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Role of Heme in Cardiovascular Physiology and Disease

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Heme is an essential molecule for living aerobic organisms and is involved in a remarkable array of diverse biological processes. In the cardiovascular system, heme plays a major role in gas exchange, mitochondrial energy production, antioxidant defense, and signal transduction. Although heme, as the prosthetic group of hemoglobin, is most commonly associated with oxygen transport, heme is also required for the function of many cardiac enzymes, including mitochondrial electron transport chain complexes and heme-containing antioxidant proteins, such as peroxidase and catalase. Despite the vital role of heme in the cardiovascular system, this molecule remains understudied outside of the erythropoietic system. This review aims to summarize the important observations of heme in cardiovascular physiology and pathology.

Heme Structure and Function

Heme consists of an iron atom and a heterocyclic tetrapyrrole ring system, referred to as a *porphyrin*. The 4 pyrrole rings of the porphyrin are cyclically linked by methene bridges. This planar network of conjugated double bonds is responsible for the characteristic absorption and fluorescence of porphyrins and provides great value for the study of heme. Porphyrins emit red fluorescence on being dissolved in mineral acids or organic solvents, allowing for simple quantification.[1] This feature of porphyrins is also exploited clinically in the field of oncology. Because tumor cells take up more porphyrins than normal cells, hematoporphyrins or related compounds are administered to cancer patients. The tumor is then exposed to an argon laser, which excites the porphyrins and causes cytotoxic effects.[2]

The function of heme-containing proteins depends strongly on the ability of heme to coordinate an iron atom inside its structure and facilitate reduction–oxidation (redox) reactions. There are several types of heme, and they differ in the composition of their side chains around the pyrrole rings. The most biologically relevant heme, heme b, consists of an iron atom and a porphyrin with 4 methyl, 2 vinyl, and 2 propionate substituent groups (also known as protoporphyrin IX [PP IX]).[3]

Heme is essential for all cells and functions as the prosthetic group for several heme-containing proteins (hemoproteins), such as hemoglobin. The function of the heme molecule is determined by the polypeptide bound to it.[4] In hemoglobin, each of the 4 heme moieties is conjugated to a globin polypeptide, forming a globular molecule made up of 4 subunits.[5] In the heme of hemoglobin, the iron atom not only coordinates with the 4 nitrogen atoms of PP IX but also binds to a histidine residue of the globin chain (referred to as the *proximal histidine*). The iron atom in the heme in hemoglobin is in the Fe(II) ferrous state to allow for reversible binding of iron with molecular oxygen, which completes the octahedral coordination of the central iron atom. Oxygenation changes the electronic state of the Fe(II)–heme complex, as reflected by the color change of blood from the dark purple of venous blood to the bright red of arterial blood. When blood is exposed to various oxidizing agents, the heme iron in hemoglobin is oxidized from the Fe(II) ferrous to the Fe(III) ferric state to form methemoglobin, which is unable to bind oxygen. Methemoglobin is normally generated in erythrocytes but is continuously reduced to ferrous hemoglobin by the NADH–methemoglobin reductase system. Congenital absence of this system is a cause of hereditary methemoglobinemia.[6] The oxidized form of heme is also known as *hemin* (ferric PP IX), the positive charge of which favors the coordination of halide ions (ie, chloride) to the ferric iron.

In addition to hemoglobin, several hundred other proteins contain heme (Table). Heme is an essential prosthetic group for hemoproteins involved in numerous cardiovascular processes, including oxygen transport (hemoglobin), oxygen storage (myoglobin), oxygen metabolism (oxidases), antioxidation (peroxidases, catalases), and electron transport (cytochromes). Although the heme-containing cytochrome P450

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Table. Cardiovascular Proteins Containing Heme as a Prosthetic Group

Function	Example Hemoproteins
Gas transport	Hemoglobin
	Myoglobin
Electron transport	Cytochrome b
	Cytochrome c
	Cytochrome c oxidase
	Cytochrome c reductase
	Succinate dehydrogenase
Enzyme catalysis	Peroxidase
	Cytochrome P450
	Nitric oxide synthase
Heme responsive factors	NPAS2
	Bach1
	eIF2α-kinase
Antioxidant defense	Catalase
Signaling	Soluble guanylate cyclase

Bach1 indicates BTB and CNC homology 1; eIF2α, eukaryotic translation initiation factor 2α; mPER2, mouse PER2; NPAS2, neuronal PAS domain protein 2.

family of enzymes is well known for its role in hepatic detoxification, several cytochrome P450 isoforms are also expressed in the heart and catalyze arachidonic acid oxidation, which attenuates myocardial ischemic injury.[7] Heme-containing proteins have also been implicated as signaling molecules (guanylate cyclase), as enzymes (cyclooxygenase, nitric oxide [NO] synthase), and in the synthesis of hormones (hydroxylases). Recently, a heme-containing mitochondrial respiratory complex, succinate dehydrogenase, has been implicated in the generation of reactive oxygen species (ROS) during ischemia–reperfusion injury in different organs, including the heart.[8] Consequently, heme is required for the maintenance of cardiovascular health, not only as a catalytic subunit of enzymes but also as a signaling molecule. Although every cell in the body requires heme, the 2 major sites of heme synthesis are the bone marrow and the liver. An important function of heme in the liver is the synthesis of cytochrome P450 enzymes, which are required in varying amounts for liver detoxification under different conditions.[9]

Heme Biosynthetic Pathway

The synthesis of heme requires several biochemical reactions both in the mitochondria and in the cytoplasm and has been studied extensively. Initially, δ-aminolevulinic acid (ALA) synthase (ALAS) catalyzes the condensation of the Krebs cycle intermediate succinyl coenzyme A and the amino acid glycine to generate the precursor ALA.[10,11] In mammals, 2 separate genes encode the enzyme. *ALAS1* is ubiquitously expressed, whereas *ALAS2* is usually present only in the red blood cell lineage.[12,13] ALA is then exported from mitochondria to the cytoplasm, where additional enzymatic reactions convert ALA to coproporphyrinogen III (CP). CP is then transported back to the mitochondria for the formation of PP IX. The last step of heme biosynthesis requires the incorporation of iron into PP IX, and this reaction is catalyzed by ferrochelatase (Figure 1). Mature heme can then be incorporated into the respiratory chain proteins or exported into the cytoplasm.[14] Both the synthesis of ALA and iron incorporation into heme have been suggested as the rate-limiting steps for heme synthesis.[15,16]

