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Testing the Iron Hypothesis in a Mouse Model of Atherosclerosis

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SUMMARY

Hepcidin, the iron-regulatory hormone and acute phase reactant, is proposed to contribute to the pathogenesis of atherosclerosis by promoting iron accumulation in plaque macrophages, leading to increased oxidative stress and inflammation in the plaque (the "iron hypothesis"). Hepcidin and iron may thus represent modifiable risk factors in atherosclerosis. We measured hepcidin expression in *Apoe*$^{-/-}$ mice with varying diets and ages. To assess the role of macrophage iron in atherosclerosis, we generated *Apoe*$^{-/-}$ mice with macrophage-specific iron accumulation by introducing the ferroportin *ffe* mutation. Macrophage iron loading was also enhanced by intravenous iron injection. Contrary to the iron hypothesis, we found that hepatic hepcidin expression was not increased at any stage of the atherosclerosis progression in *Apoe*$^{-/-}$ or *Apoe/ffe* mice and that the atherosclerotic plaque size was not increased in mice with elevated macrophage iron. Our results strongly argue against any significant role of macrophage iron in atherosclerosis progression in mice.

INTRODUCTION

Whether iron promotes atherosclerosis is an unresolved question. Multiple studies in animals and humans over the last 30 years assessed the effect of increased body iron on atherosclerosis but have yielded inconsistent results (Sullivan, 2007). Although iron is present in human atherosclerotic plaque at higher concentrations than in healthy arterial tissue (Stadler et al., 2004), it is not clear why iron accumulates in the plaque, whether it is deleterious, and whether its specific cellular and subcellular location is important for any harmful effects. The macrophage is a key cell type in the formation and fate of atherosclerotic plaque, eventually evolving into a foam cell that undergoes apoptosis. Iron catalyzes the generation of reactive oxygen species and could therefore promote lipid oxidation and contribute to atherosclerotic plaque instability (Ramakrishna et al., 2003). The liver-produced hormone hepcidin is the main regulator of body iron levels and its tissue distribution (Ganz and Nemeth, 2012). Hepcidin controls the major flows of iron into plasma: the absorption of dietary iron in the intestine, recycling of iron by macrophages that ingest old erythrocytes and other cells, and mobilization of stored iron from hepatocytes. Iron is exported from these tissues into plasma through ferroportin, the sole cellular iron efflux channel and the hepcidin receptor. Hepcidin causes endocytosis and degradation of ferroportin, leading to the retention of iron in cells and decreased flow of iron into plasma. Hepcidin, as an acute phase protein, is increased in inflammatory disorders, where it causes iron sequestration in macrophages and hypoferremia (Ganz and Nemeth, 2012). Considering the chronic inflammatory nature of atherosclerotic disease, hepcidin production and plasma concentrations would be expected to increase in atherosclerosis. In his refined "iron hypothesis," Sullivan proposed that, under the influence of the increased concentrations of hepcidin, iron is primarily sequestered in macrophages and that iron-laden macrophages within the plaque are an important promoter of atherosclerosis (Sullivan, 2009b). Iron overload that spares macrophages, as in human hereditary hemochromatosis caused by hepcidin deficiency, is not associated with increased incidence of cardiovascular diseases, including coronary artery disease, other forms of ischemic heart disease, stroke, or peripheral artery disease, except for cardiomyopathy caused by iron-mediated damage to cardiomyocytes (Sullivan, 2009a). Several mouse models of iron overload (i.e., hereditary hemochromatosis) are available, but, like their human counterparts, they accumulate iron in hepatocytes rather than macrophages and thus are inappropriate for studies on the role of excess macrophage iron in atherosclerotic plaques. The flatiron (*ffe*) mouse is the first mouse model that accumulates iron in macrophages without other confounding abnormalities (Zohn et al., 2007). The flatiron mouse has a heterozygous H32R mutation in ferroportin that causes a dominant negative mistrafficking of the iron exporter. The relative deficiency of ferroportin results in iron overload in macrophages and replicates the effect of increased hepcidin concentration on macrophages. We generated a mouse model

