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ApoE^{-/-} Mice Lacking Hemopexin Develop Increased Atherosclerosis *via* Mechanisms
That Include Oxidative Stress and Altered Macrophage Function

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Molecular and Medical Pharmacology

by

Niyati Mehta Thosani

2015

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2015

ABSTRACT OF THE DISSERTATION

ApoE^{-/-} Mice Lacking Hemopexin Develop Increased Atherosclerosis *via* Mechanisms
That Include Oxidative Stress and Altered Macrophage Function

by

Niyati Mehta Thosani

Doctor of Philosophy in

Molecular and Medical Pharmacology

University of California, Los Angeles, 2015

Professor Srinivasa T. Reddy, Chair

Objective: We previously reported that Hemopexin (Hx), an acute phase protein and a heme scavenger, is significantly increased and associated with proinflammatory HDL under atherogenic conditions. Although it is established that Hx together with macrophages plays a role in mitigating heme and ROS mediated oxidative damage to cells and tissues, the role of Hx in systemic oxidative stress, HDL function, macrophage function, and the development of atherosclerosis, is not known.

Approach and Results: We generated Hx and apoE double knockout mice (HxE^{-/-}) on a C57BL/6J background to determine the role of Hx in the development of atherosclerosis. HxE^{-/-} mice had significantly more free heme, ROS, and proinflammatory and dysfunctional HDL in their circulation, when compared to control apoE^{-/-} mice. Atherosclerotic plaque area (apoE^{-/-} = $9.72 \pm 2.5 \times 10^4 \mu\text{m}^2$ and HxE^{-/-} = $27.23 \pm 3.6 \times 10^4 \mu\text{m}^2$) and macrophage infiltration (apoE^{-/-} = $38.8 \pm 5.8 \times 10^3 \mu\text{m}^2 \mu\text{m}^2$

and $\text{HxE}^{-/-} = 103.4 \pm 17.8 \times 10^3 \mu\text{m}^2$) in the aortic sinus were significantly higher in the $\text{HxE}^{-/-}$ mice when compared to $\text{apoE}^{-/-}$ mice. Also, atherosclerotic lesions in the aortas were significantly higher in the $\text{HxE}^{-/-}$ mice when compared to $\text{apoE}^{-/-}$ mice. Analysis of polarization and phenotype revealed that macrophages from $\text{HxE}^{-/-}$ mice were more M1-like and proinflammatory. *Ex vivo* studies demonstrated that $\text{HxE}^{-/-}$ macrophage cholesterol efflux capacity was significantly reduced when compared to $\text{apoE}^{-/-}$ mice.

Conclusion: We conclude that Hx plays a novel protective role in alleviating heme induced oxidative stress, improving inflammatory properties of HDL and macrophage function and inhibiting the development of atherosclerosis in $\text{apoE}^{-/-}$ mice.

The dissertation of Niyati Mehta Thosani is approved.

Jonathan Braun

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University of California, Los Angeles

2015

DEDICATION

I would like to dedicate this work to my parents, Mr. Umesh J. Mehta and Mrs. Seema Umesh Mehta. They have provided me every opportunity to achieve all my goals. They have always been available to listen and advise.

I would also like to dedicate this thesis to my husband, Gaurav Thosani. He is patient and compassionate and has been very supportive through happy and difficult times.

Lastly, I would like to dedicate this to all my other family members and friends who have encouraged me and helped me.

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Mehta, N.U., Hama, S., Grijalva, V. and Reddy, S.T. *ApoE^{-/-} mice lacking hemopexin develop aggravated atherosclerosis via mechanisms that include altered HDL inflammatory properties and macrophage function.* Poster presentation, ATVB Meeting, San Francisco, 2015.

Mehta, N.U., Krystal, C., Hama, S., Grijalva, V., Reddy, S.T. *Hemopexin improves High Density Lipoprotein (HDL) function and inhibits the development of atherosclerosis in ApoE^{-/-} mice*. Poster presentation, American Heart Association Meeting (AHA), Texas, 2013.

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Chapter 1

Introduction

Atherosclerosis: Coronary artery disease (CAD) is the leading cause of morbidity and mortality in developed countries and is likely to attain this status worldwide, accounting for 16.7 million deaths each year (1, 2). Atherosclerosis is a chronic and systemic vascular inflammatory process that forms the pathological basis of CAD (3). Understanding the principles of the inflammatory processes is important for deciphering the complexity involved in atherosclerosis progression.

Atherogenic dyslipidemia is a major risk factor for premature development of CAD. This state represents an imbalance in favor of circulating levels of proatherogenic low density lipoprotein (LDL), rather than anti-atherogenic high density lipoprotein (HDL). Large, prospective, epidemiological studies reveal that low HDL cholesterol (HDL-C) concentrations constitute an independent predictive risk factor for CAD. CAD risk is elevated by approximately 3% in women and 2% in men for each decrement of 0.0259 mM (1mg/dl) in HDL-C (4). The imbalance between circulating levels of atherogenic lipoproteins (LDL, VLDL and intermediate density lipoprotein) relative to those of HDL is associated with induction of endothelial dysfunction, which is in turn intimately related to inflammation and oxidative stress (5); as a result, dyslipidemia, inflammation and oxidative stress are closely interrelated in the development of atherosclerosis. Oxidative stress, an integral component of inflammation, involves an imbalance that favors pro-oxidant factors such as reactive oxygen, nitrogen and chlorine species over antioxidant factors (6). This typically underlies endothelial dysfunction—a central process in the pathophysiology of atherosclerosis (Figure 1A).

Endothelial injury and activation involves both excessive penetration and intimal retention of proatherogenic, cholesterol-rich, apolipoprotein B (apoB)-containing

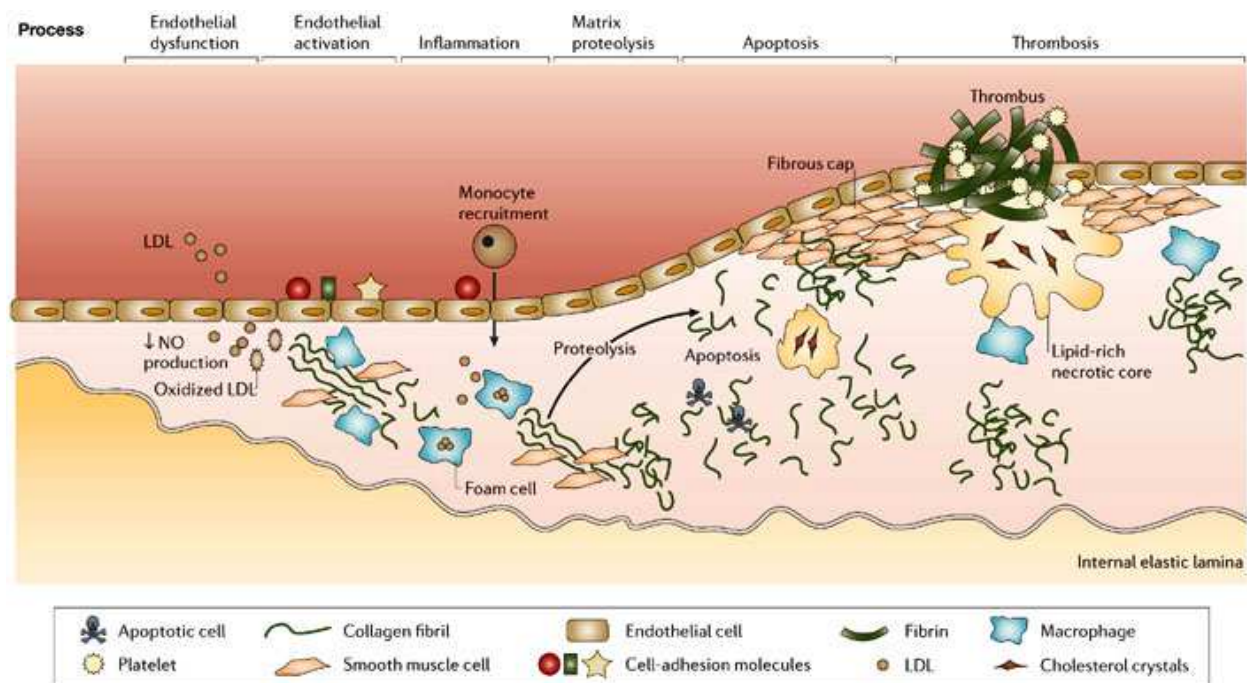
lipoprotein LDL rather than anti-atherogenic apolipoprotein A-I (apoA-I)-containing HDL. In addition, monocyte recruitment, differentiation and activation of macrophages in the arterial wall also occur. Preferential retention of LDL in the arterial wall via proteoglycan binding makes this lipoprotein a major substrate for oxidation by arterial wall cells (5, 7). After inflammatory activation, monocytes recruited to the intima express scavenger receptors that permit the uptake of modified LDL particles, such as oxidized LDL (ox-LDL). Ox-LDL has multiple proatherogenic properties, which include induction of cholesterol accumulation in monocytes as well as potent proinflammatory, immunogenic, apoptotic and cytotoxic activities (5). Cholesterol loading of monocytes leads to the formation of foam cells, and ultimately leads to the mature lipid-laden macrophages of the plaque's core (Figure 1A). Most of the proinflammatory properties of ox-LDL arise from bioactive products of LDL lipid peroxidation. As a result, LDL oxidation further propagates the inflammatory process in the arterial wall, thereby accelerating atherosclerosis (5). Atherosclerosis can be regarded, therefore, as a chronic inflammatory disease of the arterial wall, mediated in part by ox-LDL and other proinflammatory agents, such as reactive oxygen species (ROS), tissue factor pro-coagulants and cytokines produced by inflammatory macrophages and macrophage-derived foam cells. These cells can amplify local inflammation and promote thrombotic complications.