As outlined above, 3 mitochondrial transporters are required for heme biosynthesis: the exporter of ALA, the importer of CP, and the exporter of heme. A recent in vitro study suggested that feline leukemia virus subgroup C receptor (FLVCR) 1β is the mitochondrial heme exporter, although the authors did not perform a direct transport assay.[17] Another study demonstrated that ATP-binding cassette (ABC) B10, an inner mitochondrial ABC protein, participates in the export of ALA.[18] The true identity of the CP transporter has not yet been identified, but studies suggest that peripheral-type benzodiazepine receptors may be involved in the process because pharmacological blockade of the receptor reduces binding of CP to the mitochondria.[19] Nevertheless, this receptor also has a strong affinity for PP IX,[20] and the evidence for its transport activity is lacking. In addition, *ABCB6*, another mitochondrial ABC transporter, is required for the active transport of CP to mitochondria,[21] but its subcellular localization and involvement in heme biosynthesis remain controversial.[22]

Although heme is predominately synthesized in the mitochondria, some cells also exhibit the ability to uptake heme from the surrounding environment. This mode of heme acquisition has been studied extensively in *Caenorhabditis elegans*. Heme-responsive gene 1 (*HRG-1*), the mammalian homolog of which is expressed in various tissues including brain, heart, and kidney, is responsible for heme uptake from the extracellular space in *C elegans*. The function of this protein is highly conserved from worms to zebrafish.[23,24] In the mammalian system, heme acquisition is better understood in enterocytes, which directly absorb heme from ingested food. Proton-coupled folate transporter/heme carrier protein 1 (*PCFT/HCP1*) was first identified in intestinal cells as the heme transporter and is responsible for heme uptake from the intestinal lumen.[25,26] *FLVCR2* is another protein that mediates heme uptake. Cells with *FLVCR2* overexpression demonstrate heightened sensitivity to heme overload. *FLVCR2* is widely expressed throughout the body at the mRNA level, including the heart,[27] but its involvement in

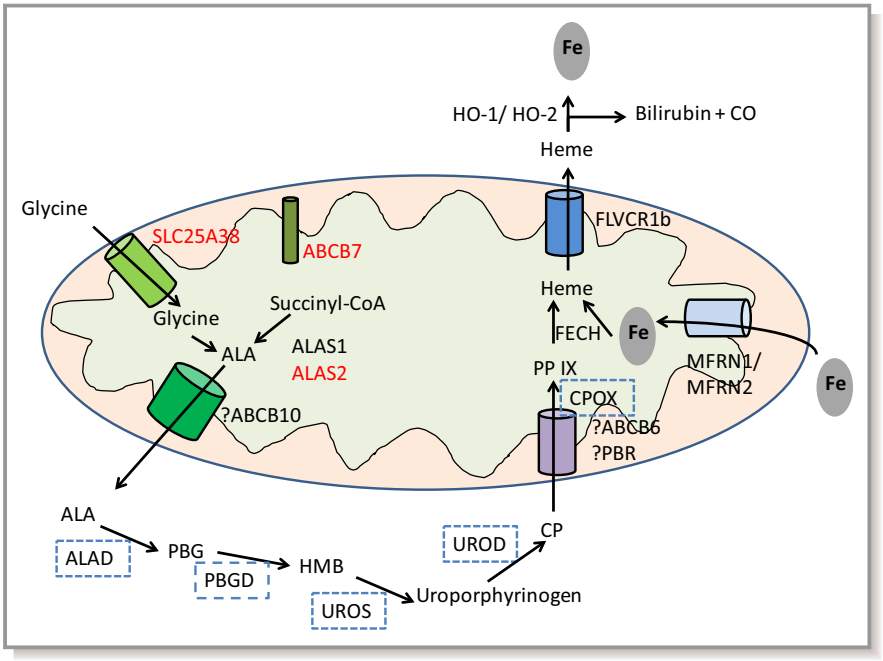

Figure 1. Heme synthesis and degradation pathways. Aminolevulinic acid (ALA) is synthesized from succinyl coenzyme A (CoA) and glycine by ALA synthase (ALAS1, ALAS2) and is subsequently exported to the mitochondria. After several enzymatic reactions, ALA is converted to coproporphyrinogen III (CP), which is transported back to mitochondria and converted to protoporphyrin IX (PP IX). Ferrochelatase (FECH) incorporates iron (Fe) into PP IX to form heme. The mature heme can then be exported out of the mitochondria by feline leukemia virus subgroup C receptor 1 (FLVCR1b). Heme is also degraded in the cytoplasm by heme oxygenases (HO-1, HO-2). The proteins in dotted boxes are associated with porphyrias. The proteins in red are associated with sideroblastic anemia. ABCB indicates ATP-binding cassette subfamily B member; ALAD, ALA dehydrogenase; CO, carbon monoxide; CPOX, coproporphyrinogen III oxidase; HMB, hydroxymethylbilane; MFRN, mitoferrin; PBG, prophobilinogen; PBGD, prophobilinogen deaminase; PBR, peripheral-type benzodiazepine receptors; SLC25A38, solute carrier family 25, member 38; UROD, uroporphyrinogen decarboxylase; UROS, uroporphyrinogen synthase.

cardiovascular physiology and pathology has not been studied. In addition, some cells exhibit PCFT/HCP1-independent heme uptake.[26] It was speculated that a heme receptor may be responsible for the heme uptake independent of *PCFT/HCP1*, but the receptor remains to be identified.[28] It is also unclear whether cardiomyocytes use this mechanism to acquire heme from the environment. Last, the redistribution of heme from one cell type to another has also been described in worms during embryogenesis.[29]