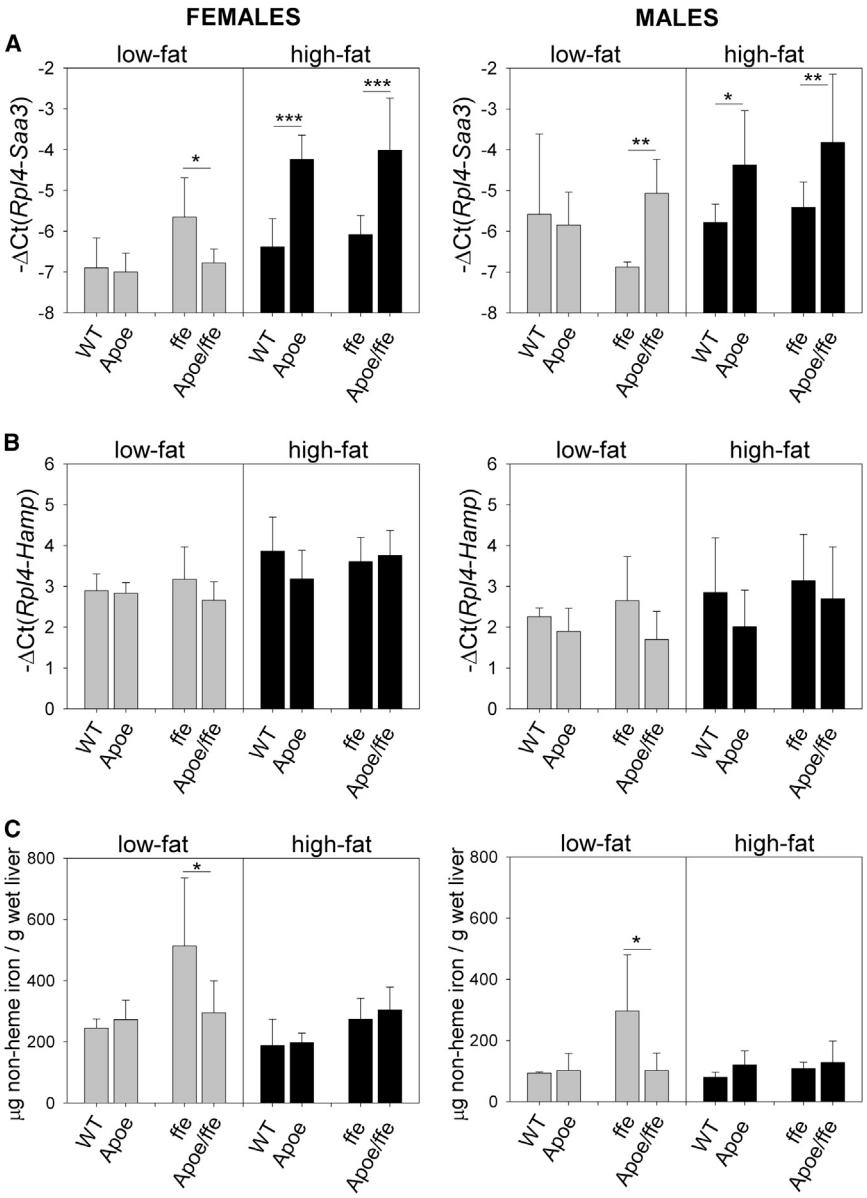

Figure 1. Saa3 mRNA, Hepcidin mRNA, and Liver Iron Analysis in Atherosclerotic Mice

(A and B) Wild-type, *Apoe*$^{-/-}$, *ffe*, and *Apoe/ffe* mice were fed for 4 months with either a standard chow (300 ppm Fe, gray bars) or the high-fat diet (300 ppm Fe, black bars). (A) Serum amyloid A-3 (*Saa3*) and (B) hepcidin (*Hamp*) mRNA levels were measured by qPCR. Values shown are means of −ΔCt (i.e., Ct *Rpl4* − Ct *Hamp or Saa3*) ± SD.

(C) Liver nonheme iron content (mean ± SD). For each gender and diet, statistical analysis was conducted to evaluate the effect of atherosclerotic genotype: *Apoe*$^{-/-}$ mice were compared to WT, and *Apoe/ffe* were compared to *ffe* mice. *p < 0.05, **p < 0.01, and ***p < 0.001 by Student's t test (n = 5–11). None of the comparisons in (B) are statistically significant.

by breeding *ffe* with *Apoe*$^{-/-}$ mice, a classical mouse model of atherosclerosis, to study *Apoe*$^{-/-}$*/ffe* mutants that were expected to have more macrophage iron than *Apoe*$^{-/-}$ mice. We examined the effect of increased macrophage iron on atherosclerosis progression and vascular calcification and also explored the effect of atherosclerosis or high-fat diet on systemic (hepatic) hepcidin production.