Free heme is also a potent proinflammatory molecule that is sensed by innate immune receptors and causes inflammation. Heme is an essential prosthetic group for hemoproteins such as hemoglobin (Hb). Heme is also involved in numerous cardiovascular processes. Heme-mediated synthesis of ROS is implicated in the

pathophysiology of atherosclerosis and CAD. Heme is also required for the function of many cardiac enzymes and heme-containing antioxidant proteins. Heme is an essential molecule for living aerobic organisms and is involved in a remarkable array of diverse biological processes and several diseases.

Figure 1

(A)



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Figure 1A: Pathogenesis of atherosclerosis: Low Density Lipoprotein (LDL) cholesterol enters dysfunctional endothelium and is oxidized by macrophage and smooth muscle cells. Release of growth factors and cytokines, and upregulation of adhesion molecules, attracts further monocytes. Foam cells (arising from lipid-laden macrophages) accumulate and smooth muscle cells proliferate, which results in the

growth of the plaque. Inflammatory cell infiltrate, smooth muscle cell death through apoptosis, and matrix degradation through proteolysis generate a vulnerable plaque with a thin fibrous cap and a lipid-rich necrotic core. Plaque rupture can cause thrombosis which might be sufficient to cause vessel occlusion.

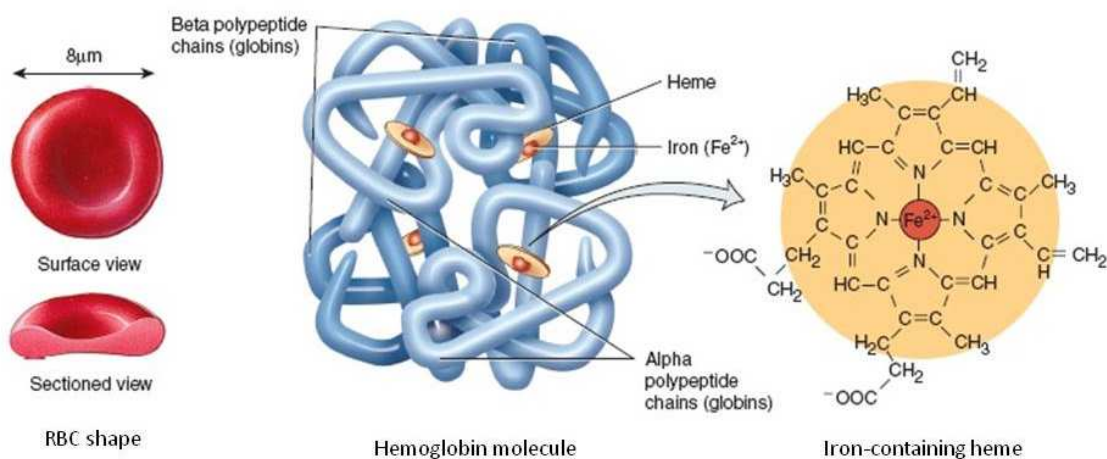
Heme: Heme is an essential prosthetic group for Hb. Heme consists of an iron atom and a heterocyclic tetrapyrrole ring system, referred to as a porphyrin (Figure 1B). There are several types of heme, and they differ in the composition of their side chains around the pyrrole rings. The function of the heme molecule is determined by the polypeptide bound to it (9). In Hb, each of the 4 heme moieties is conjugated to a globin polypeptide, forming a globular molecule made up of 4 subunits (10). The iron atom in the heme in Hb is in the Fe(II) ferrous state to allow for reversible binding of iron with molecular oxygen, which completes the octahedral coordination of the central iron atom (Figure 1B). Oxygenation changes the electronic state of the Fe(II)–heme complex, as reflected by the color change of blood from the dark purple of venous blood to the bright red of arterial blood. When blood is exposed to various oxidizing agents, the heme iron in Hb is oxidized from the Fe(II) ferrous to the Fe(III) ferric state to form methemoglobin, which is unable to bind oxygen. The function of heme-containing proteins depends strongly on the ability of heme to coordinate an iron atom inside its structure and facilitate reduction–oxidation (redox) reactions.

Although every cell in the body requires heme, the 2 major sites of heme synthesis are the bone marrow and the liver. The most rapid rates of heme synthesis occur in the erythroid cells of the bone marrow and in hepatocytes. In human erythroid cells, ~300mg of heme, which accounts for 75% of total body heme, is synthesized daily to

support hemoglobin production, whereas in hepatocytes, ~50mg of heme per day is produced and incorporated into cytochrome P450, catalases, reticuloendoplasmic cytochrome B5, and mitochondrial cytochromes (11).

As heme overload usually occurs in the bloodstream under pathologic conditions, circulating and endothelial cells are the first cell populations exposed to heme-mediated damage. Heme entraps into red blood cell membranes and shortens the erythrocyte life span, thus enhancing hemolysis (18). The lipophilic nature of heme allows it to intercalate into biologic membranes, perturbing lipid bilayers, promoting the conversion of low-density lipoprotein to cytotoxic oxidized products, and favoring iron to participate in Fenton reaction, a process during which ROS are produced (14, 15, 16, 17, 18, 19).

(B)



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Figure 1B: Structure of the hemoglobin molecule: Hb has four heme moieties and a central iron atom in ferrous state. The heme is surrounded by two alpha polypeptide chains and two beta polypeptide chains (globins).

Hemopexin: Heme binds with the highest known affinity ($K_d < \text{pM}$) to hemopexin (Hx) in circulation. Hx is a 60-kDa plasma glycoprotein composed of a single 439 amino acid–long peptide chain. It is encoded by an 11-Kb gene located on human chromosome 11 (chromosome 7 in mice) and is expressed mainly in liver. Other sites of Hx synthesis are the nervous system, skeletal muscle, retina, and kidney. The concentration of Hx in plasma is 0.5–1.2 mg/ml. This can, however, increase during inflammatory events, because Hx is an acute-phase protein (53). Hx constitutes two homologous ~200 residue domains (known as Hx domains), separated by a 20 residue linker region. The Hx domain presents as a disk-like structure composed of a four-bladed b-propeller fold (54). The two disk-like domains of Hx are oriented perpendicular to one another, and heme has been shown to bind between these two propeller domains in a pocket bounded by the linker region (54).

The only known receptor for the heme-hemopexin (heme-Hx) complex is the scavenger receptor, LDL receptor–related protein (LRP-1) or CD91, which is expressed in most cell types, thus indicating multiple sites of heme-Hx complex recovery. The important function of Hx is heme scavenging at the systemic level, consisting of the transport of heme to the liver, where it is catabolized or used for the synthesis of hemoproteins or exported to bile canaliculi. This is important both in physiologic heme management for heme-iron recycling and in pathologic conditions associated with intravascular hemolysis to prevent the pro-oxidant and proinflammatory effects of heme. Other than scavenging heme, the heme-Hx complex has been shown to be able to activate signaling pathways, thus promoting cell survival and modulate gene expression.

