MINIREVIEW

# Macrophage Iron, Hepcidin, and Atherosclerotic Plaque Stability

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**Hepcidin has emerged as the key hormone in the regulation of iron balance and recycling. Elevated levels increase iron in macrophages and inhibit gastrointestinal iron uptake. The physiology of hepcidin suggests an additional mechanism by which iron depletion could protect against atherosclerotic lesion progression. Without hepcidin, macrophages retain less iron. Very low hepcidin levels occur in iron deficiency anemia and also in homozygous hemochromatosis. There is defective retention of iron in macrophages in hemochromatosis and also evidently no increase in atherosclerosis in this disorder. In normal subjects with intact hepcidin responses, atherosclerotic plaque has been reported to have roughly an order of magnitude higher iron concentration than that in healthy arterial wall. Hepcidin may promote plaque destabilization by preventing iron mobilization from macrophages within atherosclerotic lesions; the absence of this mobilization may result in increased cellular iron loads, lipid peroxidation, and progression to foam cells. Marked downregulation of hepcidin (e.g., by induction of iron deficiency anemia) could accelerate iron loss from intralesional macrophages. It is proposed that the minimally proatherogenic level of hepcidin is near the low levels associated with iron deficiency anemia or homozygous hemochromatosis. Induced iron deficiency anemia intensely mobilizes macrophage iron throughout the body to support erythropoiesis. Macrophage iron in the interior of atherosclerotic plaques is not exempt from this process. Decreases in both intralesional iron and lesion size by systemic iron reduction have been shown in animal studies. It remains to be confirmed in humans that a period of systemic iron depletion can decrease lesion size and increase lesion stability as demonstrated in animal studies. The proposed effects of hepcidin and iron in plaque progression offer an explanation of the paradox of no increase in atherosclerosis in patients with hemochromatosis despite a key role of iron in atherogenesis in normal subjects.** Exp Biol Med 232:1014–1020, 2007

**Key words:** atherosclerosis; hemochromatosis; hepcidin; iron; macrophage

## Introduction

The "iron hypothesis" suggests a primary protective effect of sustained iron depletion or mild iron deficiency against ischemic heart disease (1–5). The proposal was initially formulated as an explanation for the sex difference in cardiovascular disease and the increase in disease following menopause. The idea, although not yet fully tested and continually debated over the last 25 years (6), has achieved standing as a plausible and testable hypothesis (7–19).

The original presentation of the hypothesis did not specify a mechanism by which diminished iron status might protect against heart disease. One of the early objections to the hypothesis was the perception that there was no plausible mechanism for such protection. With the continuing evolution of our understanding of the roles of iron in oxidative and inflammatory stress, there are now many plausible, potential mechanisms (2, 7, 18, 20–22). It has been proposed that removal of all storage iron from the body decreases the availability of redox-active iron *in vivo* (23) and may protect the vascular system through multiple

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cooperative mechanisms. There is significant experimental support for this hypothesis (reviewed in Ref. 23).

Hepcidin has recently emerged as the key hormone in the regulation of iron balance and iron recycling (24–30). The physiology of this hormone suggests a novel and specific mechanism by which iron depletion or deficiency could promote the stability of atherosclerotic plaques. The interactions proposed here involve effects of hepcidin on excessive iron deposition in the macrophages within atherosclerotic plaque with subsequent increased lipid peroxidation and progression to foam cells. Low hepcidin levels in inherited hemochromatosis may explain the paradoxical effects of iron overload in atherogenesis in patients with hemochromatosis (31). The present proposal does not exclude other possible mechanisms.

