily B member 6 (ABCB6) in heme transfer into mitochondria has been proposed. Mitochondrial heme can be exported to the cytoplasm *via* the FLVCR1β isoform. Intracytoplasmic heme can be catabolized by HO, a process assisted by FTH, which neutralizes the Fe extracted from the protoporphyrin ring of heme, storing it into multimeric ferritin complexes. Alternatively, intracytoplasmic heme can be exported from cells by the ATP-binding cassette subfamily G member 2 (ABCG2) and the FLVCR1α. Whether heme is transported to the nucleus is likely to be the case, although this has not been established. For a recent and comprehensive review on heme transport, see reference (189). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

mitochondrial *FLVCR1* expression, given that specific deletion of the *Flvcr1α* gene variant is dispensable for erythropoiesis, although necessary to prevent edema and hemorrhage (35). FLVCR1 mutations in humans are associated with the development of posterior column ataxia and retinitis pigmentosa (136), whereas *FLVCR2* mutations have been associated to proliferative brain vasculopathy, such as observed in the Fowler syndrome (47). Conversely, mutations in *ABCG2* and *ABCB6* do not impair erythroid differentiation, suggesting a redundant role for these transporters in erythropoiesis (75, 144, 189).

Systemic Fe homeostasis relies on continuous Fe intake from diet (Fig. 4), that is, average of 1–2 mg of Fe per day in humans (70). Fe is absorbed in the form of Fe^{3+}, which is then reduced by cytochrome B reductase (Dcytb) expressed in gut epithelial cells, that is, enterocytes (114). This produces Fe^{2+}, which is transported into the cytoplasm by DMT1 (68) and exported from enterocytes by FPN (45) (Fig. 1). Expression of FTH in enterocytes is required to sustain this process (176) (Fig. 1). Extracellular Fe^{2+} is delivered to TF and consequently to TFR1-expressing erythroblasts (81), where it is used in heme biosynthesis and loaded into nascent Hb in the process of erythropoiesis (Fig. 4). Dietary Fe can also be extracted from heme, which involves most probably the catabolism of heme by HO, allowing for Fe extraction and transport.

Recycling of the Fe extracted from the prosthetic heme groups of Hb in senescent RBCs is essential to maintain Fe homeostasis (Fig. 4). This is achieved by hemophagocytic Mø in the red pulp of the spleen, *via* a mechanism involving the heme transporters HRG-1 (137) and presumably FLVCR2 (189). These deliver heme from the phagocytic endosomes or plasma membranes, respectively, into the cytoplasm allowing its catabolism by HO-1, which extracts the Fe that is then exported by FPN (45). The Fe captured by TF in plasma is incorporated into TFR1-expressing erythroblasts, sustaining erythropoiesis (81) (Fig. 4). Expression of FTH by hemophagocytic Mø is most probably not required to this process, as suggested by the deletion of the mouse *Fth* allele specifically Mø (data not shown) (66).

Fe concentration in plasma is regulated by hepcidin (62), an acute-phase protein produced mainly by hepatocytes (46, 64). Hepcidin is also produced by innate immune cells, for example, neutrophils and Mø (126). This occurs in response to pathogen recognition by pattern recognition receptors (PRR), for example, toll-like receptor (TLR) 2 or 4, or in response to cytokines, for example interleukin-6 (IL-6), IL-22, and type I interferon or IL-1β (46, 64, 126). Hepcidin expression occurs *via* a mechanism regulated essentially at transcriptional level by the transcription factor signal transducer and activator of transcription 3 (STAT3) (46) as well as by SMAD proteins (64, 138), adjusting the requirement of Fe for erythropoiesis (122).

Hepcidin binds to and triggers FPN degradation (119), thus suppressing cellular Fe export and dietary Fe uptake, while retaining intracellular labile Fe systemically (119). When the levels of hepcidin in plasma increase above a certain threshold level, such as observed during inflammatory and immune reactions, cellular Fe export by FPN is no longer an available option to reduce cellular Fe overload (46, 61, 183).