RESULTS

The Effects of Atherosclerosis and Dietary Fat on Hepcidin

Inflammation and Hepcidin

To test whether the liver hepcidin production was increased by the atherosclerosis-associated systemic inflammation, we compared *Apoe*$^{-/-}$ to wild-type (WT) mice and *Apoe/ffe* to *ffe* mice after 4 months of either the low-fat or high-fat diet, both containing 300 ppm iron. The acute-phase protein serum amyloid A-3 was used as a sensitive biomarker of inflammation. On the high-fat diet, *Apoe*$^{-/-}$ mice compared to WT mice and *Apoe/ffe* mice compared to *ffe* mice had significantly increased *Saa3* mRNA (2- to 4-fold) in both genders, consistent with the severe atherosclerosis seen in mice lacking ApoE (see Figure 1A). As expected on the low-fat diet causing much milder atherosclerosis, the increase in *Saa3* was absent or less consistent in *Apoe*$^{-/-}$ or *Apoe/ffe* mice.

Hamp mRNA levels were not increased but were consistently similar in *Apoe*$^{-/-}$ mice compared to WT mice or in *Apoe/ffe* mice compared to *ffe* mice on the same diet (Figure 1B).

To determine whether a more marked inflammatory effect of atherosclerosis on hepcidin emerged with age, groups of *Apoe*$^{-/-}$ and *Apoe/ffe* mice were maintained on the high-fat diet containing 300 ppm Fe for 8 months, but no further increase in *Saa3* was observed compared to the 4-month time point (Figure S1A), indicating that inflammation was already maximal after 4 months of the high-fat diet. Hepcidin mRNA in the 8-month group compared to that in the 4-month group was not increased but rather diminished in females or remained the same in males (Figure S1B).

In addition to *Saa3*, we also examined hepatic interleukin-6 (*Il6*) and *haptoglobin* mRNA expression. *Il6* expression was close to the lower limit of detection, and for either marker, only a small and inconsistent increase was detected in atherosclerotic mice compared to nonatherosclerotic mice (Figure S2), suggesting that the systemic inflammation accompanying atherosclerosis

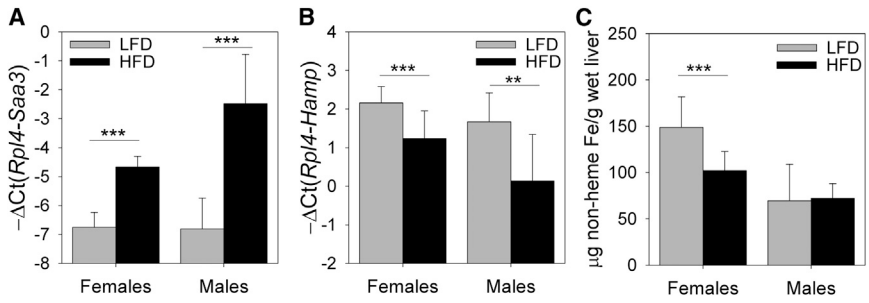

Figure 2. *Saa3* mRNA, Hepcidin mRNA, and Liver Iron Analysis in *Apoe*$^{-/-}$ Mice

(A and B) For 2 months, *Apoe*$^{-/-}$ mice were fed either the low-fat diet containing 50 ppm Fe (LFD, gray bars) or the high-fat diet containing 50 ppm Fe (HFD, black bars) (n = 6–10). F, females; M, males. (A) *Saa3* mRNA. (B) Hepcidin mRNA.

(C) Liver nonheme iron concentrations. Vertical bars show the means, and error bars show SDs. Statistical analysis was performed to evaluate the effect of worsening atherosclerosis: mice on the low-fat diet were compared to those on the high-fat diet by Student's t test. ***$p \leq 0.001$. **$p = 0.01$.

in these mouse models is relatively mild and possibly insufficient to promote hepcidin production.

Iron Stores and Hepcidin

A high-fat diet could interfere with iron absorption (Sonnweber et al., 2012; Chung et al., 2011), thereby decreasing liver iron stores and counterregulating the effect of inflammation on hepcidin. However, at 4 months, except for the difference between *ffe* and *Apoe/ffe* mice on the low-fat diet, there was no difference in liver iron content between WT and *Apoe*$^{-/-}$ mice when maintained on the same diet (either low fat or high fat, both with 300 ppm iron) or between *ffe* and *Apoe/ffe* littermates on the high-fat diet (Figure 1C). On the whole, this indicates that the lack of hepcidin increase in atherosclerotic mice cannot be explained by the counterregulation of hepcidin by liver iron stores. At 8 months, liver iron stores of *Apoe*$^{-/-}$ and *Apoe/ffe* mice were higher than at 4 months as would be expected (Figure S1C), but despite this, hepcidin mRNA did not increase (Figure S1B).