(C)

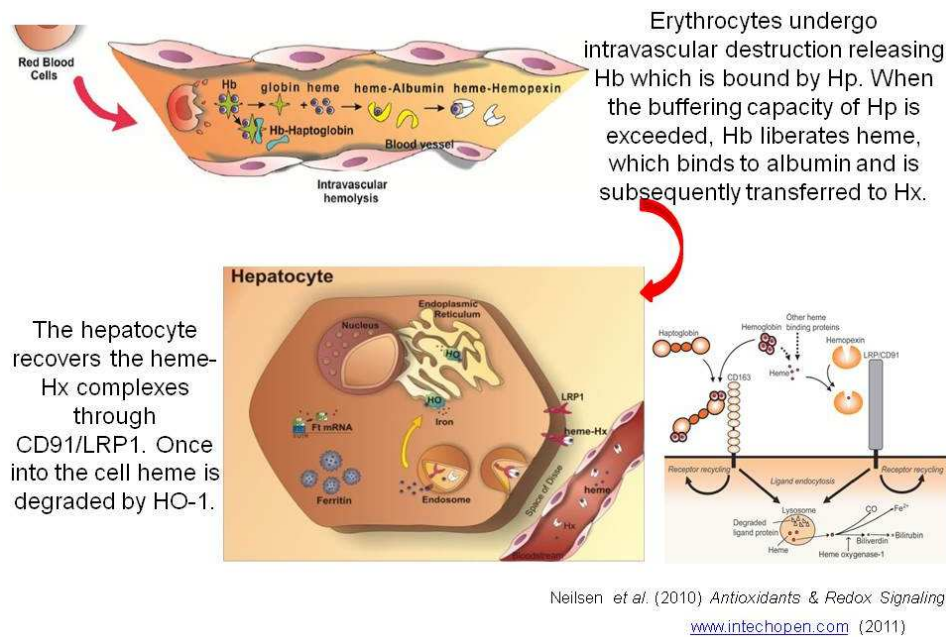


Figure 1C: Hemoglobin, heme and Hx: Fate in circulation: Hx binds to heme to form a heme-Hx complex. The complex gets cleared from circulation through receptor-mediated endocytosis by the CD91/LRP1 receptor.

Signaling role of heme-Hx: Several studies have demonstrated that the heme-Hx complex, other than mediating heme uptake, may activate intracellular signaling pathways, important for cell proliferation and survival. The heme-Hx complex supports and stimulates proliferation of human acute T-lymphoblastic cells (MOLT-3) by its ability to replace iron transferrin (64). Besides this function, heme-Hx has been demonstrated to increase serum-stimulated MOLT-3 cell growth, suggesting that the heme-Hx receptor activation triggers signaling pathways involved in the regulation of cell growth. The heme-Hx-stimulated signaling pathway has been demonstrated to involve protein kinase C (PKC) activation (64). Moreover, the heme-Hx complex has been shown to

induce the nuclear translocation of nuclear factor-kappa B (NF- κ B), a key transcription factor activated by stress and involved in the cellular responses to inflammation (60).

In Hepa cells, it was demonstrated that the heme–Hx complex increased the expression of p53 and p21, thus allowing long-term survival (60). Furthermore, the cell-cycle inhibitor p21, by binding JNK/SAPK, may prevent apoptosis. This may explain why, despite sustained elevated levels of phospho-c-Jun, Hepa cells exposed to heme–Hx do not undergo apoptosis. These findings represent additional evidence for the pleiotropic protective effects of heme–Hx and may explain why Hx-null mice showed extensive cellular damage and cell death after heme overload (62, 63). Cells expressing the heme–Hx receptor may catabolize heme and activate pathways controlling cell proliferation, survival, and apoptosis.

Protective role of Hx: Several studies demonstrated the potent antioxidant role of Hx. Heme binding to Hx has been demonstrated to reduce heme-mediated free radical production from organic peroxides (55). Furthermore, *in vitro* studies by Grinberg et al. (56) demonstrated that Hx strongly decreased the peroxidative and catalytic activity of heme by forming inactive heme– protein complexes. Interestingly, heme activities were found to be inhibited by 80–90% with Hx but only by 50–60% with either human or bovine albumin. Moreover, binding to Hx was shown to inhibit heme-catalyzed lipid peroxidation in artificial liposomes (57), rat liver microsomes (58), and plasma low-density lipoprotein (59). Finally, Eskew and coauthors (60) demonstrated that Hx has an essential role in the prevention of heme-induced oxidative damage and cell death in Hepa cells.

Much experimental evidence also supports the antioxidant function of Hx *in vivo*. Hx-null mice have been demonstrated to be particularly sensitive to heme overload and more prone to heme-induced oxidative damage and inflammation during hemolytic processes. Furthermore, *in vivo* studies showed that the most-damaged tissues with heme overload conditions are the vasculature, the kidney, and the liver (61, 62).

After heme overload, Hx-null mice in the vasculature have shown an increased induction of the adhesion molecules intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) in the endothelium and an increased vascular permeability compared with wild-type mice. This demonstrated that Hx activity is required to prevent heme-induced vasopermeabilization and endothelial activation. Furthermore, heme-overloaded Hx-null mice showed a higher expression of HO-1 in the endothelium than did wild-type animals and the induction of HO-1 before heme overload preserved endothelial integrity, thus indicating that Hx and HO-1 work in the same pathway to counteract the toxic effect of heme (62).

Besides the vasculature, Hx-null mice recovered more slowly after phenylhydrazine-induced hemolysis and had severe renal damage compared with control mice. These animals had prolonged hemoglobinuria, higher iron loading, and lipid peroxidation in the kidney than did wild-type mice (61). Increased oxidative damage in the Hx-null kidney was also evident after heme injection (62).

Finally, when Hx is lacking, heme overload resulted in a marked liver congestion accompanied by red blood cell stasis and sinusoidal dilation around the centrilobular vein. Hepatic congestion was found to be associated with abnormal iron deposits,

increased lipid peroxidation, and massive leukocyte infiltrates (62). Liver damage was also evident in compound mutant mice, lacking both Hx and haptoglobin (Hp), after intravascular hemolysis (63).

Watanabe *et al.* (92) reported that Hb was associated with HDL in mouse models of atherosclerosis. They demonstrated that Hb when associated with HDL was predominantly in the oxyHb form and could effectively consume NO and contract arterial vessels *ex vivo* (92). In a subsequent study, Watanabe *et al.* (93) reported that Hb scavenger proteins, Hp and Hx, were significantly increased in the apoA-I-containing particles of HDL both in mouse models of atherosclerosis and in CAD patients, when compared with their wild type counterparts, respectively. Watanabe *et al.* (93) further demonstrated that the association of Hb, Hp, and Hx proteins with HDL positively correlated with the inflammatory properties of HDL and systemic inflammation in CAD patients. Watanabe *et al.* (93) showed that HDL from Hp^{-/-} mice under atherogenic conditions did not accumulate Hb and was anti-inflammatory whereas HDL from Hx^{-/-} mice was proinflammatory, indicating a novel protective role of Hx in the inflammatory properties of HDL in atherogenic mice. These studies suggested for the first time that the Hb/Hx/Hp pathway, in part, modulates the inflammatory properties of HDL in both mice and humans during systemic inflammation and atherosclerosis.

Although it is established that Hx plays a role in mitigating heme and ROS mediated oxidative damage to cells and tissues, the role of Hx in systemic oxidative stress, HDL function, macrophage function, and the development of atherosclerosis, is not known.

To study the role of Hx in the development of atherosclerosis, we generated Hx^{-/-} mice on an apoE^{-/-} background (HxE^{-/-}) in order 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 also show that Hx deficiency results in increased oxidative stress and proinflammatory HDL in HxE^{-/-} mice.

