Apolipoprotein E−/− Mice Lacking Hemopexin Develop Increased Atherosclerosis via Mechanisms That Include Oxidative Stress and Altered Macrophage Function

Niyati U. Mehta, Victor Grijalva, Susan Hama, Alan Wagner, Mohamad Navab, Alan M. Fogelman, Srinivasa T. Reddy

Objective—We previously reported that hemopexin (Hx), a heme scavenger, is significantly increased and associated with proinflammatory high-density lipoprotein under atherogenic conditions. Although it is established that Hx together with macrophages plays a role in mitigating oxidative damage, the role of Hx in the development of atherosclerosis is unknown.

Approach and Results—We used Hx and apoE double-knockout mice (HxE−/−) to determine the role of Hx in the development of atherosclerosis. HxE−/− mice had significantly more free heme, reactive oxygen species, and proinflammatory high-density lipoprotein in their circulation, when compared with control apoE−/− mice. Atherosclerotic plaque area (apoE−/−=9.72±2.5×10⁴ μm² and HxE−/−=27.23±3.6×10⁴ μm²) and macrophage infiltration (apoE−/−=38.8±5.8×10⁴ μm² and HxE−/−=103.4±17.8×10³ μm²) in the aortic sinus were significantly higher in the HxE−/− mice. Atherosclerotic lesions in the aortas were significantly higher in the HxE−/− mice compared with apoE−/− mice. Analysis of polarization revealed that macrophages from HxE−/− mice were more M1-like. Ex vivo studies demonstrated that HxE−/− macrophage cholesterol efflux capacity was significantly reduced when compared with apoE−/− mice. Injection of human Hx into HxE−/− mice reduced circulating heme levels and human Hx pretreatment of naive bone marrow cells ex vivo resulted in a shift from M1- to M2-like macrophages.

Conclusions—We conclude that Hx plays a novel protective role in alleviating heme-induced oxidative stress, improving inflammatory properties of high-density lipoprotein, macrophage phenotype and function, and inhibiting the development of atherosclerosis in apoE−/− mice. (Arterioscler Thromb Vasc Biol. 2016;36:1152-1163. DOI: 10.1161/ATVBAHA.115.306991.)

Key Words: cholesterol • heme • inflammation • macrophages • monocytes
relatively little is known on Hp’s heme-scavenging counterpart Hx, which plays an important role in heme elimination.

Hx is a 60-kDa plasma glycoprotein and is considered to be a major transport vehicle of heme into macrophages, thereby inhibiting heme-mediated ROS production and preventing both heme-catalyzed and ROS-mediated oxidative damage. Cytotoxic properties of free heme are thought to be involved in many complex cellular mechanisms: release of redox-active iron, production of superoxide and hydroxyl radicals, and peroxidation of membrane lipids. Several diseases are associated with free heme and lead to a state of endothelial dysfunction. This leads to enhanced expression of adhesion molecules on the endothelium, high levels of circulating proinflammatory cytokines and activated leukocytes, and monocyte recruitment. Heme is a major source for the generation of ROS. High levels of ROS lead to lipid, protein, and DNA damage and eventually to cell death, and they too favor endothelial activation and leukocyte recruitment, thus promoting a chronic inflammatory state. The primary defense for cells against heme toxicity is currently thought to be provided by complexing of heme with Hx.19

The heme–Hx complex is taken up by cells through receptor-mediated endocytosis, LRP (low-density lipoprotein receptor-related protein)/CD91 receptor on macrophages mediates the internalization of the heme–Hx complex, which induces the expression of antioxidant hemeoxygenase enzymes (HO-1 and HO-2). Heme is then catabolized by the heme oxygenase enzymes to generate biliverdin, free iron, and carbon monoxide as reaction products. Biliverdin is processed further to bilirubin, a direct antioxidant. Another important recently discovered effect of heme internalization is the coinduction of liver X receptor (LXR) genes along with HO-1 via a common transcription factor to maintain the lipid–iron homeostasis in macrophages. Vinchi et al have shown that Hx therapy can improve cardiovascular function by preventing heme-induced endothelial toxicity in mouse models of hemolytic diseases. Thus, Hx serves to regulate the balance between free heme and bound heme and to regulate heme degradation.

The role of Hx in the development of atherosclerosis has not been studied to date. We generated Hx−/− mice on an apoE−/− background (HxE−/−) to determine the role of Hx in the development of atherosclerosis. Our results show that Hx protects against the development of atherosclerosis in apoE−/− mice. We show that Hx deficiency results in increased oxidative stress and proinflammatory HDL in HxE−/− mice. Furthermore, during our investigations to examine the effect of Hx deficiency on macrophage function, we discovered a novel role for Hx in determining macrophage phenotype.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
Hepatic Heme Detoxification Is Dysregulated in apoE−/− Mice Lacking Hemopexin
We observed significantly higher levels of heme in the serum (Figure 1A) and HDL fractions (Figure 1B) of HxE−/− mice compared with apoE−/− mice (n=8–10). Levels of bilirubin, a heme breakdown product (Figure 1C), and albumin (Figure 1D) were significantly lower in the serum of HxE−/− mice compared with apoE−/− mice. The serum samples were collected with extreme care to avoid hemolysis during retro-orbital bleeding of the mice, which could potentially account for higher heme levels. The levels of hemoglobin are slightly although not significantly elevated (Figure IIA in the online-only Data Supplement) compared with their matched control apoE−/− serum samples. The mean corpuscular hemoglobin, which is the average mass of hemoglobin per RBC, is significantly elevated in the HxE−/− mice (Figure IIB in the online-only Data Supplement), although the RBC levels were the same (Figure IIC in the online-only Data Supplement) in both the groups of mice, indicating endogenous hemolysis. Furthermore, intraperitoneal administration of human Hx (hHx) to HxE−/− mice for 24 hours significantly reduced the levels of heme in the serum of the mice (Figure 1E), suggesting that Hx regulates circulating heme levels.