Cellular heme levels are tightly controlled through a fine balance between heme biosynthesis and catabolism. In the face of excess heme, cells can either upregulate heme degradation machinery, which will be discussed in later sections, or export excess heme. *FLVCR1* is the first heme exporter identified in hematopoietic cells. Overexpression of *FLVCR1* enhances cellular heme export,[30] whereas macrophages from *FLVCR1*-null mice display cytoplasmic iron accumulation when incubated with red blood cells[31]; however, *FLVCR1* protein is not detected in the heart, based on Western blotting results.[31] Another cell-surface ABC protein, *ABCG2*, may also be involved in cellular heme regulation. Deletion of *ABCG2* results in accumulation of PP IX in erythroid cells, and mice display protoporphyria.[32] Nevertheless, human mutation of this gene does not lead to PP IX accumulation or protoporphyria, and patients do not exhibit any cardiac abnormalities.[33]

Mechanisms of Injury by Heme

The biochemical property of iron to catalyze redox reactions also enables this ion in free heme (heme not bound to protein) to generate ROS and cause cellular injury.[34] There are 2 main cellular sources of free heme: newly produced heme that has not yet been integrated into hemoproteins or heme that has been dissociated from hemoproteins under oxidative stress. Although living organisms attempt to avoid an excess of free heme by tight control of iron homeostasis, the free heme pool may increase after upregulation of heme synthesis, excess

hemolysis, elevated hemoprotein degradation, compromised integration of heme into hemoproteins, or impaired heme oxygenase activity.

Many pathological conditions, such as sickle cell anemia and ischemia–reperfusion, are associated with severe hemolysis or myolysis.[35] Tissues can be subsequently exposed to high levels of free heme (up to 20 μmol/L), overwhelming physiological mechanisms of free heme removal and leading to its toxic effects: oxidative stress, hemolysis, and inflammation (Figure 2).

Mechanisms of Injury by Heme: Oxidative Stress

Free heme is a major source of redox-active iron, which can cause cellular damage through the Fenton reaction.[36] The Fenton reaction is a 1-electron redox reaction that produces highly toxic free hydroxyl radicals from hydrogen peroxide and Fe(II) ferrous iron (Figure 2). Although all transition metals with a free coordination site have the ability to reduce peroxides to hydroxyl radicals, iron is considered the most biologically relevant metal because of its high concentration in the human body and its redox potential.[37] Heme-driven production of ROS is involved in the pathophysiology of several disorders by damaging lipid membranes, proteins, and nucleic acids; activating cell signaling pathways; altering protein expression; and perturbing membrane channels, among other toxic effects.

Because iron can be found in close proximity to nucleic acids, the hydroxyl radicals produced from the Fenton reaction can interact with the DNA deoxyribose backbone and bases. In the absence of sufficient base excision repair, this may lead to cytotoxic and mutagenic lesions.[38] Heme-mediated DNA damage is very rapid; in the presence of hemin, circular plasmid DNA is converted to linear forms within 30 minutes and ultimately is converted into DNA fragments.[39]

Free heme toxicity is exacerbated by its ability to intercalate into lipid membranes. The extreme hydrophobicity of free heme allows it to easily enter the phospholipid bilayer. Within this highly oxidizing environment, hydrogen peroxide from various sources (ie, activated leukocytes) cleaves the heme ring to release and interact with the redox-active iron, leading to amplified production of ROS in the cell. This further catalyzes the oxidation of the cell membrane and promotes lipid peroxidation, increasing membrane permeability and ultimately leading to cell death.[40,41]

Mechanisms of Injury by Heme: Hemolysis

As mentioned earlier, because of its hydrophobic nature, free heme can readily enter cell membranes. In erythrocytes,

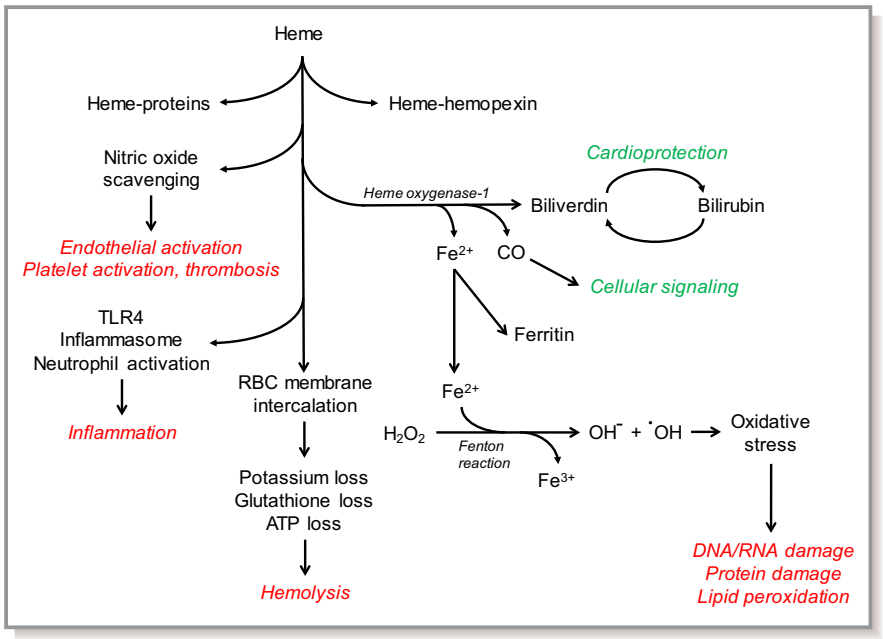

Figure 2. The many fates of heme. Heme may be incorporated into hemoproteins or scavenged by hemopexin. Free heme is also degraded by heme oxygenase-1 into carbon monoxide (CO), for cellular signaling, and biliverdin, which is cardioprotective by shuttling between biliverdin and bilirubin. Heme degradation also leads to the production of free iron (Fe), which may be stored via ferritin or may lead to oxidative damage through hydroxyl radical production through the Fenton reaction. Excess heme may also lead to inflammation, endothelial activation, platelet activation and thrombosis, and erythrocyte hemolysis. RBC indicates red blood cell.