## Hepcidin

Synthesis of hepcidin is induced by infection, inflammation, and elevated iron intake. Lower levels are associated with anemia, hypoxia, iron deficiency, and hereditary hemochromatosis (24, 26, 27, 32). Hepcidin mRNA levels also fall substantially within hours following phlebotomy (33). Shortly after an appropriate inflammatory stimulus, interleukin-6 (IL-6) is upregulated, and IL-6 in turn induces hepcidin. Hepcidin inhibits iron release from macrophages, and this inhibition results in a rapid fall in serum iron concentration. The export of iron from enterocytes and macrophages is mediated by ferroportin. Hepcidin acts by binding ferroportin on cell membranes, causing it to be internalized and degraded. In enterocytes, hepcidin-induced ferroportin degradation increases intracellular iron, which is then eventually lost into the intestinal lumen. The end result of the process is an inhibition of iron absorption by the gastrointestinal tract. In macrophages, hepcidin-induced ferroportin degradation traps iron within the cells. Retention of iron within macrophages and hepcidin-mediated inhibition of intestinal iron uptake limit the availability of iron in plasma for participation in injurious oxidative processes as well as for growth of invading microorganisms. Anemia of inflammation is a result of excessive hepcidin synthesis. Hepcidin deficiency appears to be the basis for most cases of hemochromatosis.

A recently described assay for urinary hepcidin revealed the magnitude of differences in synthesis observed in conditions that affected iron homeostasis (34). The assay used surface-enhanced laser desorption/ionization time-of-flight mass spectrometry, and the urinary hepcidin concentration was expressed as megaintensity per millimole of creatinine. The median value for normal subjects was 0.211. Median values associated with iron deficiency, hereditary hemochromatosis, and endotoxemia were 0.030, 0.016, and 5.752, respectively. In this study, urine concentrations of hepcidin were reduced by approximately 7-fold in iron deficiency and 13-fold in hereditary hemochromatosis. By contrast, there was a 27-fold increase in urinary hepcidin in an endotoxemia model in which healthy volunteers received *Escherichia coli* lipopolysaccharide intravenously in the amount of 2 ng/kg body wt. Iron deficiency and hemochromatosis are both associated with large-scale decreases in hepcidin production, despite orders-of-magnitude differences in storage iron in these conditions. The massive iron overload that can occur in hemochromatosis is predominantly localized in parenchymal cells rather than in the normal storage sites for iron within the reticuloendothelial system.

## Iron in Atherosclerotic Plaque

Smith *et al*. (35) provided indirect support for the iron hypothesis in a 1992 study that demonstrated the presence of catalytic iron in material taken from human atherosclerotic lesions. Iron deposits were confirmed in atherosclerotic lesions in the cholesterol-fed rabbit in later studies by nuclear microscopy (36) and in human lesions by conventional histologic staining (37, 38). Although they did not directly assess the effects of iron chelation with deferiprone on vascular iron concentration, Matthews *et al*. (39) observed a large and significant decrease in total aorta cholesterol concentration after chelation treatment in cholesterol-fed rabbits and suggested that "perhaps [deferiprone] functions to remove free iron in the vascular subendothelium" (39).

In a series of studies with New Zealand white rabbits fed a 1% cholesterol diet, Watt and colleagues (13, 40–43) used nuclear microscopy to demonstrate a 7-fold increase in iron concentration within newly formed atherosclerotic lesions compared to healthy artery tissue. Iron accumulation occurred at the onset of lesion formation.

Repeated bleeding sufficient to decrease iron uptake in the artery wall delayed the onset of atherogenesis. In addition, daily treatments with the iron chelator deferoxamine for 9 weeks decreased the average iron level in lesions from 95 ppm dry wt to 58 ppm dry wt ($P = 0.030$) and also significantly reduced average lesion area ($P = 0.038$). The results were interpreted as providing direct evidence of a key role of iron in initiating atherogenesis.

In another series of studies, Chau and colleagues (14, 38, 44–46) differentially screened a cDNA library of human atherosclerotic aorta and discovered that genes for the iron-storage proteins, L-ferritin and H-ferritin, are highly expressed in human atherosclerotic plaques. Similar results were obtained in a study of atherosclerotic aortas from rabbits fed a high-cholesterol diet. As lesions developed in the rabbit aortas, there was a close temporal association between lesion formation and increased ferritin gene expression (38).