Hepatic gene expression analysis was performed to determine the levels of the scavenger receptor CD91 and heme catabolizing enzymes HO-1 and HO-2. We recorded significantly lower gene expression levels of CD91, HO-1, and HO-2 in the livers of HxE−/− mice compared with apoE−/− mice (Figure 1F). Significantly lower gene expression of albumin in the liver of HxE−/− mice (Figure 1F) supported the lower levels of albumin in the serum of HxE−/− mice (despite the lower levels of albumin in the HxE−/− mice compared with the apoE−/− mice, the levels were within the normal range reported for C57BL/6J mice as shown in Materials and Methods section of this article). Together, these data indicated that lack of Hx in serum significantly affects the heme detoxifying potential of the liver. These results showed that Hx deficiency causes excess heme accumulation and reduced levels of antioxidants (bilirubin) in the circulation.

ApO−/− Mice Lacking Hemopexin Have Higher Oxidative Stress and Impaired HDL Function
Cell-free heme promotes the generation of ROS, which is involved in the pathophysiology of several disorders. We estimated levels of ROS by a cell-free assay using DCFH2 (dichlorofluorescin). The relative fluorescence units, which are reflective of the levels of ROS, were significantly higher in the serum and HDL of HxE−/− mice compared with apoE−/− mice. Individual serum samples were used to estimate ROS levels in...
circulation (Figure 2A). In addition, serum was pooled from the apoE−/− and HxE−/− mice (n=6–8), and lipoproteins were separated using fast-performance liquid chromatography. ROS levels were estimated on all the FPLC fractions. HDL fractions from HxE−/− mice exhibited significantly higher levels of ROS compared with the HDL fractions from apoE−/− mice (Figure 2B). We estimated lecithin-cholesterol acyltransferase (LCAT) activity on the same HDL fractions and observed significantly lower LCAT activity in HDL from HxE−/− mice compared with apoE−/− mice (Figure 2C). Increased heme content and ROS levels associated with HDL and reduced LCAT activity further led us to determine HDL functionality by measuring anti- versus proinflammatory activity of HDL. This was measured ex vivo by a monocyte migration assay as described in Materials and Methods section of this article. We observed a significantly higher degree of monocyte migration from the transwells treated with the conditioned media collected from human aortic endothelial cells treated with HDL from apoE−/− and HxE−/− mice (n=3–4). All genes were normalized to GAPDH. The mean value for each group of mice is indicated by the horizontal bars. The data shown are mean±SEM. **Significance: P<0.01; *Significance: P<0.05.

Figure 1. Hepatic heme detoxification is dysregulated in apolipoprotein E (apoE)−/− mice lacking hemopexin. A and E, Heme content was measured colorimetrically in the serum and (B) in pooled high-density lipoprotein (HDL) fractions of apoE−/− and Hx-null/ApoE-null (HxE)−/− mice (n=8–10). C, Bilirubin (n=8–10) and (D) albumin levels (n=20, apoE−/−; n=9, HxE−/−) were measured in the serum of apoE−/− and HxE−/− mice as described in Materials and Methods section of this article. E, HxE−/− mice at 4 weeks of age were given a single injection of human Hx (hHx) or saline intraperitoneally. Twenty-four hours later, retro-orbital bleeding was performed to obtain serum and the levels of serum heme were determined. F, Hepatic gene expression was determined by real-time quantitative polymerase chain reaction for apoE−/− and HxE−/− mice (n=3–4). All genes were normalized to GAPDH. The mean value for each group of mice is indicated by the horizontal bars. The data shown are mean±SEM. **Significance: P<0.01; *Significance: P<0.05.
control apoE<sup>−/−</sup> mice (n=7–8). However, levels of alanine transaminase and aspartate transaminase enzymes (Figure IIIA and IIIB in the online-only Data Supplement) were similar between the 2 groups of mice. Our results show that Hx deficiency in apoE<sup>−/−</sup> mice leads to accumulation of proinflammatory HDL and higher systemic oxidative stress.

Figure 2. Absence of hemopexin significantly increases oxidative stress in apolipoprotein E (apoE)<sup>−/−</sup> mice. A, Reactive oxygen species (ROS) levels were determined and represented as relative fluorescence units (RFUs) in a cell-free assay using DCFH<sub>2</sub> (dichlorofluorescin) in serum samples of Hx-null/ApoE-null (HxE)<sup>−/−</sup> and apoE<sup>−/−</sup> mice (n=7) as described in Materials and Methods section of this article. B, Lipoproteins were separated using fast-performance liquid chromatography (FPLC) from pooled serum samples obtained from apoE<sup>−/−</sup> and HxE<sup>−/−</sup> mice (n=6–8) and, cholesterol levels on the different lipoprotein fractions were obtained using a colorimetric assay. ROS levels were determined on all the FPLC fractions using a cell-free assay. C, Lecithin-cholesterol acyltransferase (LCAT) activity was measured fluorometrically on concentrated pooled high-density lipoprotein (HDL) fractions. D, HDL cholesterol (50 μg/dL) from apoE<sup>−/−</sup> and HxE<sup>−/−</sup> mice (n=3) was used to perform a monocyte migration assay as described in Materials and Methods section of this article. E, Malondialdehyde levels were determined using a colorimetric assay in livers of HxE<sup>−/−</sup> and apoE<sup>−/−</sup> mice (n=7–8). The mean value for each group of mice is indicated by the horizontal bars. The data shown are means±SEM. ***Significance: P<0.001; **Significance: P<0.01; *Significance: P<0.05.