hemin causes hemolysis by stimulating potassium loss, which leads to impaired maintenance of cation gradients across the cellular membrane and causes cellular swelling. Two phases have been reported in heme-induced hemolysis: (1) intracellular loss of potassium, followed by loss of glutathione and ATP, and (2) loss of hemoglobin.[42] Hemin has also been demonstrated to cross-link cytoskeletal proteins, such as spectrin, thereby altering erythrocyte membrane stability, although this has not yet been shown to occur in cardiomyocytes.[43] Interestingly, hemin levels are increased in the membranes of aged circulating erythrocytes compared with young circulating erythrocytes, suggesting that heme may play a physiological role in the removal of aged erythrocytes from the circulation through hemolysis.[44] Cellular heme accumulation is also likely pathological because circulating erythrocytes from patients with sickle cell anemia and β-thalassemia have increased hemin compared with healthy circulating erythrocytes.[45] Notably, the effects of heme on cell lysis are not limited to the erythropoietic system because heme administration to cardiac cells led to loss of cell integrity, sarcolemmal damage, and release of cytosolic enzymes.[46]

In sickle cell disease, extensive intravascular hemolysis leads to the release of large quantities of free heme and hemoglobin from lysed erythrocytes into the plasma. Although the plasma heme level in healthy individuals is approximately 0.2 μmol/L, patients with sickle cell experience plasma heme levels up to 20 μmol/L,[47] Severe acute hemolysis was found to be the only predictor of sudden death due to acute chest syndrome in the Cooperative Study of Sickle Cell Disease.[48] A recent study showed that low-dose hemin infusion directly caused intravascular hemolysis, autoamplification of extracellular hemin, and acute lung injury, similar to acute chest syndrome, in a mouse model of sickle cell disease.[49] Targeting specific molecules, such as hemin, may have important consequences for the prevention, diagnosis, and treatment of acute chest syndrome in sickle cell disease.

Mechanisms of Injury by Heme: Inflammation

Free heme is also a potent proinflammatory molecule that is sensed by innate immune receptors and causes inflammation in both sterile and infectious conditions. Toll-like receptors (TLRs) are membrane-spanning receptors that play a key role in innate immunity by recognizing pathogen-associated molecular patterns derived from microbes.[50] Excess free ferric heme in plasma has recently been demonstrated to activate *TLR4*, which mediates the innate immune response to bacterial lipopolysaccharide.[51] Heme is capable of binding to *TLR4* and inducing TLR4-dependent production of tumor necrosis factor. In a mouse model of sickle cell disease, intravascular hemolysis released significant amounts of heme that activated endothelial TLR4 signaling, leading to nuclear factor-κB activation, and vaso-occlusion.[52] This suggests that heme, released from plasma hemoglobin during hemolysis, may be the common link among the 3 major phenotypic features of sickle cell disease: hemolysis, vaso-occlusion, and inflammation. In the setting of cerebral hemorrhage, heme also potentiates microglial activation via *TLR4*, which in turn induces nuclear factor-κB activation by the MyD88/TRIF signaling pathway and ultimately increases inflammation.[53]

Interestingly, a recent study revealed that heme promotes macrophage necrosis through 2 synergistic mechanisms: TLR4/MyD88-dependent tumor necrosis factor production and TLR-independent generation of ROS. This suggests that heme-induced TLR4 signaling may have both proinflammatory and procyotoxic effects.[54] This observation is further strengthened by the fact that overexpression of heme oxygenase-1 (*HO-1*), a major heme-degradation enzyme that will be discussed in a later section, is protective in a mouse model of liver ischemia–reperfusion injury. The protection is due to a reduction of inflammation secondary to decreased TLR4-mediated nuclear factor-κB activation and reduced apoptosis through increased Bcl2/Bcl-xL expression.[55]

In addition to activating immune receptors on the cell surface, heme may also activate the cytoplasmic inflammasome, a large multiprotein complex composed of a member of the nucleotide-binding oligomerization–like receptor family (ie, nucleotide-binding domain and leucine rich repeat containing family, pyrin domain containing 3 [NLRP3]) that activates inflammatory caspases and interleukin-1β.[56] In a recent study, Dutra et al found that heme is a potent inducer of interleukin-1β processing through the activation of the NLRP3 inflammasome in macrophages primed by lipopolysaccharide or tumor necrosis factor. Heme-induced NLRP3 inflammasome activation was dependent on Syk phosphorylation, mitochondrial ROS generation, and potassium efflux[57]; however, it remains to be determined whether the coordinated iron of the heme ring, free iron released from heme, or both are responsible for this process. Further understanding of the molecular mechanisms of heme-induced inflammation and cell death may lead to the identification of novel therapeutic targets for sickle cell disease, disseminated intravascular coagulation, transfusion reactions, crush injuries, and sepsis.

Finally, heme has also been shown to activate neutrophils by promoting the release of neutrophil extracellular traps. Neutrophil extracellular traps are decondensed chromatin released into the plasma by activated neutrophils and are believed to destroy pathogens through a high concentration of granular enzymes (ie, neutrophil elastase) associated with them.[58] A recent paper demonstrated that the production of neutrophil extracellular traps from neutrophils activated by heme promotes acute lung injury and death in a mouse model of sickle cell disease.[59] The ability of heme to activate several

different inflammatory pathways in the absence of infection has led some investigators to refer to erythrocyte hemolysis products, including heme, as *erythroid damage-associated molecular pattern molecules*.[60]

Heme Degradation

In addition to heme export, cells also have heme degradation machinery to prevent oxidative damage associated with intracellular heme accumulation.[41,61] Heme oxygenase converts heme to biliverdin and releases free iron and carbon monoxide, thereby preventing the buildup of excess heme.[62,63] Mammals have 2 isoforms of heme oxygenase. Heme oxygenase-2 is the constitutively expressed form and is present in almost every tissue. In contrast, *HO-1* is normally present only in cells responsible for systemic heme degradation, such as macrophages in the reticuloendothelial system; however, this isoform is inducible, and its expression can increase dramatically in different organs in response to heightened levels of circulating hemoglobin.[64] The majority of heme degradation results from the catabolism of hemoglobin in senescent red blood cells, primarily in the spleen. Carbon monoxide and biliverdin are initial products of heme degradation (Figure 1). The carbon monoxide released from heme degradation can function as a signaling molecule via soluble guanylate cyclase or can bind to hemoglobin to form carboxyhemoglobin and subsequently be eliminated through expired air. Meanwhile, biliverdin is converted to bilirubin and excreted out of the body predominantly by the liver but also by the kidney.[65–67] The gradual color change of a healing bruise, from red heme to green biliverdin to yellow bilirubin, is a classic visible manifestation of heme degradation.