Subsequently, Chau and colleagues found stainable iron deposits in atherosclerotic lesions in apolipoprotein E (apoE)-deficient mice (45). Stainable iron increased with age in atherosclerotic lesions as well as in heart and liver. Stainable iron and iron content in aortic tissues were much

less in young mice that received a low-iron diet for 3 months than in littermates that were given standard iron-replete feed. Atherosclerotic lesions were likewise significantly smaller in the iron-restricted animals, and circulating autoantibodies to oxidized low-density lipoprotein (LDL) were also significantly lower. A later study by this group using the same apoE-deficient mouse model (14) indicated that decreased vascular iron content is associated with lower expression of matrix metalloproteinase–9, increased lesional collagen content, and increased plaque stability.

Davies and colleagues (17) used the minimally disruptive techniques of electron paramagnetic resonance (EPR) spectroscopy and inductively coupled plasma mass spectroscopy (ICPMS) to quantify iron in *ex vivo* carotid lesions and in healthy human arteries. Levels of iron were greater in the intima of lesions than in healthy controls (0.370 vs. 0.022 nmol/mg tissue as determined by EPR, 0.525 vs. 0.168 nmol/mg tissue as determined by ICPMS, $P < 0.05$ in both cases). Cholesterol levels in lesions correlated positively with iron accumulation. In a later study (47), iron levels in human atherosclerotic plaque correlated positively and strongly with multiple markers of protein oxidation but not with markers of lipid oxidation.

Lapenna *et al.* (48) studied the relationship between serum ferritin and low-molecular-weight iron (LMWI) in *ex vivo* carotid endarterectomy specimens. They found that LMWI in these atherosclerotic specimens correlated significantly with serum ferritin. They also reported an association of plaque LMWI with markers of lipid peroxidation in the largest subgroup of patients with <90% stenosis. In a small subgroup of patients with >90% stenosis, the association of LMWI and lipid peroxidation markers did not achieve significance. These data are consistent with the possibility that the concentration of redox-active iron within atherosclerotic lesions can be lowered by depletion of body iron stores.

Yuan and colleagues (7, 37, 49–53) also documented the presence of iron in atherosclerotic vascular tissue, suggested erythrophagocytosis as an important source of this iron, and considered the role of such iron in increasing the intralesional concentration of redox-active iron. They also explored the interactions of iron and lipoproteins as plaque macrophages progress to apoptotic foam cells. Ingestion by cultured macrophages of iron-rich ceroid derived from plaque induces apoptosis, a process that is inhibited if ceroid is treated first with an iron chelator (54). In more recent work, Yuan and colleagues have found that increased ferritin and transferrin receptor are significantly correlated with macrophage infiltration in human carotid atheroma. Transferrin receptor expression is significantly higher among patients with symptomatic carotid atherosclerosis than among asymptomatic ones.[1]

Much of the iron within plaque is associated with macrophages and foam cells. The source of the iron remains a matter of investigation; however, it seems clear that a portion of the iron in established lesions is derived from microhemorrhage within plaque (55). Iron not derived from microhemorrhage is probably present even in early lesions. In a study of developing atherosclerotic lesions in the cholesterol-fed rabbit, Pang *et al.* (38) noted increased ferritin gene expression from early stages of lesion development.

## Role of Iron-Laden Macrophages in Plaque Progression

The macrophage is a key cell type in the formation and fate of atherosclerotic plaque. The roles of the macrophage in atherogenesis and the details of the sequence of events in which a circulating monocyte develops into a resting intralesional macrophage, which becomes a foam cell that eventually undergoes apoptosis are the subjects of intense investigation. The storage and processing of iron from erythrophagocytosis and other sources by macrophages within plaque have an important role in disease progression. Loading of iron in the macrophage promotes uptake of lipids through stimulation of expression of the macrophage scavenger receptor–1 (56). Oxidative reactions associated with the acquisition of iron and lipid may facilitate macrophage apoptosis with the release of cellular contents into the lesion. The release of iron and lipid contributes to the acellular mass of the lesion and probably recruits more monocytes to amplify this process. Interruption of iron acquisition and storage in plaque macrophages by iron restriction or iron chelation inhibit lesion initiation and progression (13, 45).