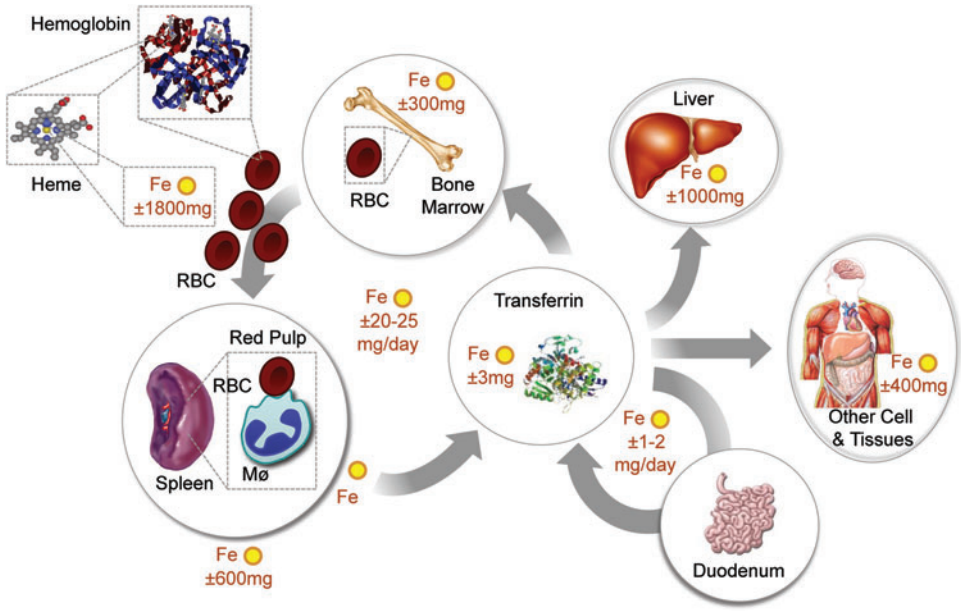

FIG. 4. Systemic Fe homeostasis in mammals. Systemic Fe homeostasis is maintained in mammals through a series of mechanisms regulating Fe absorption, its mobilization, and storage into different compartments [adapted from Hentze *et al.* (78)]. Considering that daily Fe excretion is minimal and absorption is residual compared to the amount required to sustain erythropoiesis and other vital functions, almost all the Fe used in mammals must be recycled within different compartments of the organism. The main pathways of Fe uptake and trafficking involved in the maintenance of Fe homeostasis at a systemic level are illustrated. Normal values for Fe content in different human tissues are also shown [adapted from Hentze *et al.* (78)]. Notice that 75%–80% of the bioavailable Fe exists in the form of heme as a prosthetic group of Hb. Fe recycling *via* phagocytosis of senescent RBCs by hemophagocytic macrophages (Mø) in the red pulp of the spleen insures that Fe is extracted from heme and recycled back *via* TF to provide a steady-state Fe supply for erythropoiesis in the bone marrow. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Presumably, this explains the central role played by FTH in preventing the deleterious effects of cellular Fe overload in the context of inflammation and immunity (66). The role of hepcidin in the control of Fe homeostasis will not be discussed in further detail hereby, and the reader is kindly directed to recent reviews on the subject (46, 55, 63, 64, 128).

Heme Cytotoxicity

Inflammation and immunity are associated with the production of free radicals, aimed at destroying evading pathogens and providing host resistance to infections (116). The evolutionary trade-off of this defense strategy is that free radicals can lead to oxidative stress in host tissues and eventually cause tissue damage and disease (116). Under oxidative stress, noncovalently bound heme can be released from hemoproteins, producing free heme (32, 66, 123) (Fig. 5). This phenomenon is well illustrated for Hb, which can donate its prosthetic heme groups to albumin or hemopexin (32) as well as to LDL (15, 87). Many factors associated with inflammation and immunity can act directly or indirectly to promote varying degrees of RBC lyses and as such the generation of cell-free Hb and the subsequent release of its prosthetic heme groups (Fig. 5). Moreover, other hemoproteins containing noncovalently bound prosthetic heme groups, such as myoglobin, can probably act in a similar manner, although this remains to be established.