Although heme oxygenase prevents the accumulation of heme, it also generates free iron. This free iron becomes stored in ferritin through coordinated expression of ferritin with the heme-degradation machinery. After being stored in the center of ferritin molecules, iron cannot freely interact with other molecules and generate ROS.[68,69] Each ferritin molecule is a large 24-subunit protein consisting of heavy and light chains with sequences that share ≈55% homology.[70] The heavy chain contains ferroxidase activity that catalyzes the oxidation of ferrous iron to ferric iron, allowing for iron uptake by ferritin and limiting the production of free radicals from ferrous iron and hydrogen peroxide by the Fenton reaction.[71] Recently, an RNA binding protein, poly (rC)-binding protein 1 (*PCBP1*), was shown to be required for the delivery of iron to ferritin.[72] Ferritin iron stores can be mobilized by protein degradation, and the excess iron can be released back into the circulation through the cellular iron exporter ferroportin 1, in the form of ferrous iron. Because transferrin, the major iron carrier in blood, only binds to the ferric ion and ferroportin 1 exports ferrous ion, the copper-dependent ceruloplasmin is required for iron oxidation before the association of exported iron with transferrin. Copper deficiency can thus reduce iron release from macrophages and lead to iron overload, as seen in patients with aceruloplasminemia.[73] Although most ferritin is located in the cytoplasm, small amounts are secreted into the serum to serve as iron carriers. Consequently, plasma ferritin levels, in conjunction with other serum iron parameters, are used clinically as a diagnostic marker of total body iron stores.

Regulation of Heme Synthesis and Degradation

To prevent excess heme accumulation, both the synthesis and degradation of heme are tightly regulated. Although the coding sequences of *ALAS1* and *ALAS2* are highly similar, these 2 genes are differentially regulated, which reflects their roles in heme homeostasis in different cell types. The transcriptional regulation of *ALAS2* is better understood because of its critical role in heme synthesis in developing red blood cells. Erythropoietin induces differentiation of hematopoietic stem cells to the erythroid lineage and upregulates mRNA levels of *ALAS2* via the JAK/STAT signaling pathway.[61,74] *ALAS2* is also transcriptionally regulated by *GATA1*, a transcription factor critical for specifying the erythroid lineage.[16] In addition, *ALAS2*, but not *ALAS1*, possesses an iron response element site in the 5′ untranslated region of its mRNA sequence. When cells have insufficient iron, active iron response proteins (IRPs) bind to the iron response element site and prevent the translation of mRNA. Consequently, the presence of this iron response element site allows for the coupling of heme synthesis to cellular iron levels.[75] The expression of *HO-1* is also under transcriptional and post-transcriptional regulation. Oxidative stress upregulates *HO-1* expression, and *HO-1* mRNA is stabilized by NO, heavy metals, and oxidative stress.[76,77] The regulation of *HO-1* expression is consistent with its role as an antioxidant defense enzyme.

It is not surprising that based on its pleiotropic biological effects, heme can also control the expression of several genes, including those coding for globins, heme biosynthetic enzymes, heme oxygenase, ferroportin, and transferrin receptor.[78–80] Several of these genes are regulated by heme via heme response elements and the mammalian transcription repressor Btb and Cnc homology 1 (*Bach1*).[81] Heme also regulates gene expression at the translational level; in erythrocytes, low heme activates the heme-regulated eukaryotic translational initiation factor (eIF)-2α kinase, which in turn regulates the synthesis of α- and β-globins by phosphorylating and thereby inhibiting the activity of *eIF2*.[82] Interestingly, heme levels have opposite effects on the expression of genes involved in its synthesis and degradation in different cell types. In red blood cells, heme serves as positive feedback for

its own synthesis while inhibiting its degradation; however, heme acts as negative feedback for its synthesis in nonerythroid cells by downregulating the levels of *ALAS1*. Heme biosynthesis and the circadian clock have also been recently shown to be reciprocally regulated in mammals because heme controls the activity of the core clock protein neuronal PAS domain protein 2 (*NPAS2*), and *NPAS2* transcriptionally regulates *ALAS1*.[83]

Heme in the Cardiovascular System

Heme is involved in normal function of various cell types in the cardiovascular system. In addition to cardiomyocytes, heme also modulates the function of smooth muscle and endothelial cells. Patients with sickle cell anemia or other forms of hemolytic anemia often develop pulmonary hypertension secondary to endothelial dysfunction.[84] One of the pathogenic factors is thought to be the increased circulating ferrous heme and hemoglobin that scavenge NO, a potent vasodilator, in these patients.[47,85] Interestingly, hemoglobin protects bacteria against NO toxicity by catalyzing the dioxygenation of NO to nitrate.[86] Based on the growing evidence of NO as a major regulator of vascular homeostasis in mammals, the interaction between NO and heme has become a topic of intense investigation. Despite the high affinity of hemoglobin for NO, this reaction is significantly dampened under normal physiological conditions because of the compartmentalization of hemoglobin within the erythrocyte and diffusional barriers to NO around the erythrocyte membrane.[87] In human volunteer subjects, mean arterial pressure increased after infusion of hemoglobin, and this is attributed to the decreased NO availability through the formation of nitrosohemoglobin.[88] Additional studies have also demonstrated that the binding of NO to hemoglobin is independent of oxygen tension. Consequently, circulating heme and free hemoglobin result in depletion of NO; reduction in guanylate cyclase activity; increased smooth muscle contraction; and, ultimately, vasoconstriction.[89]