Hemopexin Ablation in apoE<sup>−/−</sup> Mice Causes Increased Atherosclerosis

We investigated the effect of Hx ablation on the extent of atherosclerosis in apoE<sup>−/−</sup> mice. Six-month-old female mice (n=13 apoE<sup>−/−</sup> and n=12 HxE<sup>−/−</sup> mice) maintained on a chow diet were euthanized after overnight fasting. The HxE<sup>−/−</sup> mice...
showed a higher Oil-Red-O staining of the sinus, indicative of an increase in the plaque area in the heart, compared with the control apoE<sup>−/−</sup> mice (Figure 3A, representative images). Quantitative analysis showed a significant increase in plaque area in the HxE<sup>−/−</sup> mice compared with the apoE<sup>−/−</sup> mice. On average, a 3-fold increase in the area of plaque was observed in the double-knockout HxE<sup>−/−</sup> mice compared with its respective control (Figure 3B). To examine the extent of atherosclerotic lesions, en face analysis was performed on paraformaldehyde-fixed aortas from the 2 groups of mice (n=24 apoE<sup>−/−</sup> and n=25 HxE<sup>−/−</sup> mice). Figure 3C shows representative images of the whole aorta stained for lesions using Sudan IV. There was a significant increase in aortic atherosclerosis in the HxE<sup>−/−</sup> mice compared with the apoE<sup>−/−</sup> mice (Figure 3D). These results demonstrated that Hx deficiency in apoE<sup>−/−</sup> mice causes increased atherosclerosis. Macrophage infiltration is a hallmark for the onset of atherosclerosis. Immunohistochemistry using macrophage marker CD68 was performed on sections of aortic sinus to examine the extent of macrophage content in the mice. Morphometric analysis for antibody-stained area revealed an augmentation of macrophage accumulation in the double-knockout mice compared with control mice (Figure 3E, representative images). A 2-fold increase in...
macrophage content in the plaque area was observed in the HxE−/− mice compared with apoE−/− mice (Figure 3F). Taken together, these results demonstrated that HxE−/− mice develop augmented atherosclerosis compared with apoE−/− mice.

**BMDMs From HxE−/− Mice Have Impaired/Reduced Cholesterol Efflux Capacity Through the ABCA1 Receptor**

Hx along with macrophages play an important role in the clearance of cell-free heme from the circulation, thereby mitigating oxidative damage caused by heme. Boyle et al\(^1\) recently demonstrated that heme can transcriptionally coactivate HO-1 and LXR genes in macrophages. We used naive and differentiated bone marrow–derived macrophages (BMDMs) from 8 to 12-week-old apoE−/− and HxE−/− mice (n=3–4) without and with hHx, to determine the expression of LXR genes using real-time quantitative polymerase chain reaction gene expression analysis. Treatment of the naive cells with macrophage colony-stimulating factor (MCSF) dramatically increased the expression of LXR-α and ATP-binding cassette, subfamily A, member 1 (ABCA1), and the expression of these genes was significantly less on exposure to lipopolysaccharides (LPS) (data not shown). Interestingly, in contrast to LXR-α on exposure to LPS, levels of LXR-β were not significantly reduced in MCSF-1–treated BMDMs from HxE−/− mice compared with apoE−/− mice (Figure 4A). ABCA1, a downstream target of LXR-α was also significantly less expressed in HxE−/− MCSF-1–treated BMDMs treated with LPS; however, there was no change in the expression of ABCG1 (Figure 4A) between the 2 groups of mice. ABCA1 protein expression was significantly lower (Figure 4B) in MCSF-1–treated BMDMs from HxE−/− mice compared with apoE−/− mice. ABCA1 is an important cell surface cholesterol efflux transporter. We determined levels of ABCA1 on the cell surface of the BMDMs by flow cytometry analysis as described in Materials and Methods section of this article. We observed a significant reduction in expression of cell surface ABCA1 in HxE−/− MCSF-1–treated BMDMs compared with apoE−/− BMDMs after exposure to LPS (Figure 4C).

Because ABCA1 is a key cholesterol efflux transporter, we performed a cholesterol efflux assay using MCSF-1–treated BMDMs loaded with Ac-LDL (50 μg/mL) and measured apoA-I (10 μg/mL)–mediated cholesterol efflux. Net percent cholesterol efflux was measured as a ratio of counts in media divided by counts in media plus cells. MCSF-1–treated BMDMs from HxE−/− mice showed a significant reduction in cholesterol efflux capacity compared with apoE−/− MCSF-1–treated BMDMs (Figure 4D). Because the efflux was apoA-I mediated, we can propose that the efflux was via the ABCA1 transporter. Ac-LDL cholesterol uptake was similar in MCSF-1–treated BMDMs from both groups of mice (Figure 4D, bottom), and serum apoA-I levels estimated by ELISA were not different between apoE−/− and HxE−/− mice (Figure IIIC in the online-only Data Supplement). These results led us to hypothesize that the absence of Hx in HxE−/− mice may favor more M1-like macrophages. To test this hypothesis, we added hHx to differentiated BMDMs from apoE−/− and HxE−/− mice and determined the expression of 2 important M1 and M2 genes using real-time quantitative polymerase chain reaction. On adding hHx to the MCSF-1-LPS BMDMs, the expression of CCR-2, an important M2 macrophage marker was significantly upregulated in naive HxE−/− macrophages compared with apoE−/− naive macrophages as well as in cells treated with MCSF-1-LPS (Figure 5B). Chemokine receptor-2 (CCR-2), a well-established M1 macrophage marker was significantly increased in differentiated BMDMs from the HxE−/− mice compared with the cells from the apoE−/− mice (Figure 5B). The MCSF-1-LPS HxE−/− macrophages had significantly lower expression of F4/80, Arg-1, and anti-inflammatory IL-10 (Figure 5B) compared with their apoE−/− controls. Together with the data in Figure 4A, these results suggest that the macrophages from HxE−/− mice are more M1-like (more proinflammatory). These results led us to hypothesize that absence of Hx in HxE−/− mice may favor more M1-like macrophages. To test this hypothesis, we added hHx to differentiated BMDMs from apoE−/− and HxE−/− mice and determined the expression of 2 important M1 and M2 genes using real-time quantitative polymerase chain reaction. On adding hHx to the MCSF-1-LPS BMDMs, the expression of CCR-2, a well-established M1 macrophage marker was significantly reduced in HxE−/− cells, but not in apoE−/− cells (Figure 5C). In addition, the expression of Arg-1, an important M2 macrophage marker was significantly increased in HxE−/− MCSF-1-LPS-treated BMDMs that were also treated with hHx (Figure 5C). We also performed, immunohistochemistry analysis using a macrophage M1 marker, major histocompatibility complex (MHC) II in aortic root sections (n=6 apoE−/− and n=6 HxE−/− mice). We observed a significant increase in % MHCII-positive staining in the macrophage-stained area (CD68 positive) in the double-knockout mice.
Figure 4. Hx-null/ApoE-null (HxE)−/− macrophages have reduced cholesterol efflux capacity through the ATP-binding cassette, subfamily A, member 1 (ABCA1) transporter. A, Macrophage colony-stimulating factor (MCSF)-1 (10 ng/mL) was used to differentiate the bone marrow–derived cells isolated from apolipoprotein E (apoE)−/− and HxE−/− mice (n=3–4) as described in Materials and Methods section of this article. Real-time quantitative polymerase chain reaction analysis was performed as described in Materials and Methods (Continued)
compared with control mice (Figure 5D). These results confirm that the absence of Hx modifies macrophage polarization not only in the bone marrow but also in the atherosclerotic lesions.