While not cytotoxic *per se*, free heme can sensitize nonhematopoietic cells to undergo programmed cell death in response to proinflammatory agonists, such as demonstrated for TNF (148), among others (67, 102) (Fig. 6). This deleterious effect is driven by Fe (66), although it is not clear if Fe must be released from heme to become cytotoxic or whether cytotoxicity is exerted by Fe within the context of the protoporphyrin ring of heme or both (67).

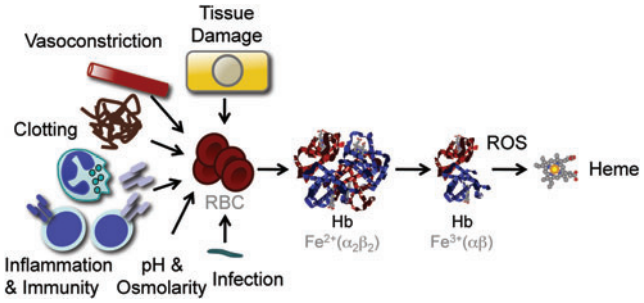

FIG. 5. Heme release from Hb. Pathogens, products associated with the activation of innate and adaptive immunity, changes in plasma pH and osmolarity, microvascular clotting, vasoconstriction, and molecules released in the context of tissue damage can act directly or indirectly to trigger varying levels of RBC lyses and concomitant Hb leakage into plasma. Upon release from RBC, Hb tetramers are dissociated into dimers favoring oxidation of their prosthetic heme groups and promoting heme release. The shear number of RBC ($2–3\times10^{13}$ in humans), their high Hb (3×10^{6} molecules/RBC), and heme (1.2×10^{7} molecules/RBC) content make that lysis of a small fraction of RBC not detectable by standard hematological analyzes can lead to the release of significant amount of heme into plasma. Presumably for this reason, Hb is the main source of free heme involved in the pathogenesis of immune-mediated inflammatory diseases, such as severe sepsis and malaria. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

The mechanism underlying Fe-heme-mediated cytotoxicity involves the sustained activation of the c-Jun N-terminal kinase (JNK) signaling transduction pathway (66). This is in keeping with the notion that JNK activation plays a central role in the mechanism *via* which cytotoxic agonists associated with the generation of free radicals induce programmed cell death, as illustrated for TNF (111) and UV radiation (170), among others (110). However, under pathophysiological conditions, TNF cytotoxicity is prevented *via* a mechanism involving the activation of NF-κB, a transcription factor that uncouples inflammation from programmed cell death (20). The cytoprotective action of NF-κB is exerted *via* the expression of the so-called protective genes (13), which include manganese superoxide dismutase (186), *A20* (40, 51, 104), *GADD45* (43, 125), and *FTH* (127), all of which control cellular accumulation of free radicals in response to TNF (89). The antioxidant effect of these NF-κB-dependent genes restrains JNK activation and as such the induction of programmed cell death in response to TNF (30, 164). The mechanism underlying this cytoprotective effect involves the activation of redox-sensitive phosphatases inhibiting JNK activation, *via* the conversion of their catalytic cysteine residues to sulfenic acid upon exposure to free radicals (89, 127). This explains why restraining the participation of Fe in the Fenton chemistry prevents sustained activation of JNK and programmed cell death in response to TNF (21, 66, 127) (Fig. 6).

The cytoprotective effect exerted by NF-κB-dependent genes and in particular by FTH was so far only observed under nonphysiological conditions, such as when specific components of the NF-κB family of transcription factors and/or when NF-κB-responsive genes are deleted *in vitro* (127). Our recent findings that the pro-oxidant effect of intracellular heme can bypass this protective pathway, sustaining JNK activation and triggering programmed cell death (66), provides evidence for a pathophysiological relevance for this phenomenon. This cytotoxic effect of free heme is strictly dependent on the accumulation of free radicals (66), most likely produced by the mitochondria. These observations provide a physiological context in support of the notion that tissue Fe overload can drive the oxidative activation of JNK and promote programmed cell death (66), a deleterious effect that plays a central role in the pathogenesis of inflammatory diseases as illustrated for severe malaria (52, 102, 148) as well as for severe sepsis (102) (Fig. 6).