The inhibitory effect of the high-fat diet on iron stores within each genotype was either small (female WT and *Apoe*$^{-/-}$) or absent (male WT and *Apoe*$^{-/-}$ and both genders of *Apoe/ffe*) (Figure 1C). Only *ffe* mice showed reproducible decrease in liver iron on the high-fat diet in both genders, suggesting that the high-fat diet may manifest its inhibitory effect under conditions where iron absorption is already stressed, as may be the case in *ffe* mice where the decreased expression of mutant ferroportin in the intestinal epithelium could limit absorptive capacity for iron. It is interesting that, even on the low-fat diet, *Apoe/ffe* mice had significantly less liver iron than *ffe* mice (Figure 1C), showing that *Apoe* ablation itself can inhibit iron absorption, possibly through its effects on lipid metabolism.

Because hepcidin expression is increased by iron loading (Ganz and Nemeth, 2012), we considered that feeding mice with 300 ppm iron for 4 months may have raised baseline hepcidin mRNA enough to render it poorly responsive to inflammation. We therefore examined *Apoe*$^{-/-}$ mice fed diets containing 50 ppm iron (close to the mouse daily iron requirement), with either low or high fat content for 2 months. *Saa3* mRNA increased on the high-fat diet compared to the low-fat diet (Figure 2A). However, hepatic *Hamp* mRNA expression was not induced but even decreased (~2-fold) in *Apoe*$^{-/-}$ mice fed the high-fat diet (Figure 2B). The mild hepcidin decrease could potentially be related to iron malabsorption on the high-fat diet (Sonnweber et al., 2012; Chung et al., 2011), although only female *Apoe*$^{-/-}$ mice had a small decrease in liver iron content (Figure 2C).

Together, the lack of hepcidin increase in atherosclerotic mice on either 50 ppm or 300 ppm iron diets, with or without the *ffe* mutation, reflects the very low intensity of the inflammatory response associated with the atherosclerotic process, insufficient to raise hepcidin significantly.

The Effects of Macrophage Iron on Atherosclerosis

The Effects of the Ferroportin ffe Mutation

To explore the effects of iron retention in macrophages on atherosclerosis, we compared *Apoe/ffe* mice with *Apoe*$^{-/-}$ mice. After 4 months on the high-fat diet, *Apoe/ffe* mice had more splenic iron than *Apoe*$^{-/-}$ mice (Figure 3A), confirming that the *ffe* mutation caused macrophage iron retention. Perls' stains of spleen sections confirmed that iron retention was confined to the macrophage-rich red pulp (Figure S3).

Contrary to our hypothesis, the size of atherosclerotic lesions in *Apoe/ffe* mice was not larger, but even smaller, than in *Apoe*$^{-/-}$ mice (Figure 3B). Furthermore, en face analysis of whole aortas, innominate artery lesions, and chondrocytes count revealed no significant effects of *ffe* in the *Apoe*$^{-/-}$ model (data not shown). However, aortic lesion calcification was modestly higher in *Apoe/ffe* mice than in *Apoe*$^{-/-}$ mice, although this did not reach statistical significance for individual genders (Figure 3C). Furthermore, the effect of iron on calcification was not consistently observed as described in the next paragraph for mice treated with parenteral iron.

To ascertain whether the *ffe* mutation promotes atherosclerosis in the absence of the atherogenic *Apoe* mutation, we compared *ffe* mice to WT mice after 4 months on the high-fat diet. Atherosclerotic lesions were very small in both groups (median lesions sizes were 219 μm^2 in WT, n = 13; and 144 μm^2 in *ffe* mice, n = 26), and neither WT nor *ffe* mice developed calcifications.

The Effects of Parenteral Iron Supplementation

To further enhance macrophage iron loading in atherosclerotic mice, we administered parenteral iron by weekly injections of 2 mg iron sucrose for 8 weeks while feeding mice the high-fat diet (50 ppm iron). Treatment with iron sucrose resulted in greater iron accumulation not only in the liver and the spleen (Figures 4A and 4B; Figure S4) but also around the aortic lesions (Figure 4D, tissue sections of *Apoe*$^{-/-}$ mice are shown, and similar iron accumulation was observed in *Apoe/ffe*). Hepatic hepcidin expression was also increased by iron sucrose treatment (Figure 4C). However, the size of atherosclerotic lesions did not increase, but even tended to decrease with iron