**Discussion**

The results presented here show that lack of Hx in the apoE−/− mouse model on a chow diet causes increased atherosclerosis. Hx is an acute phase protein that binds to heme with high affinity. To limit free heme availability and prevent free radical formation, mammals use Hx as the major heme scavenger protein. Hx binds to free heme, and the resultant heme–Hx complex is taken up by macrophages and hepatocytes through the CD91 receptor via receptor-mediated endocytosis. Heme scavenging is an important process to avoid oxidative stress and inflammation that occurs in the presence of increased levels of free heme. The results in Figure 1 show that the absence of Hx resulted in increased heme in the serum and HDL of Hx−/− mice and supplementing the mice with hHx significantly reduced serum heme levels. Hx−/− mice also showed decreased expression of the CD91 receptor and the antioxidant enzymes HO-1 and HO-2 in the liver. HO-1 is a vital enzyme for iron homeostasis and protection from oxidant stress. HO-1 catabolizes the pro-oxidant heme and generates biliverdin, free iron and carbon monoxide as reaction products. Biliverdin is further processed to bilirubin, a direct antioxidant, which increases the anti-inflammatory efficacy of the CD91–HO-1 pathway.

Epidemiological studies have revealed that moderately increased plasma levels of bilirubin can decrease the risk of developing cardiovascular diseases. Interestingly, Hx−/− mice showed reduced serum bilirubin levels compared with control apoE−/− mice suggesting that reduced heme breakdown by the CD91–HO-1 pathway may, in part, contribute to increased atherosclerosis. Albumin is known to also bind to heme and form a heme–albumin complex that can be taken up by endothelial cells. The results in Figure 1 show that Hx−/− mice had reduced levels of albumin. The cause of the decreased albumin levels in the Hx−/− mice is not evident from our studies. However, the decreased albumin levels may also contribute to the higher levels of free heme in the serum of Hx−/− mice compared with apoE−/− mice. Moreover, free heme can transiently bind to lipoproteins, such as HDL and LDL, before being transferred to Hx for scavenging. We observed a significantly higher level of heme in HDL fractions from Hx−/− mice compared with apoE−/− mice (Figure 2), indicating that in the absence of Hx free heme accumulates on transient carrier proteins, such as HDL.

Free heme activates NADPH oxidase, a major source of ROS, and ROS has been shown to induce several pathological conditions. With augmented heme in the circulation, we observed significantly increased ROS accumulation in serum, HDL, and post-HDL fractions of Hx−/− mice compared with the apoE−/− controls (Figure 2). Free heme toxicity is exacerbated by its ability to intercalate into lipid membranes. The extreme hydrophobicity of free heme allows it to enter the phospholipid bilayer. This catalyzes the oxidation of the cell membrane and promotes lipid peroxidation, increasing membrane permeability and ultimately leading to cell death. Levels of malondialdehyde can be used as a biomarker to estimate oxidative stress in vivo. Malondialdehyde levels are a measure of hepatic lipid peroxidation. Our data showed that Hx−/− mice had increased levels of malondialdehyde in their livers compared with apoE−/− mice consistent with increased heme and ROS leading to increased lipid peroxidation. Watanabe et al showed that in patients with cardiovascular disease, hemoglobin and its scavenger proteins: Hp and Hx can associate with HDL and influence the inflammatory properties of HDL. This study also reported that absence of Hx could convert HDL from anti-inflammatory to a proinflammatory HDL in C57BL/6J mice. Our results confirm these findings in apoE−/− mice lacking Hx. In a monocyte chemotactic assay, the HDL from Hx−/− mice stimulated cultured endothelial cells to produce more monocyte chemotactic protein-1 as determined by a bioassay. In addition, HDL from Hx−/− mice had significantly lower LCAT activity compared with HDL from apoE−/− mice. Our results suggest that Hx confers protection against heme- and ROS-driven HDL dysfunction and oxidative stress in apoE−/− mice.

Excess heme and ROS are implicated in various aspects of cardiovascular pathology. Increased free radicals in the circulation cause endothelial dysfunction, platelet activation, and induce smooth muscle proliferation, which are features of atherosclerosis and hypertension. Hx−/− mice on a chow diet developed increased atherosclerotic lesions in the aortic sinus and the aorta compared with apoE−/− mice. Macrophage content in the aortic sinus was also significantly higher in Hx−/− compared with apoE−/− mice as seen in Figure 3 of this study. In addition, total and LDL-cholesterol levels tended to be elevated and HDL-cholesterol levels tended to be lower in the Hx−/− mice compared with apoE−/− mice, but these changes did not reach statistical significance (Table I in the online-only Data Supplement). Collectively, these results demonstrated that Hx ablation in apoE−/− mice causes aggravated plaque formation and macrophage infiltration leading to increased atherosclerosis.