Heme sensitization to TNF-mediated programmed cell death is partially suppressed by pharmacological inhibition of caspase-3 (66, 101, 148), indicating that heme sensitizes cells to undergo a caspase-dependent form of programmed cell death, that is, apoptosis (49). However, heme sensitization to TNF-mediated programmed cell death is also suppressed by pharmacological inhibition of the receptor interacting serine/threonine kinases 1 (RIPK1) as well as by deletion of the *Rip3* gene (Raffaella Gozzelino and Ana Ribeiro unpublished), suggesting that heme sensitizes cells to undergo programmed cell death by necrosis, that is, necroptosis (175). This is in keeping with the recent finding that heme can signal *via* TLR-4 to induce TNF-mediated necroptosis in Mø (59) as well as with the notion that sustained JNK activation shifts TNF-

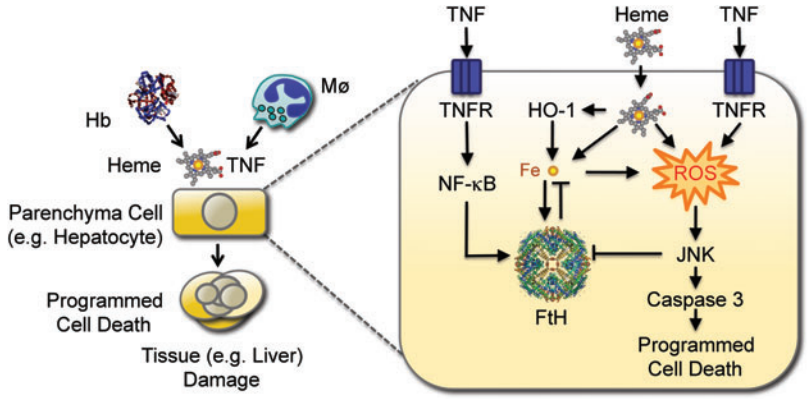

FIG. 6. Protective effect of FTH against systemic infection. Disruption of RBCs produces cell-free Hb, that upon oxidation releases heme. Non-Hb (free) heme sensitizes hepatocytes to undergo programmed cell death in response to TNF. This cytotoxic effect is mediated *via* a mechanism involving the production of free radicals (reactive oxygen species; ROS) that sustain JNK activation, leading to caspase activation and ultimately to programmed cell death by apoptosis. Labile Fe released from the protoporphyrin ring of heme catalyzes the production of free radicals *via* the Fenton chemistry, sustaining JNK activation and leading to programmed cell death. Labile Fe, however, also induces FTH expression, which neutralizes its pro-oxidant effects, suppressing JNK activation and programmed cell death. Expression of FTH can also be induced *via* NF-κB activation in response to TNF. Expression of FTH is inhibited by JNK activation, promoting the accumulation of labile Fe and the production of ROS, leading to programmed cell death. This pathological process can compromise host survival when confronted with systemic infections, as demonstrated for severe forms of malaria. JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor kappa B; TNF, tumor necrosis factor. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

mediated programmed cell death from apoptosis to necrosis (175, 177). The cytotoxic effect of free heme and in particular its ability to trigger necroptosis may be particularly relevant in the context of intracellular pathogens, as illustrated for *Mycobacterium tuberculosis* (140, 153). We suggest that heme sensitization to TNF-mediated programmed cell death acts in a rather peculiar way, in that it can probably drive, simultaneously, the activation of two antagonistic cytotoxic signal transduction pathways, that is, apoptosis and necroptosis. The mechanisms *via* which this occurs remain to be elucidated and are currently subjects of our studies.