Chapter 2

Generation of Hx^{-/-}/ApoE^{-/-} (HxE^{-/-}) mice

Introduction

To understand the role of Hx in the formation and/or accumulation of proinflammatory HDL, our laboratory obtained Hemopexin (Hx^{-/-}) knockout mice on a C57BL/6J background from Franklin G. Berger (University of South Carolina). Hx^{-/-} mice were bred on to the hyperlipidemic apoE null background (from Jackson Labs (Bar Harbor, ME) to generate the double knockout mice; Hx^{-/-} /ApoE^{-/-}(HxE^{-/-}). ApoE^{-/-} mice were used as controls. Female HxE^{-/-} and apoE^{-/-} mice (n=15-20 per group) were fed a chow diet for twenty four weeks to study the role of Hx in the development of atherosclerosis.

Results

DNA genotyping was performed for all the mice using tail DNA (Figure 2A and B). Gene knock outs were also confirmed by western blot using mouse sera (Figure 2C and D). Absence of apoE was shown by a band at 145 base pairs (bp) while a band at 155bp indicated wild type for apoE gene (Figure 2A). Hemopexin knock out was estimated by a PCR product band at 320 bp, while a wild type band was estimated at 290 bp (Figure 2B). Fig 2 C shows a western blot for apoE protein at 34 KDa. We observed bands for apoE at 34 KDa only in Bl/6J sera but not in apoE^{-/-} and HxE^{-/-} sera (Figure 2C), while Hx bands were observed at 60KDa in Bl/6J and apoE^{-/-} sera but not in HxE^{-/-} sera (Figure 2D).

Figure 2

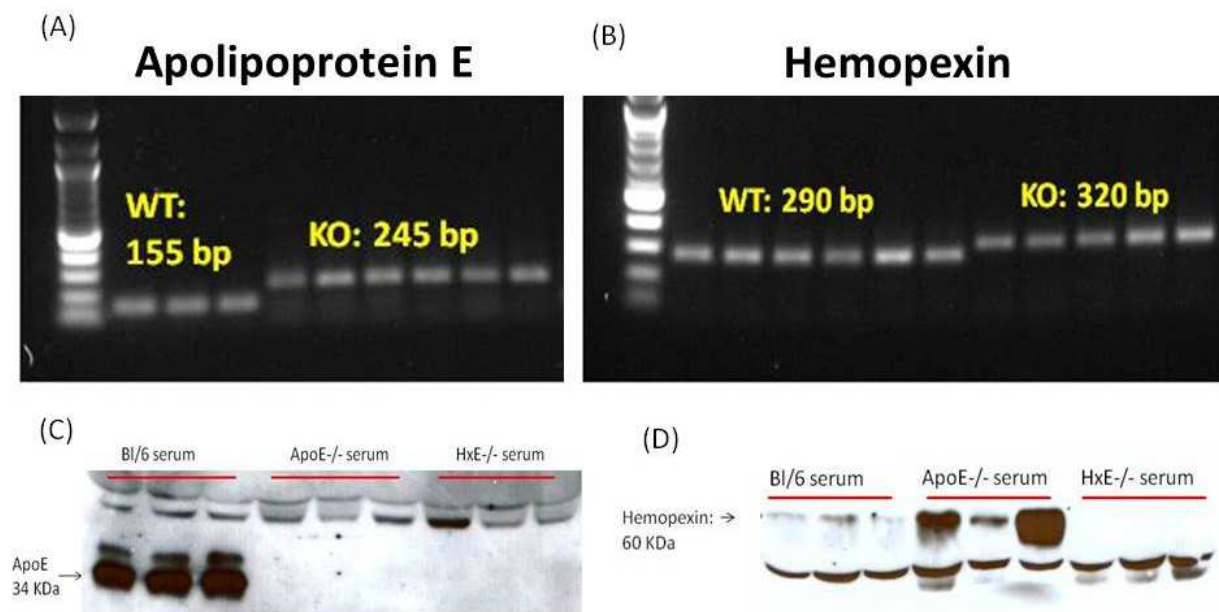


Figure 2: Generation of $Hx^{-/-}/ApoE^{-/-}$ ($HxE^{-/-}$) mice: (A and B) Absence of *apoE* and *Hx* was shown in an agarose gel. (C and D) Absence of *apoE* and *Hx* was shown by western blot analysis.

Methods

Animal Experiments – All animal experiments presented here were approved by the UCLA animal research committee. $ApoE^{-/-}$ and $Hx^{-/-}/ApoE^{-/-}$ ($HxE^{-/-}$) female mice 24 weeks of age were used in all experiments unless mentioned otherwise. Mice were fed a chow diet (Ralston Purina Mouse Chow). Serum samples were isolated from mice after transferred to new cages with fresh bedding and fasted overnight, cryopreserved in 0.2X sucrose, and freshly frozen at -80°C until use.

Genomic Polymerase Chain Reaction - The hemopexin (Hx) gene has been disrupted by the insertion of a lacZ-PGK Neomycin (Neo) cassette on the ATG start codon located in the first exon. The deletion in the genomic DNA can be detected using a touch-down PCR with the following three primers: 5'- TCC TGT GTG GCC TTT GCA GC-3', 5'- CAA CTT CGG CAA CTC TCC CG-3', 5'- GAT GCG GTG GGC TCTA TGGC-3'. Polymerase chain reaction conditions used were 94°C for 4 minutes, 20 cycles of 92°C for 40 seconds, 65°C for 45 seconds(-0.5°C/cycle), and 72°C for 45 seconds. Subsequently, 25 cycles of 92°C for 40 seconds, 55°C for 30 seconds and 72°C for 45 seconds with a final extension of 72 °C for 10 mins. The PCR product was run on a 1% agarose gel for detection of the PCR product.

The murine apolipoprotein E (apoE) gene contains 4 exons. The gene has been disrupted by insertion of a Neo cassette sequence in replacement of exons 2 and part of 3. The deletion can be detected using the following three primers: 5'-GCC GCC CCG ACT GCA TCT-3', 5'-TGT GAC TTG GGA GCT CTG CAG C-3', 5'-GCC TAG CCG AGG GAG AGC CG-3'. These primers amplify a section of exon 3 (Wildtype) and the Neo cassette and exon 3 (knock out) to yield a unique band specific to the knockout or a wild-type section of exon 3. Polymerase chain reaction conditions used were 94°C for 3 minutes, 35 cycles of 94°C for 20 seconds, 68°C for 40 seconds, and 72°C for 2 minutes with a final extension of 72 °C for 10 mins. The PCR product was run on a 1% agarose gel for detection of the PCR product.

Western blotting – Serum samples were fractionated on 4-20% pre-cast SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes (Bio-Rad, Hercules, California) using a semi-dry apparatus (Bio-Rad, Hercules, California).

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, San Diego, CA) 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) 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).

Chapter 3

Hepatic heme detoxification is dysregulated in apoE^{-/-} mice lacking hemopexin

Introduction

Heme scavenging and degradation: When red blood cells are damaged within the vascular compartment Hb escapes into the plasma, dimerizes, and is rapidly bound by Hp. Scavenging of Hb by Hp is critical for preventing Hb mediated oxidative damage. Hp is an acute phase, plasma-born glycoprotein that is produced mainly by hepatocytes, but also by other cell types including adipocytes and lung cells, and is well known for its ability to tightly bind free Hb following its release from erythrocytes (34). Hp plasma concentrations are high, ranging from 0.3 mg/mL to 3.0 mg/mL, yielding an Hb-Hp molar ratio of 400:1 and allowing effective Hb scavenging. Following its formation, Hb-Hp complex is taken up by various scavenger receptors in the liver and other tissues. One such receptor is the CD163 receptor, present on macrophages and Kupffer cells. The CD163 receptor strongly binds the Hb-Hp complex and facilitates its endocytosis and degradation (35). This activity of Hp is important for the prevention of iron loss via Hb filtration by the glomeruli and causing renal damage.

Since haptoglobins are not recycled, severe hemolytic diseases result in rapid Hp depletion and rapid accumulation of free heme. Hx is an acute phase glycoprotein that is able to bind an equimolar amount of heme with high affinity (36). Many types of cells synthesize Hx including hepatic parenchymal cells, ganglionic and photoreceptor cells of the retina, kidney mesangial cells, and cells in the central and peripheral nervous system (37-39). In adults, the serum Hx concentration ranges from 0.40 to 1.5 mg/mL, and the level of Hx increases in response to high concentrations of free heme. Hx scavenges free heme, and the resultant heme-Hx complex is taken up by the liver through receptor-mediated endocytosis (40). CD91/LRP1 is the scavenger receptor

identified for scavenging the heme-Hx complex from circulation to prevent heme mediated tissue damage (40). Heme can transiently bind to lipoproteins such as HDL and LDL (21), albumin (189), α 1-microglobulin (190) before being transferred to Hx for scavenging.