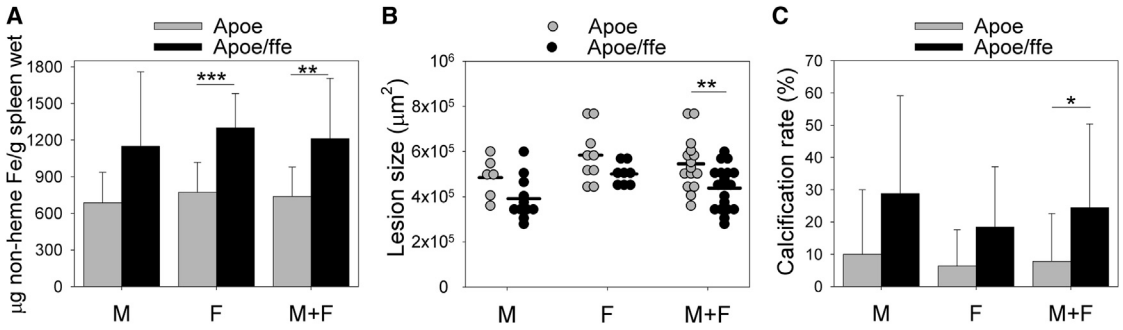

Figure 3. Despite Iron-Loaded Macrophages, *Apoe/ffe* Mice Do Not Develop More Severe Atherosclerosis than *Apoe*$^{-/-}$ Mice

(A and B) *Apoe*$^{-/-}$ (n = 15) and *Apoe/ffe* (n = 19) mice were fed the high-fat diet with 300 ppm Fe for 4 months. (A) Nonheme spleen iron content was measured to confirm macrophage iron loading associated with *ffe* mutation. Means and SDs are shown. (B) Atherosclerotic lesion size is given in squared micrometers (horizontal lines represent the means).

(C) Calcification count (mean percentage of slides showing calcification ± SD). For each gender, *Apoe*$^{-/-}$ mice were compared to *Apoe/ffe* mice using Student's t test (*p < 0.05; **p < 0.01; ***p < 0.001).

treatment, in both *Apoe*$^{-/-}$ and *Apoe/ffe* mice (Figure 4E), and no lesion calcifications were observed. There was also no difference in lesion size when *Apoe*$^{-/-}$ mice were compared to *Apoe/ffe* mice on the same treatment. Hepatic *Il6* and *Saa3* mRNA levels were unchanged by parenteral iron supplementation (Figure S5).

DISCUSSION

We report that liver hepcidin, the central regulator of iron homeostasis, is not influenced by inflammation in a standard mouse model of atherosclerosis (*Apoe*$^{-/-}$) after 2, 4, or 8 months on the high-fat diet. The results argue against the hypothesis that increased systemic hepcidin promotes macrophage iron retention in atherosclerosis. Furthermore, we report that increased macrophage iron, achieved either through a genetic mutation in the iron exporter ferroportin or through parenteral iron administration, also fails to promote atherosclerosis in mice. Although increased cellular iron would be expected to result in greater oxidative stress, one possible explanation for the lack of effect of macrophage iron loading on atherosclerosis is that iron is effectively chaperoned in macrophages by ferritin, and macrophage antioxidant defenses are very efficient. Compared to parenchymal cells such as hepatocytes and cardiac myocytes, macrophages are highly resistant to iron-induced damage, as evidenced by the lack of clinical consequences of the pure loss-of-function mutations in ferroportin (classical ferroportin disease) (Le Lan et al., 2011).

By measuring liver iron content, we demonstrated that the absence of systemic (hepatic) hepcidin increase in atherosclerotic mice was not due to inhibitory effects of the high-fat diet on iron absorption (Sonnweber et al., 2012). The absence of hepcidin increase was also not due to anemia, as a subset of *ffe*, *Apoe*$^{-/-}$, and *Apoe/ffe* mice in which a sufficient sample was available for hemoglobin measurements, all had normal hemoglobin (data not shown). Instead, the absence of hepcidin increase may be related to the relatively mild systemic inflammation caused by atherosclerosis in our model and in most human atherosclerosis. Although hepatic *Saa3* mRNA was elevated in our model, other markers of inflammation, including hepatic *Il6* and *haptoglobin* mRNA, were not consistently increased. In humans, measurements of serum IL-6, the most important inflammatory driver of hepcidin (Ganz and Nemeth, 2012), show statistically significant differences when thousands of patients are studied but also a very large overlap between atherosclerotic heart disease cases and controls [Reykjavik study, $\log_e$(IL-6) = 0.78 ± 0.74 versus 0.65 ± 0.76; British Regional Heart Study, 0.97 ± 0.62 versus 0.79 ± 0.63) (Danesh et al., 2008). Thus, the mild inflammation in the mouse models used in our study is not out of line with human disease characteristics. Although plaque inflammation is central to the atherosclerotic process, systemic inflammation is not an invariant feature of atherosclerosis. It requires very sensitive markers, such as high sensitivity C-reactive protein in humans or Saa3 in mice, to be reliably detectable.