A reduction in NO availability by plasma heme also promotes platelet activation and thrombosis. Loss of NO leads to endothelial activation, a state characterized by increased expression of adhesion molecules, such as intracellular adhesion molecule 1, vascular cell adhesion molecule 1, and E-selectin.[90] In addition, cell-free heme promotes the generation of ROS,[91,92] which also leads to endothelial activation. The changes in adhesion molecule expression on the surface of endothelial cells promote platelet adhesion and aggregation, suggesting that heme plays a role in platelet activation through NO scavenging. Indeed, NO donor drugs (ie, S-nitrothiols) that increase systemic levels of NO have been demonstrated to inhibit platelet aggregation.[93] Furthermore, administration of heme often causes thrombophlebitis even in healthy volunteers, suggesting that heme causes vascular inflammation and obstruction.[94] Finally, the addition of cell-free hemoglobin to human serum causes a dose-dependent decrease in a disintegrin and metalloprotease with thrombospondin motifs 13 (*ADAMTS13*), an enzyme essential for clot formation.[95]

In addition to inducing endothelial dysfunction and platelet activation, excess heme can induce smooth muscle proliferation and contribute to cardiovascular pathology. Turbulent blood flow may cause hemolysis and release free heme.[96] Free heme activates NADPH oxidase, a major source of ROS in the cytoplasm, and ROS from heme has been shown to induce smooth muscle proliferation, a pathological feature of atherosclerosis and hypertension.[97–99] The smooth muscle proliferation can be further potentiated by inhibition of heme oxygenase.[100] It is thought that the induction of heme oxygenase expression by free heme results in production of carbon monoxide, which can be antiproliferative through blocking T-type Ca^{2+} channels.[101] The induction of *HO-1* does not fully reverse smooth muscle proliferation in pathological conditions; therefore, it is unknown whether further induction of *HO-1* expression in the disease setting offers protection against smooth muscle proliferation. Alternatively, effective modulation of free heme levels can be another approach against smooth muscle proliferation in diseases such as atherosclerosis or hemolytic anemia.

It is also shown that heme in the red blood cells facilitates the formation of NO from nitrite under low oxygen tension.[102,103] The resulting NO causes vasodilation, and this mechanism may increase blood flow to relatively hypoxic areas. The formation of NO from nitrite has been observed in human volunteers infused with either physiological or supraphysiological levels of nitrite.[104] Because it is also present in the circulation under basal conditions, nitrite is believed to be a storage form of NO in the circulation.[102,104] The relevance of basal levels of nitrite in pathological conditions requires further investigation.

Disorders

Altered Heme Synthesis: Porphyrias

The porphyrias are a group of metabolic disorders caused by a partial deficiency in a specific heme biosynthetic enzyme, leading to the accumulation of particular heme biosynthetic intermediates in various tissues of the body. The most common porphyria, porphyria cutanea tarda, is due to acquired inhibition of hepatic uroporphyrin decarboxylase.[105] The porphyrias have been classically organized based on the primary site of initial overproduction and accumulation of heme intermediates (hepatic versus erythropoietic) and on clinical manifestations (acute versus cutaneous). Acute por-

phyrias typically present with nonspecific neurovisceral symptoms, most commonly abdominal pain. Meanwhile, the cutaneous porphyrias affect sun-exposed skin and are further divided into those causing acute, nonblistering photosensitivity and those causing chronic, blistering photosensitivity. The heme synthetic enzymes responsible for several porphyrias are illustrated in Figure 1. In the clinic, heme is a generic term for heme preparations (ie, lyophilized hematin, heme arginate) used as therapies for acute porphyrias. When administered intravenously, heme preparations bind to circulating albumin as heme-albumin and allow for uptake by hepatocytes and renewal of the regulatory heme pool in the liver. This increase in the hepatic heme pool represses hepatic *ALAS1* expression, thereby reducing the levels of ALA and heme synthetic intermediates in the urine and plasma.[106]

Altered heme Synthesis: Sideroblastic Anemias

Anemia often reflects the inability of erythroid precursor cells to support adequate heme synthesis. Although dietary iron deficiency contributes to a significant portion of anemia cases, some anemia cases are caused by certain genetic mutations, leading to iron accumulation in the mitochondria. Erythroid precursors with mitochondrial iron accumulation are termed *ringed sideroblasts* based on the characteristic morphology after Prussian blue staining.[107] Ringed sideroblastic anemia can be caused by 2 categories: disruption in heme synthesis and mutations in the iron-sulfur (Fe/S) cluster maturation pathway.

Sideroblastic Anemias Due to Heme Synthesis Disruption

Multiple mutations in the heme synthesis pathway are associated with sideroblastic anemia. Two major causes of this category will be discussed: sideroblastic anemia associated with *ALAS2* mutation and sideroblastic anemia associated with *SLC25A38* mutation.

Mutations in *ALAS2*, the key heme synthetic enzyme in erythroid cells, lead to X-linked sideroblastic anemia. Many different mutations of *ALAS2* affecting protein expression, stability, binding to cofactors, enzyme activities, and mitochondrial localization have been described in these patients. In addition to defective red blood cell production, patients develop diabetes and heart failure secondary to altered systemic iron regulation.[108] Because *ALAS2* covalently binds to pyridoxine, supplementation of pyridoxine often alleviates the symptoms.[107]

A small group of patients presenting with sideroblastic anemia is refractory to pyridoxine supplementation. These patients do not have mutations in the *ALAS2* gene, and the inheritance pattern of the disease is autosomal recessive. The disease in these patients is caused by mutations of a mitochondrial carrier protein, *SLC25A38*,[109,110] that may play a role in the first step of heme synthesis by importing glycine, a starting material for heme synthesis, into mitochondria.[109]

Sideroblastic Anemias due to Fe/S Cluster Maturation Defects

In addition to disruption of heme synthesis, mutations in Fe/S cluster maturation pathways are associated with sideroblastic anemia. The sideroblastic anemia caused by *ABCB7* (X-linked sideroblastic anemia with ataxia) and *Glrx5* mutations will be described.