Cells have heme degradation machinery to prevent oxidative damage associated with intracellular heme accumulation (22, 41). Heme oxygenase (HO) enzymes are involved in the catabolism of heme, thus preventing the buildup of excess heme (42, 43). Mammals have 2 isoforms of HO. Heme oxygenase-2 (HO-2) is the constitutively expressed form and is present in almost every tissue. In contrast, Heme oxygenase-1 (HO-1) is normally present only in cells responsible for systemic heme degradation, such as macrophages in the reticuloendothelial system; however, this isoform is inducible, and its expression can increase dramatically in different organs in response to heightened levels of circulating heme and Hb (44). Heme oxygenases catabolize heme to equimolar amounts of biliverdin, carbon monoxide, and free iron, followed by the conversion of biliverdin into bilirubin by biliverdin reductase. The majority of heme degradation results from the breakdown of hemoglobin in senescent red blood cells, primarily in the spleen. Although excess heme is detrimental, heme degradation exhibits protective effects because of the production of carbon monoxide and biliverdin. The carbon monoxide released from heme degradation can function as a signaling molecule via soluble guanylate cyclase which in turn leads to vasodilation and inhibits platelet aggregation (45). It also activates the Akt signaling pathway, which provides protection in various stress models, including doxorubicin-induced cardiomyopathy and liver ischemia–reperfusion (46, 47). Meanwhile, biliverdin is converted to bilirubin and

excreted out of the body predominantly by the liver but also by the kidney (50-52) and hence is protective against oxidative stress (48, 49). The gradual color change of a healing bruise, from red heme to green biliverdin to yellow bilirubin, is a classic visible manifestation of heme degradation. Although HO prevents the accumulation of heme, it also generates free iron. This free iron becomes stored in ferritin through coordinated expression of ferritin with the heme-degradation machinery.

Hx is required for heme-dependent activation of heme oxygenase 1: During intravascular or extravascular hemolysis, Hx binds and transports cytotoxic free heme, to hepatocytes and macrophages where it can be efficiently catabolized. The heme that is transported from the blood stream to the liver and macrophages by Hx is catabolized by the anti-inflammatory gene, HO-1 (74, 75). Heme has been shown to induce the expression of HO-1 by inactivating the transcription repressor Bach1 through direct binding (76). However, the source of heme for the regulation of the Bach1-HO-1 axis had been unclear. Since extracellular heme exists as a complex with Hx in serum under the physiological conditions, Hada *et al.* (76) examined the effects of (recombinant) rHx-bound heme on HO-1 expression and Bach1 in Hepa-1c1c7 liver cells as well as THP-1 macrophage cells. Hada *et al.* demonstrated that rHx-bound heme was internalized into the cells via endocytosis, resulting in HO-1 expression and inactivation of Bach1, suggesting that i) induction of HO-1 expression is Hx-dependent and ii) Hx plays an antiatherogenic role. It should be noted that Hx has also been shown to exert antiatherogenic activity in a non-HO-1 dependent mechanism (77). Liang X, et al. (77) reported that Hx limits (Toll-Like Receptor) TLR4 and TLR2 agonist-induced

macrophage cytokine production directly through a mechanism distinct from HO-1 that does not involve binding and clearing of free heme (77).

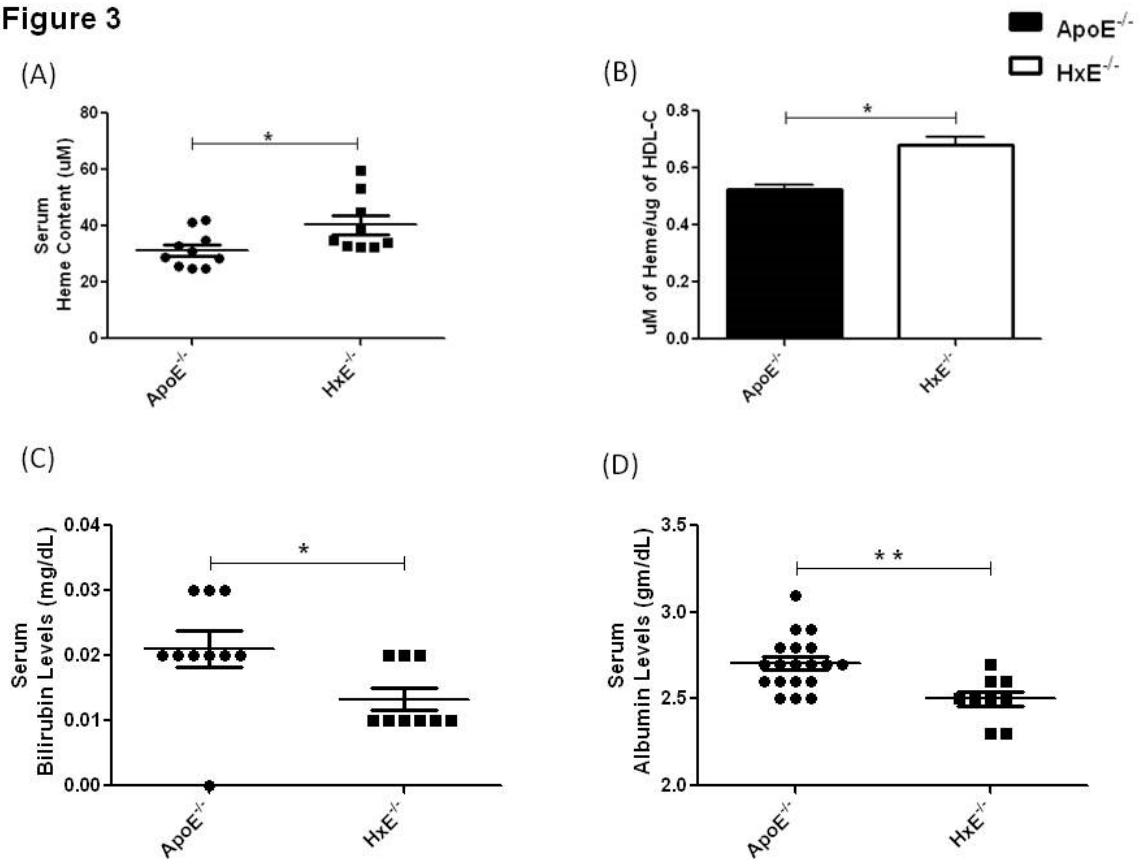
To study the effect of Hx deletion in apoE^{-/-} mice on heme degradation and detoxification we performed the following experiments.

Results

We observed significantly higher levels of heme in the serum (Figure 3A) and HDL fractions (Figure 3B) of HxE^{-/-} mice compared to apoE^{-/-} mice (n = 8 - 10). Levels of bilirubin, a heme breakdown product (Figure 3C) and albumin (Figure 3D) were significantly lower in the serum of HxE^{-/-} mice compared to apoE^{-/-} mice. The serum samples were devoid of any noticeable hemolysis that may have occurred during retro-orbital bleeding of the mice, which could potentially account for higher heme levels. However, HxE^{-/-} serum samples were visibly redder (Figure 3E) compared to their matched control apoE^{-/-} serum samples indicating endogenous hemolysis. 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 to apoE^{-/-} mice (Figure 3E-G). Interestingly, we also observed that absence of Hx strongly induced the gene expression of the Hb scavenger protein, Hp (Figure 3H) and the Hb-Hp scavenger receptor CD163 (Figure 3I) in the livers of HxE^{-/-} mice. Significantly lower gene expression of albumin in the liver of HxE^{-/-} mice (Figure 3J) supported the lower levels of albumin in the serum of HxE^{-/-} mice (despite the lower levels of albumin in the HxE^{-/-} mice compared to the apoE^{-/-} mice, the levels were within

the normal range reported for C57BL/6J mice as shown in the Materials and Methods). 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 in the serum and lipoproteins and a reduction in circulating anti-oxidants.