Monocytes originate from bone marrow–derived progenitor cells, and at early stages of monocyte development may
Figure 5. Bone marrow–derived macrophages from the Hx-null/ApoE-null (HxE)^−/− mice are proinflammatory. A, Real-time quantitative polymerase chain reaction (RT-qPCR) analysis was performed using (A) naive and macrophage colony-stimulating factor (MCSF)-1 (10 ng/mL)+interleukin (IL)-4 (10 ng/mL) treated bone marrow cells or (B) naive and MCSF-1 (10 ng/mL)+lipopolysaccharides (LPS; 10 ng/mL) treated bone marrow cells isolated from HxE^−/− and apolipoprotein E (apoE)^−/− mice (n=3–4) as described under Materials and (Continued)
be regulated by cellular cholesterol content in a manner that can affect atherosclerosis. Mice, with monocyte progenitor cells genetically engineered to have defective cholesterol efflux because of deficiency of ABCA1 and ABCG1 transporters, showed an increase in atherosclerosis.46 We found a significant reduction in ABCA1 gene and protein expression in the HxE−/− BMDMs compared with apoE−/− BMDMs (Figure 4). PPAR (peroxisome proliferator-activated receptor) is known to induce the expression of ABCA1 in macrophages through a transcriptional cascade mediated by the nuclear receptor LXR that hetero dimerizes with retinoic-X-receptor.47–49 LXR-α, a well-established transcriptional regulator for ABCA1, was also significantly downregulated in the HxE−/− BMDMs. The functional consequences of the reduced gene and protein expression of ABCA1 was demonstrated by a cholesterol efflux assay. BMDM isolated from HxE−/− mice were found to have reduced apoA-I–mediated cholesterol efflux (Figure 4). Interestingly, in an independent study it was shown that after heme internalization in macrophages HO-1 and LXR expression was increased, foam cell formation was prevented and the export of cholesterol to HDL was promoted.23–46 This coordination of iron and lipid metabolism provides protection from oxidant stress and lipid overload. These findings are also consistent with our finding of reduced expression of LXR-α and ABCA1 in BMDMs from HxE−/− mice. Interestingly, when the BMDMs from HxE−/− mice were supplemented with hHx, we observed a significant increase in the expression of LXR-α and ABCA1 genes (Figure 4). This reduction in LXR genes in HxE−/− BMDMs may contribute to increased foam cell formation because of reduced cholesterol efflux capacity, which may play a role in the increased atherosclerosis observed in our studies.

In pathological conditions such as aging, atherosclerosis, and diabetes mellitus, excess amounts of ROS in the bone marrow microenvironment may impair stem and progenitor cell function, and cause hematopoietic dysfunction.47,48 Thus, both dysregulation of ROS and a more oxidative environment may have deleterious effects on the bone marrow microenvironment. We observed that BMDMs from HxE−/− mice were more classically activated M1-like macrophages compared with the apoE−/− macrophages, which were more M2 in phenotype. Classically activated macrophages or M1 are important components of the host defense in the fight against various pathogens; M1 macrophages are thought to be more proinflammatory.49–52 In addition, proinflammatory M1 macrophages have been shown to exhibit reduced expression of ABCA1.51 Cytokines such as IL-4 can antagonize classical macrophage activation and induce the development of alternatively activated or anti-inflammatory M2 macrophages.52 It is well established that the expression of FIZZ1 and Ym1 is induced in alternatively activated macrophages when compared with classically activated macrophages, whereas tumor necrosis factor-α and CCR-2 are highly expressed in M1 macrophages.53–56 BMDMs from HxE−/− mice showed significantly reduced expression of Ym1 and FIZZ1 and significantly increased expression of tumor necrosis factor-α and CCR-2, when compared with apoE−/− mice (Figure 5). Myeloid-derived suppressor cells are considered to be an immature population of myeloid cells associated with infections or tumors that are capable of suppressing proinflammatory responses. Mouse myeloid-derived suppressor cells have been found to express F4/80, IL-4R-α, Arg-1, IL-10, and TGF-β among many other markers.56 BMDMs from HxE−/− mice showed a significant downregulation of F4/80, Arg-1, and IL-10 compared with apoE−/− BMDMs (Figure 5). Interestingly, when hHx was added to the BMDMs from HxE−/− mice, there was a significant reduction in expression of CCR-2, and a significant increase in expression of Arg-1. Our data suggest that BMDMs from HxE−/− mice promote proinflammatory responses compared with BMDMs from apoE−/− mice. In addition, immunohistochemistry results for MHCII, a well-established M1 macrophage marker, confirm that the absence of Hx results in more M1-like macrophages in atherosclerotic lesions. It has been shown that M1 macrophages have reduced expression of ABCA1, which has important implications in the development of atherosclerosis. We have performed immunohistochemistry using antibodies against both CD68 and ABCA1 on serial atherosclerotic lesion sections and quantified the data as ABCA1 expressed specifically in the macrophage area (Figure IV in the online-only Data Supplement).

It should be pointed out that (1) the lesions were elevated in HxE−/− mice, and (2) CD68-positive staining is significantly elevated in HxE−/− lesions. Therefore, the significant increase in ABCA1 per lesional macrophage area was not informative of naive macrophages. The results using BMDM from the 2 groups of mice in Figures 4 and 5 demonstrate that lack of Hx alters ABCA1 expression and function. Taking into account the different gene expression profiles of BMDMs from HxE−/− and apoE−/− mice with and without hHx treatment and the MHCII immunohistochemistry results, we suggest that the lack of Hx from birth in apoE−/− mice influences the bone marrow microenvironment resulting in macrophages in the lesions that are more proinflammatory or M1-like.

There has been extensive evidence from in vitro, animal, and human studies relating Hp to cardiovascular disease and diabetes mellitus.5,8 We have previously demonstrated that Hp can bind directly to HDL and seems to be responsible for the increased amount of the hemoglobin complex and reactive oxygen species associated with the HDL in mice. The amount of total lipid peroxides associated with HDL was also found to be increased in individuals with diabetes mellitus and the Hp 2-2 compared with the Hp 1-1 genotype.57 It was suggested...
that Hp's role in oxidative stress and HDL function might provide proof of concept for the hypothesis of dysfunctional HDL in diabetes mellitus. Because Hp and Hx are part of the hemoglobin/heme scavenging complex and in light of the results from our current studies, it is likely that Hx will have a significant role in diabetes mellitus and related cardiovascular complications.

In conclusion, our results demonstrate, for the first time, that mice lacking Hx are more susceptible to atherosclerosis and suggest that targeting free heme in the circulation may be a novel approach in preventing and treating oxidative stress-mediated atherosclerosis.