Cytoprotective Effect of Heme Catabolism by HO-1

Although heme oxidation can release Fe from its protoporphyrin ring (18), this process is strongly catalyzed by HO (165). Of particular relevance to this process is the stress-responsive HO-1 isoform whose expression is regulated essentially at the transcriptional level *via* several stress-responsive transcription factors (36), including NRF2 (5). Under steady-state conditions, NRF2 is constitutively targeted for proteolytic degradation by the 26s proteasomal pathway, driven by its binding to the Kelch-like ECH-associated protein (Keap1) (84). Keap1 has several redox-sensitive cysteine residues that form disulfide bounds when exposed to free radicals, altering its tertiary structure and disrupting its interaction with NRF2 (85, 93). While this allows for NRF2 nuclear translocation, it is not sufficient *per se* to promote the transcription of the *HMOX1* gene encoding HO-1 (84). The reason for this is that HMOX1 transcription is constitutively repressed by the binding of BRCA-1 associated carboxy C-terminal helicase (BACH-1) to DNA Maf responsive elements (MARE) present in its promoter (163). BACH-1, however, is a heme sensor targeted for 26s proteosomal degradation upon cognate heme binding (5, 83). This releases the MARE in the *HMOX1* promoter and allows nuclear NRF2 to drive *HMOX1* transcription (5, 83). Although NRF2 appears to play a central role in the transcriptional regulation of the *HMOX1* gene, several other stress-responsive transcription factors also contribute to the regulation of HO-1 expression, as reviewed elsewhere (36, 67). More recently, HO-1 expression was also shown to be under the control of several micro-RNAs, including miR-155 and miR122, which promote HO-1 expression by inhibiting BACH-1 (133, 134), whereas miR-217, miR-377, and miR-378 inhibit *HMOX1* translation *via* direct interaction with the 3′UTR in *HMOX1* mRNA (19, 154).

HO-1 confers cytoprotection to different forms of programmed cell death (157, 179), including apoptosis and/or necroptosis driven by heme and TNF (67, 102, 148). This cytoprotective effect is driven by heme degradation *per se* as well as by its end products, including the gasotransmitter CO (27) and the antioxidants biliverdin/bilirubin (147). CO can exert cytoprotective effects *via* the modulation of cellular signal transduction pathways, including the p38 mitogen activating protein kinase (MAPK) (26, 27, 152, 159). In addition, CO can bind Fe in the heme pockets of hemoproteins, inhibiting heme release and preventing the its cytotoxic effects, as illustrated for Hb, to which CO binds avidly to suppress its oxidation as well as heme release (73, 123). While the conversion of biliverdin into bilirubin, catalyzed by biliverdin reductase, may contribute to the antioxidant and cytoprotective effect of heme catabolism by HO-1 (147), this is probably not sufficient to *per se* to override the pro-oxidant effect of the labile Fe produced *via* heme catabolism. It is also possible that the cytoprotective effect of HO-1 is mediated to some extent, in the absence of its catalytic activity, *via* regulation of gene transcription consequent to C-terminal cleavage and nuclear translocation (39, 109).

We have argued that cytoprotective effects of HO-1 contribute critically to its salutary effects exerted in a variety of immune-mediated inflammatory diseases (156, 158), including the rejection of transplanted organs (155), autoimmune diseases (37), subclinical recurrent abortions (191), or infectious diseases, such as severe malaria (52, 123, 148) and sepsis (102). In the context of systemic infections, induction of HO-1 expression by the infected host provides protection against the cytotoxic effects of free heme released from Hb (52, 67, 123, 148). This cytoprotective effect is essential to support host survival and yet it does not appear to exert a negative impact on the pathogen, a phenomenon referred to as disease tolerance (116).

The relative importance of this interplay, between heme and HO-1, to the pathogenesis of severe forms of malaria is strongly supported by the finding that sickle Hb, a mutation in the beta chain Hb that confers protection against *Plasmodium* infection in human populations (31), does so *via* a mechanism that relies on the induction of HO-1 expression, through the activation of the transcription factor NRF2 (Fig. 7) (53, 142). Moreover, the production of the gasotransmitter CO, such as induced by sickle Hb, prevents heme release from Hb, conferring protection against severe forms of malaria (53, 123, 142). This protective effect acts irrespectively of pathogen load, and as such is said to confer disease tolerance to malaria (53, 116) (Fig. 7).