Figure 3



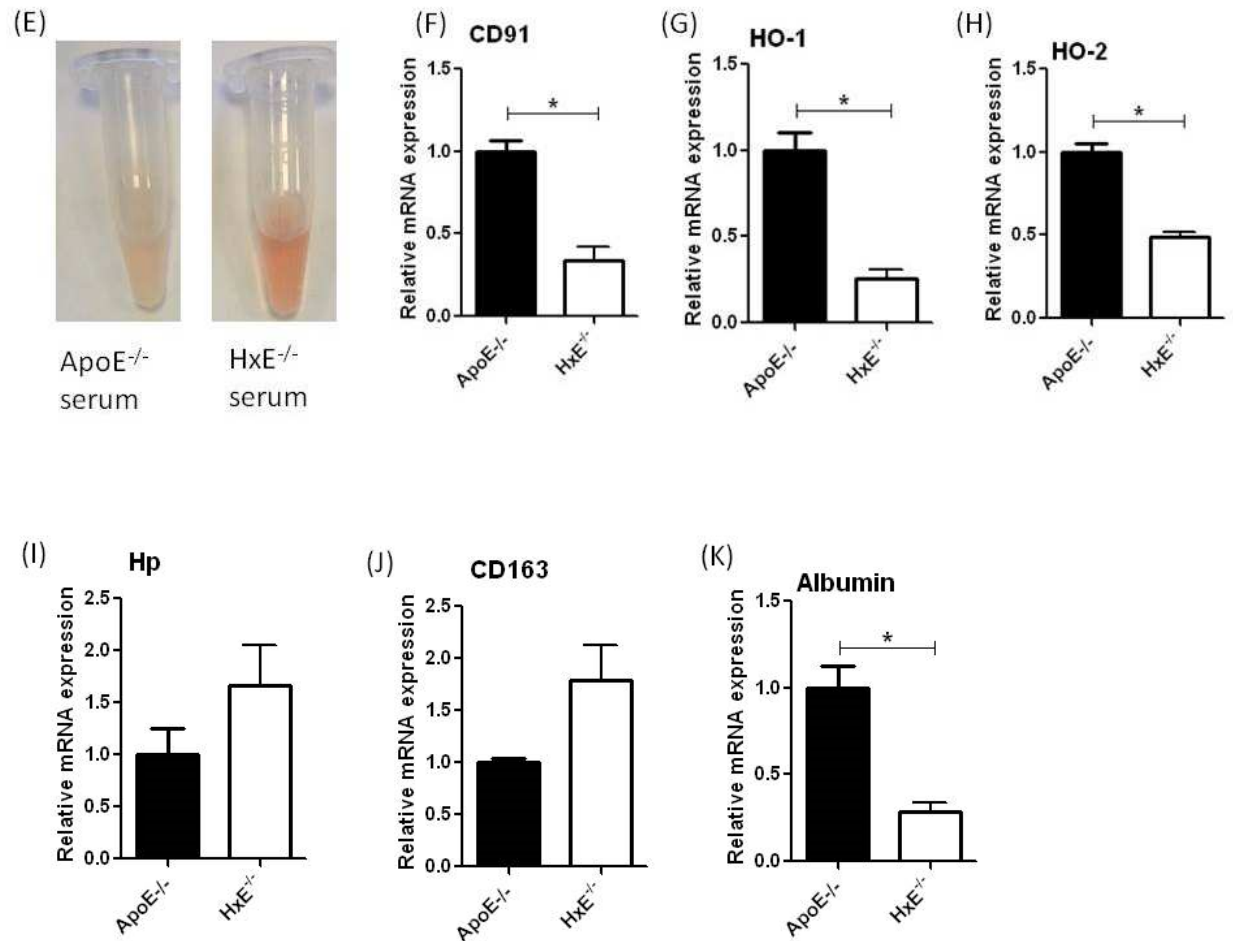


Figure 3: Hepatic heme detoxification is dysregulated in apoE^{-/-} mice lacking hemopexin: Heme content was measured colorimetrically in the (A) serum and (B) pooled HDL fractions of apoE^{-/-} and HxE^{-/-} mice (n = 8 - 10). (C) Bilirubin (n = 8 - 10) and (D) albumin levels (n = 20, apoE^{-/-}; n = 9, HxE^{-/-}) were measured in the serum of apoE^{-/-} and HxE^{-/-} mice as described under Materials and Methods. (E) HxE^{-/-} serum samples were redder than apoE^{-/-} serum samples. (F-K) Hepatic gene expression was determined by real time-quantitative PCR (RT-qPCR) 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 $\pm$ SEM. ** Significance: $p < 0.01$; * Significance: $p < 0.05$.*

Discussion

Hx is an acute phase protein that binds to heme with high affinity (30). To limit free heme availability for 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 the macrophages and hepatocytes through the CD91 receptor via receptor-mediated endocytosis (31). Heme scavenging is a very important process to avoid oxidative stress and inflammation that occurs in the presence of increased levels of free heme. Our results in Figure 2 show that the absence of Hx resulted in heme buildup in the serum and HDL of HxE^{-/-} mice. HxE^{-/-} mice also showed reduced expression of the CD91 receptor and down regulation of the anti-oxidant 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 (17, 18). Biliverdin is further processed to bilirubin, a direct antioxidant (18), which reinforces the anti-inflammatory efficacy of the CD91-HO-1 pathway.

Epidemiological studies have also revealed that moderately increased plasma levels of bilirubin can decrease the risk of developing cardiovascular diseases (32). Interestingly, HxE^{-/-} mice showed reduced serum bilirubin levels compared to control apoE^{-/-} mice suggesting that reduced heme breakdown 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 (20). Our results in Figure 3 show that

HxE^{-/-} mice had reduced levels of albumin. The cause of the decreased albumin levels in the HxE^{-/-} mice is evident from our studies. However, the decreased albumin levels may also contribute to the higher levels of free heme in the serum of HxE^{-/-} mice compared to apoE^{-/-} mice. Moreover, heme can transiently bind to lipoproteins such as HDL and LDL (21), before being transferred to Hx for scavenging. We observed a significantly higher level of heme in HDL fractions from HxE^{-/-} mice compared to apoE^{-/-} mice (Figure 3) indicating that in the absence Hx free heme accumulates on transient carrier proteins such as HDL.

Methods

Lipoprotein isolation - Lipoprotein samples were isolated from pooled or individual sera (~100-125 µl) 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. HDL fractions (#17-20) were pooled and concentrated using filter columns (Amicon Centrifugal Units). Concentrated HDL was used for heme content estimation.

Heme content – Individual mouse serum samples or HDL isolated from serum using FPLC were assayed for heme content using the QuantiChrom™ Heme Assay Kit (DIHM-250, BioAssay Systems, Hayward, CA). 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 = 62.5uM) 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. 150 μl of individual serum samples were tested on the Beckman Olympus AU400 machine. Alkaline phosphatase, alanine transaminase, albumin, and direct bilirubin were estimated. Values for serum albumin for C57BL/6J mice have been reported (1).

RNA Extraction, Reverse Transcription, and Quantitative-PCR- 30 mg of liver tissue was homogenized and lysed for RNA isolation by adding Buffer RLT plus and B-Mercaptoethanol (1:100). RNA was isolated using the manufacturer's protocol (RNEasy Mini kit, Qiagen). 40 μl of RNA was eluted and quantified using Nanodrop. cDNA synthesis was carried out using 1 μg of RNA and iScript cDNA Synthesis Kit (Biorad). The cDNA was diluted 1:20 to use for quantitative-PCR. The reverse transcription reaction contained 2 ng of reverse transcribed total RNA, 0.5 μM of forward and reverse primers each, and 10 μl of Master Mix SYBR Green in a final volume of 20 μl . Samples underwent the standard PCR protocol. 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.

Statistical Analysis: Numerical data are expressed as means ($\pm\text{SEM}$) as indicated in the figure legends. Differences between experimental groups were evaluated for statistical significance using unpaired two-tail Student *t* -test (non-parametric) using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). Significance was determined as indicated in the figure legends.

- I) Zaias J, Mineau M, Cray C, Yoon D, and Altman NH. Reference Values for Serum Proteins of Common Laboratory Rodent Strains. Journal of the American Association for Laboratory Animal Science. 2009;48:387-390.