Some patients with sideroblastic anemia demonstrate neurological defects in addition to anemia. These patients often have early onset ataxia, whereas the sideroblastic anemia can be mild.[111] Genetic studies mapped the disease mutation to the *ABCB7* gene, which encodes a mitochondrial inner membrane ATP binding-cassette protein.[112,113] Cells with *ABCB7* deficiency have cytoplasmic Fe/S cluster maturation defects and mitochondrial iron accumulation.[114] Although the exact function of *ABCB7* is unclear, it likely transports a substance required for the maturation of cytoplasmic Fe/S clusters, which serve as cofactors for many cytoplasmic proteins. The Fe/S cluster is an important cofactor for cytoplasmic aconitase, also known as *IRP1*, and loss of the protein-bound Fe/S cluster activates *IRP1*. Consequently, *ABCB7* mutation leads to increased *IRP1* activation, which in turn represses *ALAS2* expression through *IRP1* binding to iron response element sites in the 5′ untranslated region of its mRNA. The end result is a reduction of heme synthesis that may account for the anemia in patients with X-linked sideroblastic anemia with ataxia.[115]

In the zebrafish mutant shiraz, severe anemia and reduced expression of the glutaredoxin 5 (*Glrx5*) gene was observed,[116] and homozygous mutation of *Glrx5* has also been identified in a patient with late-onset anemia.[117] *Glrx5* deficiency in a human erythroid cell line impairs Fe/S cluster maturation, which in turn results in activation of the IRP/iron response element system, inhibition of *ALAS2* production, and subsequent anemia. Furthermore, erythroid precursor cells also express a variant of ferroportin 1 that is not downregulated by the IRP system. Consequently, in erythroid precursor cells with *Glrx5* deficiency, ferroportin 1 levels remain high, leading to cellular iron export and further exacerbating the iron deficiency in the cytoplasm and the anemia.[118]

Altered Heme Degradation

HO-1 deletion in mice results in significant iron accumulation on treatment with inflammatory stimuli.[119] Patients with *HO-1* deficiency present with persistent hemolytic anemia, persis-

tent inflammation, and severe growth retardation.[120,121] A lymphoblastoid cell line derived from a patient also demonstrated increased sensitivity to heme in culture.[120] This increased sensitivity may be explained by 2 mechanisms: Heme accumulation results in oxidative damage, and cells with *HO-1* deficiency cannot generate bilirubin, a potent antioxidant.

As noted earlier, impaired *HO-1* expression can lead to endothelial dysfunction and smooth muscle proliferation, both of which are observed in atherosclerosis.[97,122] Patients with *HO-1* deficiency have increased oxidized low-density lipoprotein cholesterol[123] and develop fatty streaks and fibrous caps at a relatively young age.[124] This observation is consistent with increased ROS production through heme accumulation and lack of antioxidants generated from heme degradation. In addition, deletion of *HO-1* in ApoE knockout mice further exacerbates the development of atherosclerosis. Furthermore, vein grafts are more prone to restenosis in *HO-1* knockout mice because of increased neointimal formation, increased calcification, and smooth muscle cell death. The increase in cell death may be the result of sensitivity to oxidative stress; smooth muscle cells from *HO-1* knockout mice are more sensitive to H_2O_2-induced cell death.[125]

Protective Effect of Heme Modulation

Although excess heme is detrimental, heme degradation exhibits protective effects because of the production of carbon monoxide and biliverdin. Carbon monoxide can activate guanylate cyclase, which in turn leads to vasodilation and inhibits platelet aggregation.[126] It also activates the Akt signaling pathway, which provides protection in various stress models, including doxorubicin-induced cardiomyopathy and liver ischemia–reperfusion.[127,128] Moreover, biliverdin can be converted to bilirubin, and the redox cycling between biliverdin and bilirubin is protective against oxidative stress.[129,130]

The cytoprotective effect of *HO-1* overexpression has been demonstrated in multiple injury models, including acute pancreatitis,[131] brain and liver ischemia–reperfusion injury,[132,133] and various cardiovascular disorders. *HO-1* transgenic mice are protected against the development of diabetic cardiomyopathy in the streptozotosin-induced diabetes model and display normalized levels of phosphorylated Akt and AMP-activated protein kinase.[134] Overexpression of *HO-1* through intramyocardial injection of adeno-associated virus also prevented ischemia–reperfusion damage in rat hearts and reduced cardiac remodeling.[135] Induction of *HO-1* expression by cobalt PP IX or adenoviral overexpression prevented angiotensin II–induced hypertrophy in rats and in neonatal rat cardiomyocytes. The protective effect against angiotensin II–induced hypertrophy is attributed to the production of bilirubin because incubating cells with bilirubin recapitulated the protective effect of *HO-1* induction.[136] HO-1 overexpression and incubation of neonatal rat cardiomyocytes with biliverdin or carbon monoxide–releasing molecule suppressed the activation of extracellular signal–regulated kinases (ERK1/2) and the calcineurin/nuclear factor of activated T cells signaling pathway,[137] both of which are involved in cardiac hypertrophy. *HO-1* overexpression has not been shown to exert protective effects in some other models. In mice with aortic pressure overload–induced heart failure, *HO-1* overexpression failed to preserve cardiac function despite decreased interstitial fibrosis. *HO-1* overexpression also exacerbated heart failure in normal aging.[138]

In addition to cardiomyocytes, heme degradation can protect against endothelial dysfunction. Dysfunctional endothelium in vitro and in vivo generates higher levels of ROS, demonstrates increased cell death, and accumulates advanced glycation products. Induction of *HO-1* expression by treating animals with hemin normalized the ability of blood vessels to relax in a rat model of type 2 diabetes. This effect is attributed to the beneficial role of carbon monoxide, a heme breakdown product, because treating type 2 diabetic rats with carbon monoxide–releasing molecules also normalized vascular function.[139] In addition to the generation of heme breakdown products, *HO-1* induction by hemin occurs concurrently with downregulation of cyclooxygenase 1 and NADPH oxidase in spontaneously hypertensive rats and with downregulation of cyclooxygenase 2 in type 2 diabetic rats.[139,140] Cyclooxygenases catalyze the formation of prostaglandins, potent vasoconstrictors which prevent the relaxation of vessels; therefore, downregulation of cyclooxygenase also contributes to the normalization of vascular function by reducing the production of vasoconstrictors. Pharmacological induction of *HO-1* expression also increases the expression and phosphorylation of endothelial NO synthase, which can replenish the NO levels in the vasculature and allow vessels to relax properly.[141]

Hemoglobin and Heme Scavengers

Several pathological conditions, such as sickle cell anemia, ischemia–reperfusion injury, and β-thalassemia, lead to severe hemolysis. When red blood cells are damaged within the vascular compartment, hemoglobin escapes into the plasma, dimerizes, and is rapidly bound by the serum protein haptoglobin. The haptoglobin–hemoglobin complex is recognized by the hemoglobin scavenger receptor CD163 on the surface of monocytes and macrophages. The CD163 receptor strongly binds the haptoglobin–hemoglobin complex and facilitates its endocytosis and degradation.[142] Because haptoglobins are not recycled, severe hemolytic diseases result in rapid haptoglobin depletion and rapid accumulation of free heme.