Fe, Heme, and Infection

Microorganisms, including invading pathogens, are sensed by host PRR, which trigger inflammatory responses aimed at restricting microbial growth and when necessary achieve their clearance, while limiting tissue damage (115). Through the perspective of a microbial organism, a successful infection depends strictly on its capacity to divert components of host metabolism into its own metabolic pathways (88). One of the strategies *via* which inflammatory responses limit the growth of invading pathogens is through the deployment of mechanisms limiting microbial access to key metabolic components such as Fe, which are essential for microbial growth (33, 183). These host defense mechanisms act in a concerted manner to provide systemic (i) neutralization of circulating Fe, (ii) inhibition of cellular Fe delivery, and (iii) intracellular Fe retention (7).

Systemic neutralization of circulating Fe in response to infection is mediated *via* a mechanism involving lactoferrin, a potent soluble Fe chelator secreted by activated neutrophils, which limits Fe availability to pathogens while exerting antimicrobial activity (24, 173, 174, 180). Pathogens evolved strategies to counter this host defense mechanism, *via* the expression of siderophores, that is, low-molecular-weight Fe-binding complexes that can extract Fe from lactoferrin as well as from TF (120). These virulence factors are themselves countered by siderocalin/lipocalin-2, a host acute-phase Fe-binding protein that sequesters siderophores and decreases susceptibility to infection (58). Pathogens also evolved mechanisms to circumvent this host defense strategy, including the production of stealth siderophores, precluding siderocalin binding (1, 135).

Expression of natural resistance-associated Mø protein 1 (Nramp-1) in late phagolysosomes is another host defense strategy that reduces Fe availability to intracellular bacteria (34, 88, 172, 178), conferring resistance to infection (60). Moreover, Nramp-1 modulates immune responses to microbial organisms, regulating cytokine production and recruitment of phagocytic cells to the site of the infection (60).

Pathogens can also acquire Fe from host heme, using HO-1 homologues, including ChuS (161), HemO (192), HugZ (69), and HmuO (185), for Fe extraction. While essential to the establishment of host–microbe interaction, the strategies employed by microbes to acquire Fe from the host and those used by the host to starve microbes from Fe will not be reviewed in further detail hereby as these have been recently reviewed elsewhere (33).

Systemic intracellular Fe retention by the infected host is achieved through the action of hepcidin, *via* a mechanism involving the phosphorylation, internalization, and subsequent lysosomal degradation of the Fe cellular exporter FPN

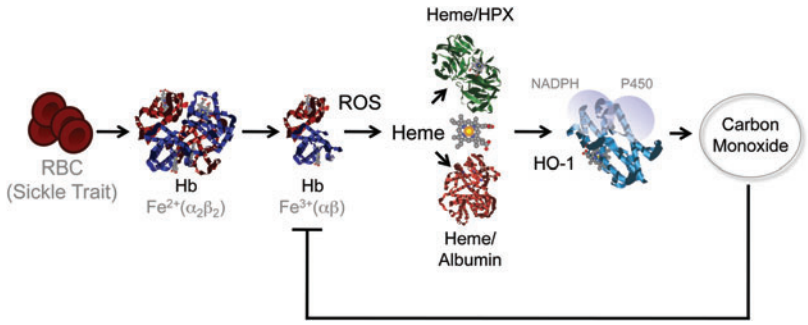

FIG. 7. Induction of heme catabolism by sickle cell trait confers disease tolerance to malaria. Sickle cell disease is a molecular disease caused by a single-point mutation in the β chain of Hb (β6Glu > Val). When present in the heterozygous form, the Hb β6Glu > Val sickle mutation is not pathogenic, conferring a survival advantage against malaria (sickle cell trait). This protective effect acts *via* the accumulation of low (noncytotoxic) levels of free heme in plasma that induce the expression of HO-1 *via* a mechanism involving the activation of the transcription factor NF-E2-related factor 2 (NRF2) (not illustrated). The CO produced *via* heme catabolism by HO-1 binds to cell-free Hb and prevents the accumulation of free heme following *Plasmodium* infection, thus suppressing the pathogenesis of severe forms of malaria. This protective effect does not interfere with parasite load revealing that sickle Hb confers disease tolerance to malaria. CO, carbon monoxide. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