Chapter 4

ApoE^{-/-} mice lacking hemopexin have higher levels of oxidative stress and impaired HDL function

Introduction I

Heme and ROS: The biochemical property of iron to catalyze redox reactions also enables this ion in free heme (heme not bound to protein) to generate ROS and cause cellular injury (20). There are 2 main cellular sources of free heme: newly produced heme that has not yet been integrated into hemoproteins or heme that has been dissociated from hemoproteins under oxidative stress.

Heme-driven production of ROS is involved in the pathophysiology of many diseases by damaging lipid membranes, proteins, and nucleic acids; activating cell signaling pathways; altering protein expression; and perturbing membrane channels, among other toxic effects. In conditions of hemolysis, tissues can be exposed to high levels of free heme (up to 20 $\mu\text{mol/L}$), overwhelming physiological mechanisms of free heme removal and leading to its toxic effects: oxidative stress and inflammation. Free heme toxicity is exacerbated by its ability to intercalate into lipid membranes. The extreme hydrophobicity of free heme allows it to easily enter the phospholipid bilayer of cell membranes. Within this highly oxidizing environment, hydrogen peroxide from various sources cleaves the heme ring to release and interact with the redox-active iron, leading to amplified production of ROS in the cell. This further catalyzes the oxidation of the cell membrane and promotes lipid peroxidation (higher levels of malondialdehyde), increasing membrane permeability and ultimately leading to cell death (21, 22).

Watanabe *et al.* (92) reported that Hb is associated with HDL in mouse models of atherosclerosis. In a subsequent study, Watanabe *et al.* (93) reported that Hb scavenger proteins, Hp and Hx, are significantly increased in the apoA-I-containing

particles of HDL both in mouse models of atherosclerosis and in CAD patients, when compared with their wild type counterparts, respectively. They further demonstrated that the association of Hb, Hp, and Hx proteins with HDL positively correlates with inflammatory properties of HDL and systemic inflammation in CAD patients (93). These studies suggested for the first time that the Hb/Hx/Hp pathway, in part, modulates the inflammatory properties of HDL in both mice and humans during systemic inflammation.

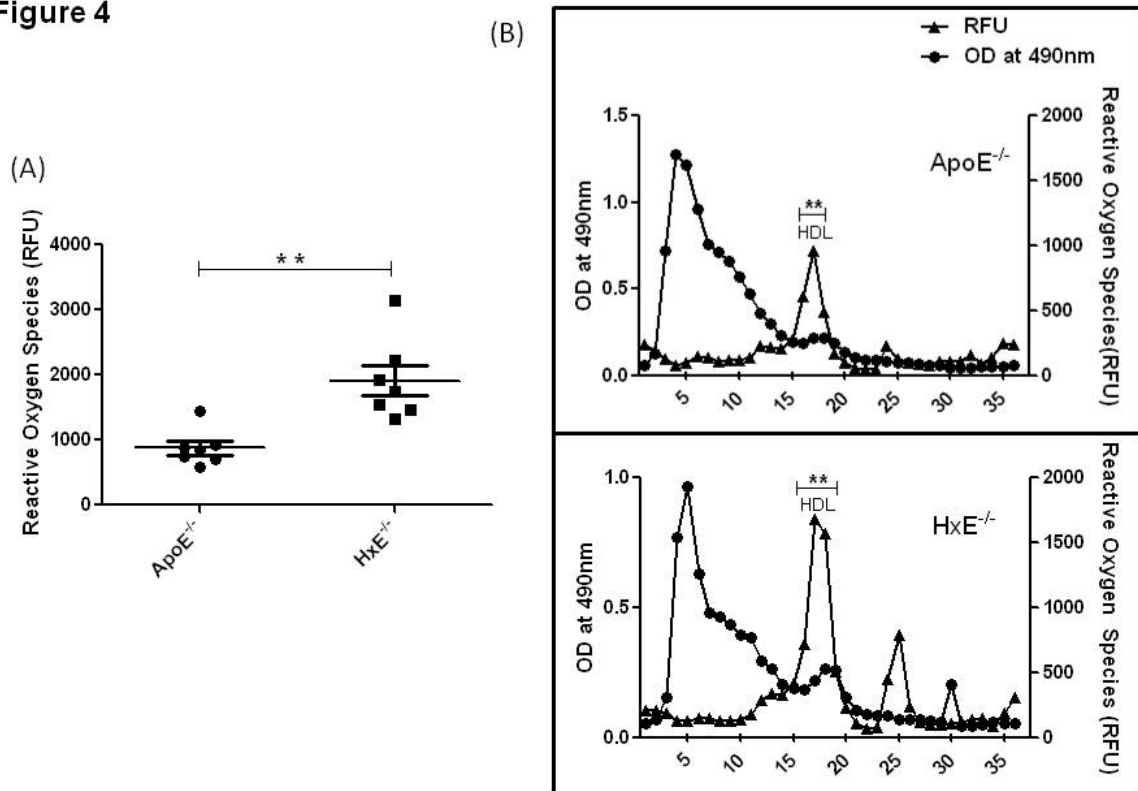
To determine the effect on ROS production due to higher levels of heme in HxE^{-/-} mice, we performed the following experiments.

Results I

Cell-free heme promotes the generation of ROS, which is involved in the pathophysiology of several disorders (22, 23). We estimated levels of ROS by a cell free assay using dichloro-dihydro-fluorescein diacetate (DCFH-DA). The relative fluorescence units (RFUs) reflective of the levels of ROS, were significantly higher in the serum and HDL of HxE^{-/-} mice compared to apoE^{-/-} mice. Individual serum samples were used to estimate ROS levels in circulation (Figure 4A). Additionally, serum was pooled from the apoE^{-/-} and HxE^{-/-} mice (n=6-8) and lipoproteins were separated using FPLC. ROS levels were estimated on all the FPLC fractions. HDL fractions from HxE^{-/-} mice exhibited significant higher levels of ROS compared to the HDL fractions from apoE^{-/-} mice (Figure 4B). We demonstrated that apoE^{-/-} mice lacking hemopexin had higher oxidative stress in the livers as measured by significantly higher malondialdehyde levels (Figure 4C) compared to the control apoE^{-/-} mice (n=7-8). However, levels of alanine transaminase (ALT) and aspartate transaminase (AST) enzymes (Figure 4D and E)

were similar between the two groups of mice indicating liver function was unaffected. Increased heme content and ROS levels associated with HDL led us to determine HDL functionality by measuring its anti- vs proinflammatory activity.