Asleh *et al.* (57) suggested that hemoglobin within plaque derived from microvascular hemorrhage is cleared more slowly from plaques associated with the haptoglobin (Hp) 2-2 genotype than from plaques associated with the Hp 1-1 genotype. The Hp 2-2 genotype is associated with an increased risk of atherosclerotic cardiovascular disease. In a more recent study these investigators created a type-2 Hp allele in the apoE-deficient mouse and explored the effect of the Hp 2-2 genotype on iron, lipid peroxidation, and macrophage accumulation in plaque (58). They found (58) that "intra-plaque hemorrhage generates greater iron deposition in mice with the Hp 2-2 genotype, leading to increased oxidation of lipids and other cellular constituents of the plaque." Slower clearance of hemoglobin-derived iron within lesions, in this case because of an Hp polymorphism, caused increased lipid peroxidation and macrophage accumulation in atherosclerotic lesions (58).

An analogous pattern may occur with polymorphisms in heme oxygenase 1 (HO1), an inducible enzyme that catalyzes the rate-limiting step in heme catabolism. HO1 has a key function in mobilizing macrophage iron derived from senescent erythrocytes back to the marrow to support erythropoiesis. Alterations in the activity of HO1 influence the rate of clearance of hemoglobin derived iron from

[1] Yuan XM. Personal communication, 2007.

macrophage storage sites. In the HO1-deficient mouse (HO1-/-), conspicuous iron loading is seen in Kupffer cells, hepatocytes, hepatic vascular tissue, and renal cortical tubules (59). In humans, HO1 promoter polymorphisms that cause weaker upregulation of the enzyme are associated with increased cardiovascular disease (60, 61). In spleen macrophages, HO1 is a component of a mediated iron efflux system (62). In a kidney ischemic injury and nephrotoxicity model, Pittock *et al*. (63) have shown that HO1-/- mice show a marked upregulation of monocyte chemoattractant protein-1 (MCP1), even in animals not subjected to oxidative stress.

Taken together, these findings are consistent with the concept that slower clearance of hemoglobin-derived iron associated with HO1 polymorphisms causing diminished upregulation may promote atherosclerotic plaque progression. Statins have recently been found to induce HO1 in murine macrophages (64). It was suggested that this induction may be the basis for the protective actions of statins that are independent of their cholesterol-lowering effect. The proposals presented here raise the possibility that statins may stabilize atherosclerotic plaques by inducing intralesional HO1, facilitating iron mobilization, and thereby lowering plaque iron levels.

Macrophages are commonly viewed as a safe repository of stored iron; however, under the influence of high hepcidin levels or other factors that slow the mobilization of iron, macrophages may acquire a load of iron sufficient to be toxic to itself and surrounding cellular elements. Iron-overloaded macrophages excrete ferritin and iron (50). The rate of ferritin and iron excretion from iron-loaded macrophages is increased by the presence of oxidized LDL and decreased by high-density lipoprotein (50). The combination of increased availability of redox-active iron derived from ingested iron and increased lipid load results in the formation of highly toxic material including iron-laden ceroid and other highly cytotoxic lipidaceous material (54). With the death of the iron-laden macrophages, the interior of atheromatous plaque becomes increasingly cytotoxic for existing cellular elements and new monocytes recruited to the destabilizing lesion.