(46, 63). The trade-off to this host defense strategy is deregulation of host Fe homeostasis with cellular Fe retention interrupting Fe recycling and reducing the levels of circulating Fe available not only to microbes but also to host erythropoiesis (88). In keeping with this notion, high levels of hepcidin in plasma and low levels of FPN are associated with impaired erythropoiesis, anemia, and hypoferrimia (46), as well as with cellular and tissue Fe overload (88). Moreover, systemic inhibition of FPN expression also causes intracellular Fe accumulation leading to oxidative stress and tissue damage (7). It should be noted that deregulated Fe metabolism, such as driven by the sustained production of hepcidin is not specific to infection, but rather to inflammation. As such it can be associated to a variety of immune-mediated inflammatory diseases as well as with cancer progression (162, 169), where it leads to poor prognosis, unfavorable outcome, and metastasis (130).

The deleterious effects of sustained hepcidin expression are exacerbated under inflammatory conditions associated with hemolysis and the generation of cell-free Hb. While cell-free Hb can provide some level of resistance against bacterial pathogens (23), the evolutionary trade-off of this defense mechanism is the release of its prosthetic heme groups (32). This leads to systemic heme loading into host cells, eventually causing oxidative tissue damage and disease (52, 67).

Heme induces the expression of HO-1, which inhibits hepcidin expression *via* a mechanism mediated by CO (150), presumably restoring FPN expression and hence Fe cellular export. NO can also induce FPN expression (118), contributing to the antimicrobial activity of this gasotransmitter (22, 118), while allowing Fe to recycle as to be used in erythropoiesis. Despite these salvage pathways, high levels of hepcidin still promote the systemic accumulation of intracellular Fe. Moreover, while cytoprotective *per se*, heme catabolism by HO-1 produces intracellular labile Fe (14, 16, 50, 67, 102, 123, 148), which cannot be readily exported due to inhibition of FPN expression enforced by hepcidin (46). Therefore, under inflammatory conditions, the intracellular pool of labile Fe must be neutralized by the induction of FtH expression (14, 21, 48, 167).

FTH and Infection

Ferritin is an acute-phase protein induced during systemic infections, *via* a mechanism regulated at both transcriptional and post-transcriptional levels (167) (see Fe and Heme Homeostasis section). *FTH* expression is regulated at a transcriptional level by NF-κB (100, 127), a transcription factor that plays a central role in the regulation of inflammation and immunity. This argues strongly for the integration of Fe metabolic adaptation as an intrinsic component of inflammation and immunity, presumably contributing to host protection against infection (46, 116). *FTH* transcription is also regulated by the transcription factor NRF2 (112, 129), suggesting yet another level of integration between cellular adaptation to oxidative stress and Fe metabolic adaptation during infection. The transcription factor hypoxia-inducible factor alpha and heat shock factor 1 also regulate FTH expression in *Caenorhabditis elegans* (2), arguing for yet a broader level of integration between Fe metabolic adaptation and cellular responses to different forms of stress associated with inflammation and immunity. Whether this level of integration is evolutionary conserved, as to be extrapolated from *C. elegans* to mammals, remains to be established.

A functional effect of ferritin in the outcome of systemic infections has only recently started to be elucidated. FTH acts *via* a mechanism involving its ferroxidase activity to provide metabolic adaptation to tissue Fe overload during systemic infections, as demonstrated for malaria (66) and severe sepsis (Sebastian Weiss and Rasmus Larsen, unpublished) in mice. Expression of FTH prevents intracellular Fe accumulation from sustaining JNK activation and hence from sensitizing nonhematopoietic cells to undergo programmed cell death (66). This finding is in line with previous demonstration of a cytoprotective effect of FTH *in vitro* (14, 41, 127, 187) and *in vivo* (21). The cytoprotective effect of FTH against heme is apparently at odds with the notion that FTH cannot target Fe inside heme (66). An alternative interpretation, however, is that the cytotoxic effect of heme acts *via* a mechanism driven by labile Fe released from, which can be targeted by FTH, and not by the Fe contained within its protoporphyrin ring, which cannot be targeted by FTH (67). This is in keeping with the notion that labile Fe fuels the activation of JNK in response to TNF (127), a cytotoxic effect repressed by the ferroxidase activity of FTH (66, 127).