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Disclosures

M. Navab, A.M. Fogelman, and S.T. Reddy are principals in Bruin Pharma and A.M. Fogelman is an officer in Bruin Pharma.

References

Hemopexin deficiency causes increased atherosclerosis


Highlights

- Hemopexin deficiency aggravates the development of atherosclerosis in apolipoprotein E null mice.
- Heme scavenging and regulation of macrophage phenotype/function seem to be the primary mechanisms by which Hx protects against increased atherosclerosis.
- Hemopexin could be a novel treatment for alleviating heme-mediated oxidative damage and atherosclerosis.
Apolipoprotein E−/− Mice Lacking Hemopexin Develop Increased Atherosclerosis via Mechanisms That Include Oxidative Stress and Altered Macrophage Function
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Hemolysis → Hb → Heme → Scavenger Buffer → Albumin HDL/LDL α1-microglobulin → Hemoxepin → Circulation → CD91 → heme → HO-1 → Biliverdin → Bilirubin → CO → Iron

- Hemoxepin Scavenges Heme
- Macrophage Metabolize Heme

- Oxidative Stress
- Dysfunctional HDL
- Abnormal Macrophage Function
- Aggravated Atherosclerosis
Table I: Serum analysis

Higher levels of free fatty acids were found in HxE−/− mice compared to apoE−/− mice. Significantly lower levels of glucose and HbA1c levels were found in HxE−/− mice compared to apoE−/− mice. NS: Not significant.
<table>
<thead>
<tr>
<th>Primer #</th>
<th>Gene Symbol</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<td>1</td>
<td>YMI</td>
<td>5'-ACT TTG ATG GCC TCA ACC TGG ACT -3'</td>
<td>5'-TGG AAG TGA GTA GCA GCC TTG GAA-3'</td>
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<td>Arg1</td>
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<td>5'-AGA GAT GCT TCC AACT GCA CAG ACT -3'</td>
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<td>Fizz1</td>
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<td>IL-10</td>
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<td>TNF-α</td>
<td>5'-TACTGAACCTCCTGGGAGATTGGTCC-3'</td>
<td>5'-CAGCCTTGTCTCTTGAAGAAGACC-3'</td>
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Table II: QPCR primer list.
Figure I: Genotyping HxE<sup>−/−</sup> mice

(A) Absence of apoE was shown by a band at 245 base pairs (bp) while a band at 155bp indicated wild type for ApoE gene in an agarose gel. (B) Hemopexin knock out was determined by a PCR product band at 320 bp, while a wild type band was observed at 290 bp. (C) Western blot for ApoE protein at 34 KDa was observed in C57BL/6J mice serum. (D) Western blot for Hx protein at 60KDa was observed in C57BL/6J mice and apoE<sup>−/−</sup> serum.
Figure II: Hb and RBC levels in apoE<sup>−/−</sup> and HxE<sup>−/−</sup> mice

RBC panel analysis was performed on samples obtained from the two groups of mice. (A) Hb levels (B) Mean corpuscular Hemoglobin, and (C) Red blood cell number. * Significance: p<0.05
Figure III: Serum analysis

(A) Alanine Transaminase (ALT) levels and (B) Aspartate Transaminase (AST) levels were determined in serum samples of apoE\(^{-/-}\) and HxE\(^{-/-}\) mice (n = 7 - 9) (C) Serum apoA-I levels in apoE\(^{-/-}\) and HxE\(^{-/-}\) mice (n = 5) were determined using ELISA as described in Materials and Methods. The mean area for each group of mice is indicated by the horizontal bar.
Figure IV: Immunohistochemistry staining for ABCA1 in aortic lesions

(A) ABCA1 positive staining was performed in the aortic sinus for macrophage content as described in Materials and Methods HxE<sup>-/-</sup> (n = 6) and apoE<sup>-/-</sup> (n = 6) mice. Each point represents the mean ABCA1 positive area per mouse (3 sections per mouse). The mean area for each group of mice is indicated by the horizontal bars. (B) IgG staining control for the CD68 antibody. (C) IgG staining control for both MHCII and ABCA1 antibodies. Scale bar = 500µm. * Significance: p<0.05
MATERIALS AND METHODS

Animal experiments – All animal experiments presented here were approved by the UCLA animal research committee. The Hx<sup>-/-</sup> mice were generously provided to us by Franklin Berger from the University of South Carolina in 2008. The Hx<sup>-/-</sup> mice were bred and backcrossed on to the apoE<sup>-/-</sup> mice on a C57BL/6 background purchased from Jackson Labs (Bar Harbor, Maine) for more than ten generations prior to generating Hx<sup>-/-</sup>/ApoE<sup>-/-</sup> (HxE<sup>-/-</sup>) double knockout mice. ApoE<sup>-/-</sup> and HxE<sup>-/-</sup> female mice 24 weeks of age were used in all experiments unless specified. Mice were fed a chow diet (Ralston Purina Mouse Chow). Serum samples were isolated from mice after being transferred to new cages with fresh bedding and fasted overnight, cryopreserved in 0.2X sucrose, and freshly frozen at -80 °C until use.

LCAT activity – Pooled HDL from mouse serum samples was used to measure LCAT activity with a commercial fluorometric assay kit (Roar Biomedical Inc., New York, NY) according to the manufacturer's instructions. Briefly, 4 μl samples were incubated with a fluorescently labeled substrate for 2.5 h at 37 °C. The conversion of cholesterol (LM1= 470 nm) to cholesteryl ester (LM2=390 nm) was determined in a fluorescence microplate reader (SPECTRAmax GEMINI, Molecular Devices Co., Sunnyvale, CA). Substrate hydrolysis by LCAT resulted in an increase in emission at 390 nm and a decrease in emission at 470 nm. The change of ratio of the two intensities (470/390) was used to express LCAT activity.

Lipoprotein isolation - Lipoprotein samples were isolated from pooled or individual sera (~100-125ul) by a fast protein liquid chromatography (FPLC) system consisting of dual Superose 6 columns in series (Amersham Bioscience). Fractions were collected with PBS at a flow rate of 0.5 mL/min and fractionated every 1 mL. Each fraction was assayed for cholesterol (Thermo Scientific) according to manufacturer’s protocols. HDL fractions (#17-20) were pooled and concentrated using filter columns to concentrate (Amicon Centrifugal Units). Concentrated HDL was used for all other assays.