During intravascular hemolysis, the release of large amounts of hemoglobin and free heme into the circulation may overwhelm the buffering capacity of the heme degradation and storage machinery. To limit free heme availability for free radical formation and bacterial pathogens, mammals use the heme scavenger hemopexin (Hx). Induced by several of the same stimuli that cause hemolytic stress, Hx is an acute-phase glycoprotein that is able to bind an equimolar amount of heme with high affinity.[143] Hx scavenges free heme, and the resultant heme–Hx complex is taken up by the liver through receptor-mediated endocytosis.[144] After heme overload by tail vein injection of hemin, Hx knockout mice display excess plasma heme levels, which promote ROS production, endothelial activation, and vascular permeability. This proinflammatory and procoagulant state stimulates the adherence of leukocytes, platelets, and erythroid cells to the endothelial cell surface, favoring clot formation and, ultimately, the development of thrombi.[145,146]

The rationale for using Hx as a therapy is based on the idea that by restocking the stores of heme scavengers, Hx may chelate circulating free heme and prevent its toxic oxidizing effects. Indeed, in mouse models of sickle cell disease and thalassemia, treatment with purified human Hx twice a week for 1 month reduced iron accumulation in the aortic endothelium and heart, decreased oxidative stress, improved cardiovascular function, and limited the induction of adhesion molecules in the vasculature compared with untreated animals.[145]

Deleterious Effect of Ectopic Heme Induction

Despite the essential role of heme in cardiac pathology, the process of heme synthesis has only recently been studied in the context of heart failure. Recently, a global metabolomics analysis of the cardiac changes that occur in vivo during hypertrophy and heart failure using mouse models of pressure overload–induced cardiac hypertrophy and coronary ligation, respectively, demonstrated increased heme levels in both hypertrophied and infarcted murine hearts.[147] In addition, our laboratory has demonstrated that both cytosolic and mitochondrial heme levels are increased in failing human hearts. Feedback inhibition was observed for the heme synthetic enzymes except for the rate-limiting enzyme *ALAS2*, which was increased in heart failure and the expression of which was previously reported to be restricted to the hematopoietic system. In cultured H9c2 cardiomyoblasts, overexpression of *ALAS2* led to increased heme, oxidative stress, and cell death. *ALAS2* expression and heme levels were also regulated in vitro by hypoxia and erythropoietin, 2 pathways that are commonly altered in heart failure. Furthermore, increased heme levels were associated with elevated oxidative stress and loss of viability in cultured cardiomyoblasts. Meanwhile, *HO-1* levels were unaltered in the failing human hearts, suggesting that HO-1–dependent antioxidation is unlikely to protect hearts from heme toxicity.[61] Consequently, the increase in heme levels in heart failure appears to be a maladaptive process; however, this study only provided an association among *ALAS2* induction, elevated cardiac heme levels, and heart failure. Furthermore, although there are case reports of cardiomyopathy associated with mutations in the *ALAS2* gene, these patients also displayed increased systemic iron levels, and the cardiomyopathy may be secondary to iron toxicity.[148,149] The generation of cardiac-specific *ALAS2* overexpression and knockout mouse models would allow direct testing of the hypothesis that heme accumulation through *ALAS2* expression is deleterious to the heart.

Potential Approaches to Modulate Heme

As discussed earlier, heme is a potent inducer of *HO-1*, so excess heme can also induce its own degradation. In animal studies, a heme analog cobalt PP IX was shown to induce *HO-1* expression.[136] Furthermore, recent studies demonstrated that *HO-1* is induced through activated AMP-activated protein kinase. Transgenic models with constitutively activated endothelial AMP-activated protein kinase have higher expression of *HO-1* and are protected against endothelial damage and vascular dysfunction. The protection is attributed to better re-endothelialization after injury by endothelial progenitor cells.[150] Furthermore, activation of AMP-activated protein kinase by metformin in cultured human endothelial cells also protected endothelial cells against cytokine-induced cell death, and *HO-1* was found to be required for this protective effect by AMP-activated protein kinase activation.[151] Because metformin is a clinically approved medication, several clinical trials investigated whether metformin treatment improved endothelial function. Similar to in vitro and animal studies, metformin improved endothelial function in type 2 diabetic patients[152–154]; however, because insulin resistance is also associated with endothelial dysfunction,[155,156] it is difficult to determine whether this protection is due to a direct effect of metformin on endothelial cells or secondary to improved insulin sensitivity.

In addition to metformin, the angiotensin-converting enzyme inhibitor ramipril induces *HO-1* expression both in vitro and in vivo and reverses the heightened oxidative stress induced by high glucose.[157] Additional efforts should be devoted to determine whether this protective effect is ramipril specific or a general feature of angiotensin-converting enzyme inhibitors.

Conclusion

Altered heme homeostasis has long been associated with hematological diseases, but most of the known diseases are

associated with insufficient heme production. Only recently has the field started to recognize the detrimental effects of excess heme on cardiac physiology. Although heme is critical for the function of a wide variety of proteins, it is also recognized that free heme accumulation causes cellular damage through altering membrane permeability and increasing oxidative stress and is associated with heart failure. The causative role of heme overproduction in the development of cardiomyopathy and heart failure should be investigated in greater detail. Currently, there are effective ways to upregulate heme-degradation machinery in vitro and in animals, but it is still unclear whether these agents can achieve similar effects in large animal studies and in humans. In addition, the side effect profile of these agents should be characterized more extensively. We should note that although activating the heme-degradation machinery may be protective against some injury, heme degradation may be detrimental in other injury processes. Consequently, additional studies are necessary to determine whether the heme synthesis and degradation pathways serve as viable therapeutic targets for cardiac pathologies.

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