Our study cannot exclude the possibility that hepcidin is locally increased in macrophages and adipocytes in the plaque environment and may promote macrophage iron accumulation locally. However, because the manipulations of macrophage iron content in our model (designed to mimic the effect of either systemic or local hepcidin excess) failed to alter the atherosclerotic phenotype, there was no compelling rationale to study the local production of hepcidin in the plaque.

In addition to introducing *ffe* ferroportin mutation on to an *Apoe*$^{-/-}$ background, we enhanced macrophage iron loading by injecting *Apoe*$^{-/-}$ and *Apoe/ffe* mice on the Western diet with iron sucrose, a common adjunctive therapy used in patients with chronic kidney diseases, also predisposed to accelerated atherosclerosis (Johnson et al., 2010). Iron sucrose injections load macrophages effectively because the compound is first processed by macrophages in order to release iron from the carrier. To balance out the losses, mice normally require about 35 µg of iron per gram of diet and consume about 4 g of food per day (although not all of the dietary iron is absorbed), thus acquiring less than 1 mg of iron per week. We injected 16 mg of iron over 2 months. Nevertheless, despite nonphysiologically high concentrations of macrophage iron, we did not observe any worsening of the atherosclerotic phenotype, as the lesions

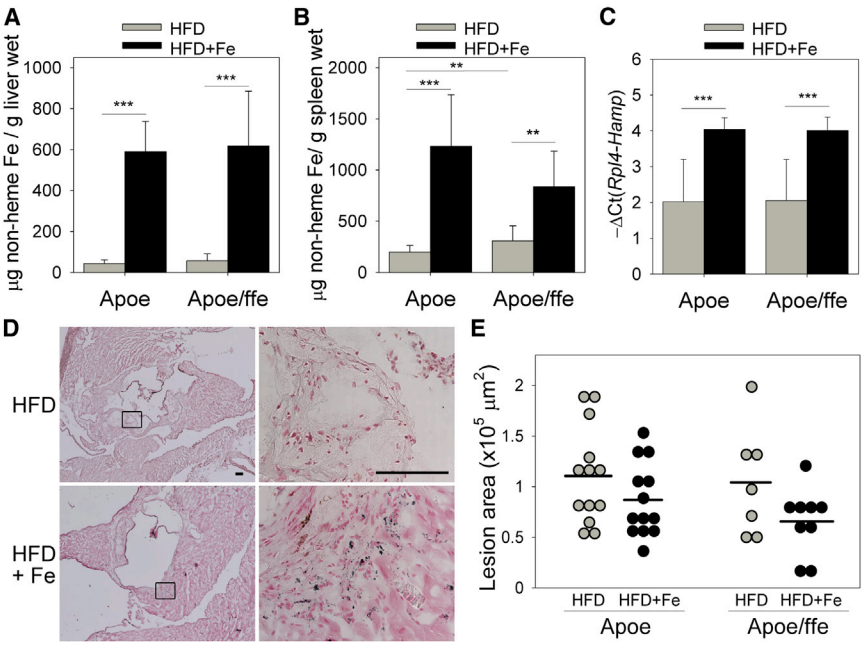

Figure 4. Parenteral Iron Administration Does Not Worsen Atherosclerosis in *Apoe*$^{-/-}$ and *Apoe/ffe* Mice

(A and B) *Apoe*$^{-/-}$ and *Apoe/ffe* mice were fed the high-fat diet for 2 months (50 ppm Fe). One group received weekly iron sucrose injections for 8 weeks (16 mg iron total, HFD+Fe) (n = 13 for *Apoe*$^{-/-}$ and 9 for *Apoe/ffe*), and the other group did not (HFD) (n = 13 for *Apoe*$^{-/-}$ and 7 for *Apoe/ffe*). (A) Nonheme liver iron and (B) spleen iron content were quantitated to confirm macrophage iron loading induced by iron sucrose injections.

(C) Hepatic *Hamp* mRNA expression. For (A), (B), and (C), means and SDs are shown.

(D) Histological examination of iron loading in the aortic lesions. Tissue iron was detected by enhanced Perls' stain (green). Scale bars, 100 µm. Illustrative tissue sections from *Apoe*$^{-/-}$ mice are shown; *Apoe/ffe* animals had an equivalent iron loading pattern.