Heme content – Individual mouse serum samples or HDL isolated from serum using FPLC as mentioned earlier were assayed for heme content using the QuantiChrom™ Heme Assay Kit (DIHM-250). It is a colorimetric determination of total heme at 400nm. The assay was performed according to the manufacturer’s protocol. Briefly, serum samples were mixed with the heme reagent. Additionally, calibrator wells (of known heme content = 62.5μM) and blanks were run on the same plate and incubated for 5 minutes at room temperature. The OD was measured at 400nm and a calculation was performed to generate the µM of heme in every sample measured. Heme content on HDL samples was normalized to HDL-C values.

Measurements of serum metabolites- Serum samples were analyzed for metabolites to test liver function and heme catabolism. 150μl of individual serum samples were tested on the Beckman Olympus AU400 machine. Alkaline phosphatase, alanine transaminase, albumin, total protein and direct bilirubin were estimated. Values for serum albumin for C57BL/6J mice have been reported (1).
**Cell free assay for ROS estimation**- ROS content in serum and lipoproteins was determined with 2,7,7′-dichlorofluorescein diacetate (DCFH₂) (Invitrogen). Serum or HDL from each mouse was incubated with DCFH-DA (10 μg/mL) in methanol for 30 min at 37°C. The presence of ROS was detected by measuring fluorescence intensity at 485nm/525nm and expressed in relative fluorescence units (RFUs).

**Monocyte Chemotactic Assay**- Monocyte chemotaxis assay was performed as previously described (2). This assay is a measure of the ability of test HDL to activate human aortic endothelial cells (HAECs) in an arterial wall co-culture system and cause a monocyte migration using chemotaxis. In general, HAECs (Human Aortic Endothelial Cells) were untreated or treated with TNF-α as the positive control, 0.1%BSA as negative control and test HDL from apoE⁻/⁻ and HxE⁻/⁻ mice for 4 hours. Solutions of HDL (50μg/ml) or TNF-α (10ng/ml) in phosphate-buffered saline were diluted with culture medium 199 (M199) containing 10% lipoprotein- deficient serum (LPDS) and added to the wells. This allowed for the collection of supernatants that were tested for monocyte chemotactic activity. At the end of incubation, the supernatants were collected from the co-cultures, diluted 20-fold, and assayed for monocyte chemotactic activity. Briefly, the supernatants were added to a standard Neuroprobe chamber (NeuroProbe, Cabin John, MD), with isolated human peripheral blood monocytes added to the top. The chamber was incubated for 60 min at 37°C. After the incubation, the chamber was disassembled and the non-migrated monocytes were wiped off. The membrane was then air dried and fixed with 1% glutaraldehyde and stained with 0.1% crystal violet dye. The number of migrated monocytes was determined microscopically and expressed as the mean SD of 12 standardized high power fields counted in quadruple wells.

**Atherosclerotic lesion analyses** - Heart and proximal aorta from mice were obtained. The heart was embedded in OCT compound and stored in -80 °C until use. Serial 10 μm-thick cryosections from the middle portion of the ventricle to the aortic arch were collected, mounted on pre-coated slides and stained with Oil Red O and hematoxylin. The lipid-containing area on each section centered on the aortic valves was determined in a blinded fashion, using the Biorevo BZ-9000 series (Keyence) microscope. The mean value of the lipid staining areas per mouse was calculated. *En face* analyses of the aorta were performed. After perfusion-fixation, the aorta was dissected out and cut longitudinally from heart to the iliac arteries, pinned on a black wax pan under water, and stained with Sudan IV solution. The image of the aorta was captured using a SONY DXC-970MD color video camera, and the image analysis was performed using the Image-Pro plus program (Media Cybernetics) in a blinded fashion. The area covered by atherosclerotic lesions divided by the area of the entire aorta was calculated and compared.

**Immunohistochemistry** - Fresh-frozen aortic root cryosections (10 μm-thick) were stored in -20°C until ready for immunohistochemistry. Briefly, slides were air-dried for 20 minutes at room temperature. Slides were fixed in ice-cold acetone for 20 minutes at -20°C. Slides were rinsed with PBS and were blocked for 2 hours at room temperature. Rat anti-mouse CD68, (1:1000; MCA 1957 AbD Serotec), rabbit polyclonal anti-mouse MHCII (1:1000; NBP1-76296, Novus Biologicals) and rabbit polyclonal anti-mouse ABCA1 (1:1000; NB400-105, Novus Biologicals) was used with an overnight incubation at 4°C. Next day rinse with
PBS and apply Dako EnVision+ System – HRP Labelled Polymer anti-rabbit (Dako, K4003) for MHCII and ABCA1 and incubate at room temperature for 30 minutes. Apply rabbit anti-rat immunoglobulins (Dako, Z0455 at the dilution of 1:1000) for CD68 and incubate at room temperature for 30 minutes. After rinsing with PBS then apply Dako EnVision+ System – HRP Labelled Polymer anti-rabbit (Dako, K4003) and incubate at room temperature for 30 minutes. Rinse all three antibodies with PBS and apply DAB (3,3'-Diaminobenzidine) on slides for 2 minutes for visualization. Rinse slides with tap water. Counterstain with Harris’ Hematoxylin. Air dry, and mount with media. Immunostaining was quantified in a blinded fashion the Biorevo BZ-9000 series (Keyence) microscope. The area for CD68 positive staining was calculated and compared. Subsequently, the area positive for MHCII or ABCA1 respectively was divided by the area positive for CD68 staining and a percent was calculated and compared to establish macrophages positive for MHCII or ABCA1 expression respectively.

**Western blotting** – Serum or BMDM cell lysate samples were fractionated on 4-20% precast SDS-polyacrylamide gels and electro-blotted onto nitrocellulose membranes (Bio-Rad) using a semi-dry or wet transfer apparatus (Bio-Rad). Membranes were blocked with phosphate-buffered saline, 0.1% Tween, and 5% nonfat dried milk for 1 hour, and then incubated overnight at 4°C with mouse Hx at 1:50 (Abgent), ABCA1 antibody at 1:1000 (Novus Biologicals #NB400-105), GAPDH antibody at 1:5000 (Sigma), and mouse apoE at 1:1000 (Meridian Life Science, Inc) in phosphate-buffered saline, 0.1% Tween. Membranes were washed 6 times (5 minutes/wash) with phosphate-buffered saline, 0.1% Tween, and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (GE Healthcare) was used at 1:5,000 dilution in phosphate-buffered saline, 0.1% Tween, and 2% nonfat dried milk for an additional 1 hour. Membranes were washed again and proteins were detected using chemiluminescence (Millipore).