Deletion of the *Fth* allele in mice promotes tissue Fe overload, oxidative stress and damage in response to systemic infections, despite normal levels of HO-1 expression (67). This suggests that FTH is required to support the salutary effects of HO-1 in the context of systemic infection (148), uncoupling heme catabolism from Fe-driven cytotoxicity (14, 66). Moreover, when HO-1 expression is deleted, induction of FTH expression can be impaired (66), which may explain the high lethality of these mice in response to systemic infections (66, 148). To what extent the protective effect of FTH is required to sustain the salutary effects of HO-1 against other immune-mediated inflammatory diseases remains to be established.

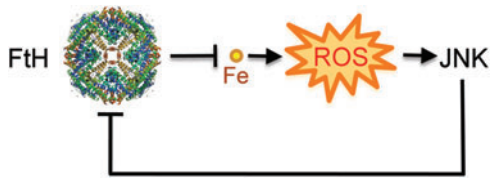

FIG. 8. FTH prevents pro-oxidant labile Fe from sustaining JNK activation. FTH controls JNK activation indirectly *via* a mechanism that prevents labile iron from partaking in the production of free radicals *via* the Fenton chemistry. This is consistent with previous studies showing that reducing free radical production is sufficient *per se* to control JNK activity, *via* inhibition of redox-sensitive phosphatases regulating JNK activity (not illustrated). Presumably, the mechanism underlying the cytotoxic effect of JNK activation involves the inhibition of FTH expression, which promotes cellular Fe overload, accumulation of free radicals, and programmed cell death. This functional cross talk between FTH and JNK controls the host metabolic adaptation to tissue Fe overload during systemic infections. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

The combination of heme and TNF produced during systemic infections inhibits the expression of FTH *via* a mechanism that involves JNK activation, as demonstrated *in vitro* (66, 127) as well as *in vivo* (66) (Fig. 8). This reduction in FTH expression leads to cellular Fe overload and oxidative stress, ultimately involved in the mechanism *via* which JNK triggers programmed cell death during systemic infections (66, 127). This observation reveals the existence of a functional link between JNK activation and metabolic adaptation to cellular Fe overload during infection, in which TNF acts *via* JNK activation to promote tissue Fe overload (66) (Fig. 8). Moreover, inhibition of FTH expression also contributes to explain how JNK activation in response to TNF reinforces the accumulation of free radicals, an effect that can shift programmed cell death from apoptosis to necrosis (177). The molecular mechanism *via* which JNK activation inhibits FTH expression and deregulates Fe homeostasis remains, however, to be elucidated (Fig. 8).

Conclusion

Host defense strategies limiting Fe availability to pathogens should be considered as an integral component of inflammation and immunity providing host resistance to infection. Moreover, metabolic adaptation to tissue Fe overload, as conferred by the expression of FTH should be taken into account as an integral component of host protection against infection, promoting disease tolerance to infection (66, 116).

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Abbreviations Used

BACH-1 = BRCA-1 associated carboxy C-terminal helicase
CO = carbon monoxide
Dcytb = cytochrome B reductase
DMT1 = divalent metal transporter 1
Fe = iron
FLVCR = feline leukemia virus C receptor
FPN = ferroportin
FTH = ferritin H (heart/heavy) chain
FTL = ferritin liver/light chain
Hb = hemoglobin
HO = heme oxygenases
HRG-1 = heme responsive gene-1
IL = interleukin
IRE = iron responsive elements
IRP = iron regulatory protein
JNK = c-Jun N-terminal kinase
Keap1 = Kelch-like ECH-associated protein
LRP-1 = low-density lipoprotein receptor-related protein 1
MAPK = mitogen activating protein kinase
MARE = Maf responsive elements
Mø = macrophage
NF-κB = nuclear factor kappa B
Nramp-1 = natural resistance-associated macrophage protein 1
NRF2 = NF-E2-related factor 2
RBC = red blood cell
RIPK = receptor interacting serine/threonine kinase
STEAP = six-transmembrane epithelial antigen of the prostate
TF = transferrin
TFR = transferrin receptor
TLR = toll-like receptor
TNF = tumor necrosis factor
UTR = untranslated mRNA regions