(E) Atherosclerotic lesion size is given in squared micrometers (horizontal lines represent the means). For each genotype (either *Apoe*$^{-/-}$ or *Apoe/ffe*), statistical analysis was performed to evaluate the effect of iron sucrose injections. Furthermore, for each condition (either HFD or HFD+Fe), *Apoe*$^{-/-}$ mice were compared to *Apoe/ffe* mice. **p = 0.01 and ***p $\leq$ 0.001, by Student's t test. No statistically significant difference between the groups was observed in (E).

tended to be smaller than in untreated mice, in both *Apoe*$^{-/-}$ and *Apoe/ffe* genotypes.

Our study did not specifically test whether depletion of macrophage iron, such as in frank iron deficiency or with hepcidin lowering agents, would be protective. Recently, Saeed and colleagues reported that pharmacological suppression of hepcidin synthesis through inhibition of the bone morphogenetic protein (BMP) pathway decreased atherosclerosis in a model similar to ours (Saeed et al., 2012). However, inhibitors of BMP signaling would be expected to have profound effects on many other biological processes potentially involved in the formation of the atherosclerotic plaque (Cai et al., 2012). Indeed, inhibition of BMP signaling in mice by overexpression of the BMP inhibitor MGP (Matrix gla protein) reduced atherosclerotic lesion size, intimal and medial calcification, and inflammation (Yao et al., 2010). Saeed et al. did not demonstrate whether replacement of hepcidin reverses the effect of the BMP inhibitor on atherosclerotic plaque size. Rather, they evaluated ex vivo foam cell formation and cholesterol efflux after mice were treated in vivo with the BMP inhibitor with or without a 2-day hepcidin injection. In these specific conditions, hepcidin addition to the BMP inhibitor increased foam cell formation and decreased cholesterol efflux compared to the BMP inhibitor by itself. A hepcidin-only condition was not tested. Further work using hepcidin-specific antagonists is required to examine whether pharmacological hepcidin suppression by itself affects progression of atherosclerosis.

Other studies that analyzed the effect of an iron-deficient diet on atherosclerosis progression did not observe a consistent result: an iron-deficient diet (11 ppm compared to 85 ppm iron) modestly reduced atherosclerotic lesions in *Apoe*$^{-/-}$ mice after 3 months (Lee et al., 1999), but not after 5 months (Lee et al., 2003). A different model of reducing iron load using iron chelation in *Apoe*$^{-/-}$ mice (daily injections of desferrioxamine for 10 weeks) modestly decreased the atherosclerotic lesion size (Zhang et al., 2010), but so did dietary iron loading using 20,000 ppm iron diet for 6, 12, or 24 weeks (Kirk et al., 2001). Although dietary iron loading causes preferential iron accumulation in hepatocytes rather than macrophages, in this study, both liver iron and spleen iron increased (~10-fold and ~3-fold, respectively). All of the aforementioned studies used *Apoe*$^{-/-}$ mice of the same background strain as our study. We surmise that any effect of macrophage iron on atherosclerosis is so small that contradictory conclusions may be reached perhaps because of differences in study design. Taken together with our results, the inconsistent effects of iron in atherosclerotic mouse models do not support the hypothesis that iron is an important aggravating factor in the pathogenesis of atherosclerosis.

EXPERIMENTAL PROCEDURES

Animals and Diets

Experiments were conducted in accordance with guidelines by the National Research Council and were approved by the University of California, Los Angeles. *Apoe*$^{-/-}$ mice on a C57BL/6J background and WT C57BL/6J mice were purchased from The Jackson Laboratory. *ffe*$^{H32R/+}$ mice (Zohn et al., 2007) were obtained from Dr. Jerry Kaplan, University of Utah. As *ffe* mice were on a C3H/HeJ background, we used marker-assisted accelerated backcrossing to transfer the *ffe* mutation (chromosome 1) on to a C57BL/6 background to match the background of *Apoe*$^{-/-}$ mice. After backcrossing, *ffe*$^{H32R/+}$ mice were mated with *Apoe*$^{-/-}$ mice to generate *Apoe*$^{+/-}$ *ffe*$^{H32R/+}$ mice, and subsequent breeding of these mice to *Apoe*$^{-/-}$ mice generated

Apoe$^{-/-}$ *ffe*$^{H32R/+}$ (referred to as *Apoe/ffe* for simplicity), as well as *Apoe*$^{-/-}$ littermate controls. Similarly, *ffe*$^{H32R/+}$ mice and WT littermate controls were generated from crossing *ffe*$^{H32R/+}$ to each other. Because of known gender differences in iron status (Courselaud et al., 2004), where females have higher iron stores and hepcidin expression than males, the two genders were analyzed separately. Gender-related differences in atherosclerosis severity in mice may also exist, with females exhibiting larger lesion sizes in many studies, although this remains controversial (Meyrelles et al., 2011).