## Do Low Levels of Hepcidin Inhibit Lesion Progression?

It is proposed that hepcidin can promote progression of atherosclerotic plaque by slowing or preventing the mobilization of iron from macrophages within atherosclerotic plaque. Levels of hepcidin associated with modest loads of stored iron may be sufficient to effectively trap iron within macrophages in existing plaques and allow the progression to cell death and release of contents into lesions. As a given lesion evolves toward the unstable state, the lesion itself may become more of an inflammatory stimulus and thus may augment upregulation of hepcidin synthesis. Intralesional iron overload in plaque macrophages could be associated with small amounts of systemic stored iron because of increased inflammation-induced hepcidin upregulation. The hypothesized minimally proatherogenic level of hepcidin is approximately that level associated with iron deficiency anemia or homozygous hemochromatosis, as these two conditions are associated with very low levels of macrophage iron. Lesser degrees of iron depletion are also associated with the loss of iron from plaque macrophages. Hepcidin-induced trapping of iron within plaque macrophages may be an important mechanism for promotion of cardiovascular disease by systemic inflammation. Hepcidin appears to exaggerate the effects of either modest iron loading or modest iron depletion in macrophages. High, induced levels of hepcidin reinforce retention with iron loading, whereas low levels facilitate release with iron depletion. There may also be pathologic consequences of a hepcidin-dependent increase in intracellular iron content in other vascular cellular elements that express ferroportin (e.g., endothelial cells; Ref. 65). In the case of endothelial cells, iron content appears to be a key regulator of vascular reactivity (5, 10, 18).

## No Increase in Atherosclerosis in Hemochromatosis: A Paradox Resolved?

A long-standing criticism of the iron hypothesis has been the contention that atherosclerotic heart disease is not a feature of homozygous hemochromatosis. Excessive iron can increase myocardial injury, especially following ischemia and reperfusion. In fully developed iron-overload cardiomyopathy, direct damage to heart muscle can occur without an occlusive event. Given the state of understanding of iron metabolism at the time, this suggested the possibility of an increase in clinically apparent cardiovascular events in hemochromatosis heterozygotes (66), even in the absence of significant structural atherosclerotic disease. However, these early proposals concerning heterozygotes were incomplete as they were developed years before the discovery of the mutations responsible for most cases of hemochromatosis and even longer before the emergence of hepcidin as the key hormone in iron homeostasis.

Some of the implications of disease expression in hemochromatosis for the iron hypothesis were presented in 2001 in a paper by Sullivan and Zacharski (31). Under the heading "Low macrophage iron in hemochromatosis: A protective factor against atherosclerosis?", the following was noted (31):

> There is evidence for a macrophage defect in hemochromatosis leading to a constriction rather than an expansion of the macrophage/reticuloendothelial iron pool. This may be a factor that tends to protect homozygotes from foam cell formation and thus, to a degree, some specific protection against atherosclerosis. In heterozygotes, the tendency to increased iron loading may be partially opposed with respect to atherogenesis because of a partial effect in the macrophage.

The original proposal concerning cardiovascular disease in hemochromatosis (66) has been followed by a number of studies (31) that, taken together, are consistent

with a degree of protection against structural atherosclerotic lesions in hemochromatosis homozygotes (67, 68) and perhaps also in heterozygotes, as noted, for example, in a study by Franco *et al*. (69).

The emerging details of the physiology of hepcidin make it clear that there is no necessary contradiction between the suppression of atherogenesis by iron deficiency and a similar effect in cases of homozygous hemochromatosis associated with low hepcidin levels. Macrophages empty of iron are characteristic of both iron deficiency and most cases of hemochromatosis. Diminished atherosclerosis in both iron deficiency and massive inherited iron overload would not be a paradox, if low levels of hepcidin promote plaque stability.

## Progression to Unstable Plaques Through a Positive Feedback Loop

As atherosclerotic plaques progress in complexity and become unstable, the plaques themselves may increasingly become an inflammatory stimulus. Reported associations of IL-6 with atherosclerotic disease (70, 71) may reflect a generalized inflammatory state. However, it appears likely that, at some stages of progression (perhaps an early stage), inflamed atherosclerotic tissue may upregulate hepcidin production through upregulation of IL-6. If hepcidin-induced retention of iron within plaque macrophages contributes to the intensity of the inflammatory stimulus, there is the possibility for positive feedback to occur. The result could be progressively higher production of hepcidin that in turn augments macrophage iron retention and iron-associated inflammation.