**Malondialdehyde estimation assay**- Liver lipid peroxidation was measured with a colorimetric assay kit Bioxytech LPO-586 (OXIS International, Portland, USA) according to the manufacturer’s protocol. Briefly, liver tissue was homogenized with ice-cold buffer. 0.5M BHT in acetonitrile was added to prevent sample oxidation. The supernatant was collected and immediately used for the assay. The kit uses a chromatogenic reagent which reacts with the lipid peroxidation products malondialdehyde and 4-hydroxynonenal at 45°C, yielding a stable chromophore with maximum absorbance at 586 nm.

**Isolation of bone marrow cells**- Bone marrow cells were isolated from apoE−/− and HxE−/− mice. Briefly, tibia and femur were obtained from both hind limbs. The bone marrow was flushed using 10ml DMEM media (Life Technologies) supplemented with glucose, glutamine and sodium pyruvate and 10% heat-inactivated fetal bovine serum, 1% penicillin-streptomycin (complete media). Cell suspension was mixed well and seeded in 10cm or 6-well plates depending on the application. Cells were cultured in complete DMEM media with MCSF-1 (10 ng/mL; Gemini Bio-products #300-847P) in 5% CO2 at 37 °C.

**Cholesterol Efflux in BMDMs**- Cellular cholesterol efflux was performed as described previously with minor modifications (3). Freshly isolated bone marrow cells were differentiated in 10cm dishes in DMEM media (Life Technologies) supplemented with glucose, glutamine and sodium pyruvate and 10% heat-inactivated fetal bovine serum, 1%
penicillin-streptomycin (complete media) with MCSF-1 (10ng/ml; Gemini Bio-products #300-847P). Differentiated bone marrow derived macrophages (BMDMs) were lifted with 5mM EDTA and re-plated as 200,000 cells/well in 24-well tissue culture plates. Cells were loaded with $^{3}$H-cholesterol (1μCi/mL), 25μg/ml acetylated-LDL (Lee Biosolutions, St. Louis, MO) in DMEM media (serum-free) supplemented with 50mM glucose, 2mM glutamine, sodium pyruvate, 0.2 % fatty acid free bovine serum albumin (BSA) (DGGB) (Sigma, St Louis, Missouri, USA) and MCSF-1 (10ng/ml) overnight to allow cell cholesterol pools to equilibrate. LPS (10ng/ml, Enzo Life Sciences # ALX-581-008-L002) and LXR agonist (1μM, T0-901317, Sigma) were used as stimulants depending on the treatment regimen. After washing cells with 0.2% BSA the next day, cells were incubated with apoA-I (10μg/ml; Lee Biosolutions, St. Louis, MO) and MCSF-1 (10ng/ml) for 24 at 37°C and 5% CO₂. Cholesterol efflux was expressed as the percentage of radioactivity released from the cells in the medium relative to the total radioactivity in cells plus medium. Non-specific efflux was subtracted from apoA-I-specific efflux as background.

RNA Extraction, Reverse Transcription, and Quantitative-PCR- Bone marrow derived cells were freshly isolated and used as naïve cells or differentiated in 6-well plates with IL-4 (10ng/ml, Biorad # 574302) or LPS (10ng/ml) or hHx (45µg/ml, #16-16-080513, Athens Research and Technology, Georgia) and MCSF-1 (10ng/ml; Gemini Bio-products #300-847P) in DMEM complete media. Cells were subsequently harvested for RNA and protein. RNA isolation was done by adding Buffer RLT plus and B-Mercaptoethanol (1:100). Cell lysates were immediately frozen at -80C. RNA was isolated using the manufacturer’s protocol (RNEasy Mini kit, Qiagen). 40ul of RNA was eluted and quantified using Nanodrop. cDNA synthesis was carried out using 1ug of RNA and iScript cDNA Synthesis Kit (Biorad). The cDNA was diluted 1:20 to use for quantitative-PCR. The reverse transcription reaction contained 2ng of reverse transcribed total RNA, 0.5uM of forward and reverse primers each, and 10ul of Master Mix SYBR Green in a final volume of 20ul. Samples underwent the standard PCR protocol. QPCR primers are listed in Table II of the supplemental methods. Threshold cycle number for genes of interest was normalized to housekeeping gene GAPDH. Data were expressed as a -fold change in mRNA expression relative to control values.

Flow cytometry- To prepare single-cell suspensions for flow cytometry, differentiated BMDMs were treated with 5mM EDTA solution. Then cells were gently lifted off the plates by gentle pipetting and transferred into flow test tubes already placed on ice. After single-cell suspensions were collected, they were incubated for 30 minutes on ice with the following: CD11b-PerCPCy5.5 (1:400) from eBioscience #45-0112-80, F4/80-e450 (1:400) eBioscience #48-4801-80 for identifying the macrophage population. ABCA1 (1:400) Novus Biologicals #NB400-105 was incubated with the cells for 1 hour and anti-rabbit IgG (H+L) Alexa Fluor-488 (1:200) Thermo Scientific #A-11070 was used a secondary fluorophore labelled antibody. Cells were washed twice before analysis on the BD LSR-II flow cytometer (Beckman Coulter). Cell sorting was performed on the BD FACSAria-II high-speed cell sorter and data were analyzed with FlowJo software (TreeStar).
**Statistical Analysis:** Numerical data are expressed as means (±SEM) as indicated in the figure legends. All statistical analysis was carried out using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). Significance was determined as indicated in the figure legends. Differences between two experimental groups were evaluated for statistical significance using unpaired two-tail Student \( t \)-test (non-parametric). We have used the non-parametric Kruskal Wallis one way ANOVA test for comparing more than two groups of data. All data passed normality testing. We used the D'Agostino-Pearson omnibus normality test. Data sets smaller than 8 passed the normality test under the Kolmogorov-Smirnov test. For data sets that did not pass the normality test we excluded them from the analysis.