All mice were initially maintained on standard chow (Diet 8604, Harlan Teklad). For atherosclerotic plaque assessment, we placed 6-week-old *Apoe*$^{-/-}$ and *Apoe/ffe* littermates on the Western diet (Research Diets, diet no. D12079B, 21% fat [WT/WT], 0.21% cholesterol [WT/WT]), which was custom made to contain 300 ppm iron (equivalent to the iron content of standard chow). Another set of *Apoe*$^{-/-}$ and *Apoe/ffe* mice were compared after 2 months on the classic Western diet (50 ppm iron) with or without weekly injections of 2 mg iron sucrose (Venofer, Vifor [International]) to enhance macrophage iron loading (eight injections total, equaling 16 mg of iron). Plaque size was also examined in WT and *ffe* littermates that were fed the high-fat diet (300 ppm iron) for 4 months.

For hepcidin assessment, the following groups of mice were used (all placed on specific diets at the age of 6 weeks): WT and *ffe*$^{H32R/+}$ littermates, and *Apoe*$^{-/-}$ and *Apoe/ffe* littermates, all fed for 4 months either standard chow (300 ppm iron) or the Western diet with 300 ppm iron. A set of *Apoe*$^{-/-}$ and *Apoe/ffe* littermates were also compared after 8 months on the Western diet containing 300 ppm iron. A set of *Apoe*$^{-/-}$ mice were fed for 2 months the classic Western diet (50 ppm iron) or the low-fat version of the diet (50 ppm iron).

For all mice, liver and spleen were harvested and divided into flash-frozen samples in liquid nitrogen for RNA and iron measurement and in 10% formalin for paraffin embedding.

Lesion Quantification

The mice were euthanized, and hearts were perfused with 10% buffered formalin via the left ventricle for 4 min. The heart and proximal aorta were excised, and the apex and lower half of the ventricles were removed. The remaining specimen was embedded in O.C.T. Compound (Tissue-Tek, Fisher Scientific), frozen on dry ice, and stored at −80°C until sectioning. Serial cryosections were prepared through the ventricle until the aortic valves appeared. From then on, every fifth 10-μm section was collected on slides coated with poly-D-lysine until the aortic sinus was completely sectioned. Sections were stained with hematoxylin and Oil Red O, which specifically stains lipids. Slides were examined by light microscopy, and the atherosclerotic lesion area was quantified with computer-assisted image analysis (Image-Pro Plus, Media Cybernetics) as described elsewhere and averaged over 40 sections (Qiao et al., 1994; Wang et al., 2004).

En Face Analysis of Aortas

The aorta, including the ascending arch, thoracic, and abdominal segments, was dissected, gently cleaned of the adventitia, and stained with Sudan IV 5. The surface lesion area was quantified with computer-assisted image analysis (Image-Pro Plus, Media Cybernetics) as previously described (Qiao et al., 1994; Wang et al., 2004).

Calcification Quantification

Sections of the aortic roots were stained with hematoxylin and examined by light microscopy. Forty sections per mouse were examined, and the sections with visible calcification were counted (Qiao et al., 1994). Results are expressed as a percentage of slides with visible calcifications.

Measurement of Iron Parameters

Spleen and liver nonheme iron concentrations were determined as described elsewhere (Ramos et al., 2012), using acid treatment followed by a colorimetric assay for iron quantitation (Sekisui Diagnostics). Deparaffinized sections were stained with Perls' Prussian blue stain for nonheme iron enhanced with the SG peroxidase substrate kit (Vector Labs) and counterstained with nuclear fast red.

Quantitation of mRNA Levels

Total RNA from mouse liver was extracted using Trizol (Invitrogen). cDNA was synthesized using iScript (Biorad). Quantitative PCR (qPCR) reactions were prepared with iQ SYBR Green Supermix (Biorad), and primers were indicated in Table S1 and run in duplicate on a MyiQ Instrument (Biorad). *Hamp*, *Saa3*, *Il6*, and *haptoglobin* mRNA expression were normalized by the reference gene *Rpl4* or *Hprt*. Results are expressed as ΔCt ± SD (i.e., the cycle threshold differences between reference and target genes within each group of mice).

Statistical Analysis

The statistical significance of differences between groups was evaluated using Sigmaplot 11.0 package (Systat Software). The Student's t test was used to compare two groups of normally distributed data. The Mann-Whitney rank-sum test was used to compare data that were not normally distributed. A p value < 0.05 was considered as statistically significant. Data are represented as mean value ± SD.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.11.009.

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