The FeAST trial has provided indirect evidence of increased IL-6 production associated with atherosclerotic plaque (19, 72). This was a randomized, controlled trial of mild iron reduction therapy in patients with peripheral vascular disease with a mean observation period of 3.5 years. No overall cardiovascular benefit was found. However, in the youngest age quartile randomized to iron reduction (ages 43–61 years), there was a 54% reduction in total mortality ($P = 0.019$), which was the primary endpoint, and a 57% reduction in death plus nonfatal myocardial infarction (MI) and stroke ($P < 0.001$), which was the secondary endpoint, in comparison with control patients. The mortality benefit in the youngest quartile was seen in association with a phlebotomy-induced reduction in serum ferritin from a mean entry value of 122.5 ng/ml to 79.7 ng/ml at the end of the study, a 35% decrease.

FeAST participants had increased IL-6 levels at baseline, and IL-6 was positively correlated with serum ferritin (70). In a sample of FeAST participants randomized to iron reduction therapy, the average pretreatment concentration of serum IL-6 (19 pg/ml) differed significantly from the average value of 4 pg/ml in a healthy reference control group ($P < 0.01$). In the trial, phlebotomy was used to lower serum ferritin levels, although the study design explicitly prevented achievement of iron deficiency or even full iron depletion. Perhaps because of insufficient iron reduction, there was no overall effect of phlebotomy on IL-6 levels (70). However, among those in the upper quartile for IL-6, there was a significant reduction after 12 months of the phlebotomy protocol. In this group, IL-6 fell from an average of 57 pg/ml to 13 pg/ml ($P = 0.03$). In the high IL-6 group, the healthy control level of 4 pg/ml was not reached. Another study has reported an IL-6 concentration of 1.4 pg/ml in iron deficiency (30). The FeAST trial did not measure iron levels in plaque or the effects of the intervention on intralesional iron.

Baseline plasma IL-6 concentration has been reported to be positively associated with future risk of MI in the Physicians' Health Study (71). IL-6 values in this study gave a rough perspective on the magnitude of the concentrations observed in the FeAST participants. Among these healthy subjects (71), the lowest IL-6 quartile had a range $<1.04$ pg/ml, and the highest a range $>2.28$ pg/ml. The fully adjusted relative risk for MI in the highest quartile of the total cohort was 2.3. IL-6 is thus another example of a cardiac risk factor that can be modified by lowering iron status (16). Because of study design constraints that limited the amount of iron removal, FeAST participants on average fell short of achieving the IL-6 concentrations associated with the lowest risk of MI (70). How IL-6 might act to increase MI risk is not established (71, 73). It is possible that IL-6 increases risk in part by its induction of hepcidin.

## Concluding Comment

Human carotid atherosclerotic lesions contain 3- to 17-fold more iron than healthy control arteries (17), depending on the method of measurement. Iron deficiency is associated with the disappearance of stainable iron from tissue, as commonly observed, for example, in iron-stained bone marrow biopsies from iron-deficient patients. The findings of Watt and colleagues (43) and of Chau and colleagues (14, 45) confirm that plaque macrophage iron can be mobilized by dietary iron restriction, phlebotomy, or iron chelation.

Long clinical experience with the effects of iron deficiency and the results of animal experiments indicate that excess plaque iron should be able to undergo mobilization from human atherosclerotic plaques *in vivo* in response to iron deficiency. Iron-deficient erythropoiesis, if sufficiently intense and prolonged, can mobilize and relocate virtually all stored iron in the body to maturing erythroid precursors in the marrow. In iron deficiency, the process of mobilization is facilitated by extreme downregulation of hepcidin. Future clinical studies are needed to determine the duration and degree of induced iron deficiency required for restoring iron levels in atherosclerotic vessel segments to the much lower level seen in healthy vascular tissue. Studies are also needed to determine the level of hepcidin downregulation that is associated with relocation of stored iron from intralesional macrophages to

erythroid precursors. The most important remaining question is whether iron-depleted lesions in humans will also decrease in size and show increased stability, as previously demonstrated in animal studies (13, 14, 45).


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