Figure 4



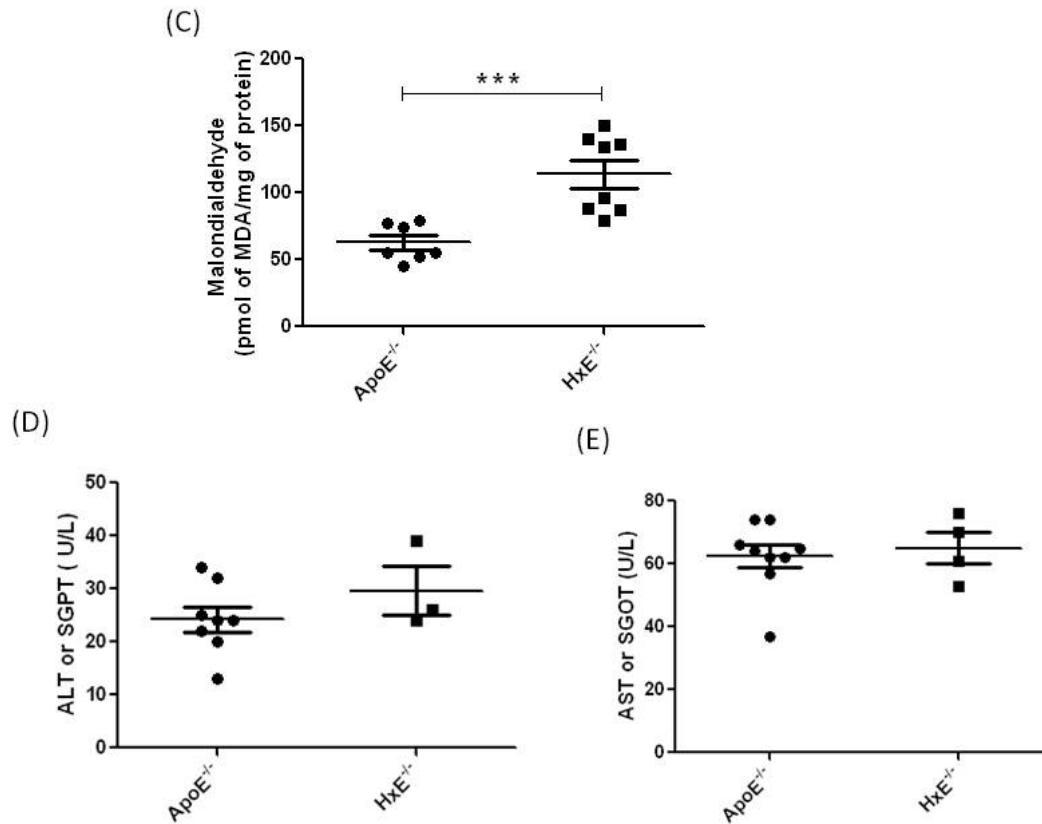


Figure 4: Absence of hemopexin significantly increases oxidative stress in *apoE*^{-/-} mice: (A) ROS levels were determined and represented as relative fluorescence units (RFUs) in a cell free assay using DCFH-DA in serum samples of *HxET*^{-/-} and *apoE*^{-/-} mice (*n* = 7) as described in Materials and Methods. (B) Lipoproteins were separated using FPLC from pooled serum samples obtained from *apoE*^{-/-} and *HxET*^{-/-} 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) Malondialdehyde levels were determined using a colorimetric assay in livers of *HxET*^{-/-} and *apoE*^{-/-} mice (*n* = 7 - 8). (D) Alanine Transaminase (ALT) levels and

*(E) Aspartate Transaminase (AST) levels were determined in serum samples of apoE^{-/-} and Hx^E mice (n = 7 - 9). The mean value for each group of mice is indicated by the horizontal bars. The data shown are mean ± SEM. *** Significance: p<0.001; ** Significance: p<0.01; * Significance: p<0.05.*

Introduction II

HDL and its function: Epidemiological data have demonstrated an inverse relationship between HDL levels and the risk for CAD, which is independent of the LDL-C levels. However, in an individual, the predictive value of an HDL-C level is far from perfect. Careful review of the literature, however, reveals that, even in the original Framingham study (which established the importance of HDL-C levels in predicting coronary events), more than 40% of events occurred in subjects with normal HDL-C levels (94-98). Evolving knowledge that a significant number of cardiovascular events occur in subjects with normal levels of both LDL-C and HDL-C (94, 99) has fueled a search for additional biomarkers with better predictive value. A recent study on Mendelian randomization analysis revealed that a single nucleotide polymorphism in the endothelial lipase gene (LIPG Asn396Ser) associated with increased HDL-C levels in the population did not decrease the risk of chronic heart failure, despite a 13% reduction expected from the increased HDL-C levels (101). Recent data provide evidence that high HDL levels are not always protective and that under certain conditions may even confer an increased risk. HDL functionality is genetically defined but can also be modified by several environmental and lifestyle factors, such as diet, smoking or certain pharmacologic interventions. As a result, other HDL-associated factors have been investigated, including the quality and function of HDL in contradistinction to the level of HDL-C

alone. Regarding their quality, HDL particles are highly heterogeneous and contain varying levels of antioxidants or pro-oxidants, which results in variation in HDL function.

In atherosclerotic conditions HDL can lose its usual atheroprotective characteristics and even paradoxically assume proinflammatory properties (102, 103). Many studies and failed clinical trials have high-lighted an emerging body of evidence that HDL may not always be anti-inflammatory. Van Lenten *et al.* (104) were the first to demonstrate that HDL could exist as an anti-inflammatory molecule or a proinflammatory molecule, depending on the context and environment. In addition, it is also shown that an atherogenic diet in mice that are genetically susceptible to atherosclerosis (but not in mice genetically resistant to atherosclerosis) converts HDL from anti-inflammatory to proinflammatory (105). Thus, a new concept has arisen, which stresses the importance of HDL functionality, rather than HDL concentration per se, in the assessment of cardiovascular risk.

Plasma HDL, which has a density of 1.063– 1.21 g/ml, is a heterogeneous group of discoid and spherical particles that differ in physicochemical properties, intravascular metabolism and biological activity (112). HDL particles possess multiple anti-atherogenic activities, which include reverse cholesterol transport from the arterial wall to the liver for excretion, and anti-oxidative, anti- inflammatory, antiapoptotic, antithrombotic, anti-infectious and vasodilatory actions (113). Studies have suggested the HDL particle number and size (ie, subfraction distribution) are better predictors of CAD risk than HDL-C (101, 106) and that HDL function may be more important in preventing CAD than HDL-C levels alone (107-109). As a result, the anti- or proinflammatory nature and function of HDL may be a more sensitive predictor of

atherosclerosis than HDL-C levels alone (102, 110,111). Thus, results understanding and identifying the factors affecting the formation and buildup of proinflammatory HDL has gained recent importance.

It has been postulated that HDL functions to promote reverse cholesterol transport. Recent studies support this role for HDL but also indicate that HDL is a modulator of systemic inflammation. HDL has the ability to protect against cellular accumulation of cholesterol, oxidative stress and inflammation in the arterial wall. In the absence of inflammation, HDL has a complement of antioxidant enzymes that work to maintain an anti-inflammatory state. In the presence of systemic inflammation, these antioxidant enzymes can be inactivated and HDL can accumulate oxidized lipids and proteins that make it proinflammatory. Animal studies and small-scale human studies suggest that measures of the quality and novel functions of HDL might provide an improved means of identifying subjects at increased risk for atherosclerotic events, compared with the current practice of only measuring HDL-C levels.

Antioxidant activity: HDL antioxidant activity is typically observed as inhibition of LDL oxidation (97). ApoA-I is a major component of the anti-oxidative activity of HDL and can prevent LDL oxidation, delay it, or both, by the removal of oxidized phospholipids from LDL, artery wall cells or both (97). Paraoxonase 1 (PON1), platelet-activating factor acetyl hydrolase (also known as lipoprotein- associated phospholipase A2), glutathione peroxidase and lecithin: cholesterol acyltransferase (LCAT) are enzymatic components of HDL that can hydrolyze LDL-derived oxidized phospholipids (97). During an acute-phase response, there seems to be an increase in the formation of these oxidized lipids that results in the inhibition of these HDL associated enzymes and an

association of acute phase proteins such as ceruloplasmin with HDL that renders HDL proinflammatory (121,122). Furthermore, HDL associated enzymes, including PON1, platelet-activating factor acetyl hydrolase and LCAT, can become dysfunctional, depleted or both under inflammatory conditions, (97) in metabolic diseases involving low HDL levels, such as metabolic syndrome and type 2 diabetes , (123-125) and in CHD (96).

Anti-inflammatory: Exciting data obtained in a rabbit model of acute arterial inflammation demonstrate that the anti-inflammatory activity of HDL can be expressed as decreased cytokine-mediated expression of adhesion molecules, diminished neutrophil infiltration within the arterial wall and reduced generation of reactive oxygen species (112, 126). The ability of HDL to inhibit adhesion molecule expression could be mediated by apolipoproteins, particularly by apoA-I, but also by phospholipids, including sphingosine-1- phosphate and sphingosyl phosphorylcholine (127). The anti-inflammatory action of HDL could involve the following features: hydrolysis of proinflammatory oxidized lipids by HDL- associated enzymes, which is mechanistically similar to HDL anti-oxidative activity; (97, 112, 118) inhibition of cytokine-induced adhesion molecule expression in endothelial cells associated with induction of transforming growth factor,(128) and attenuation of interleukin-6 production; (129) and inhibition of synthesis of platelet-activating factor and attenuation of monocyte chemo attractant protein-1 expression (97).

ApoA-I and SAA exchange: HDL undergoes pronounced structural and functional modification in dyslipidemia associated with an acute-phase response and inflammation (131, 132). HDL levels of apoA-I might be reduced because of decreased apoA-I

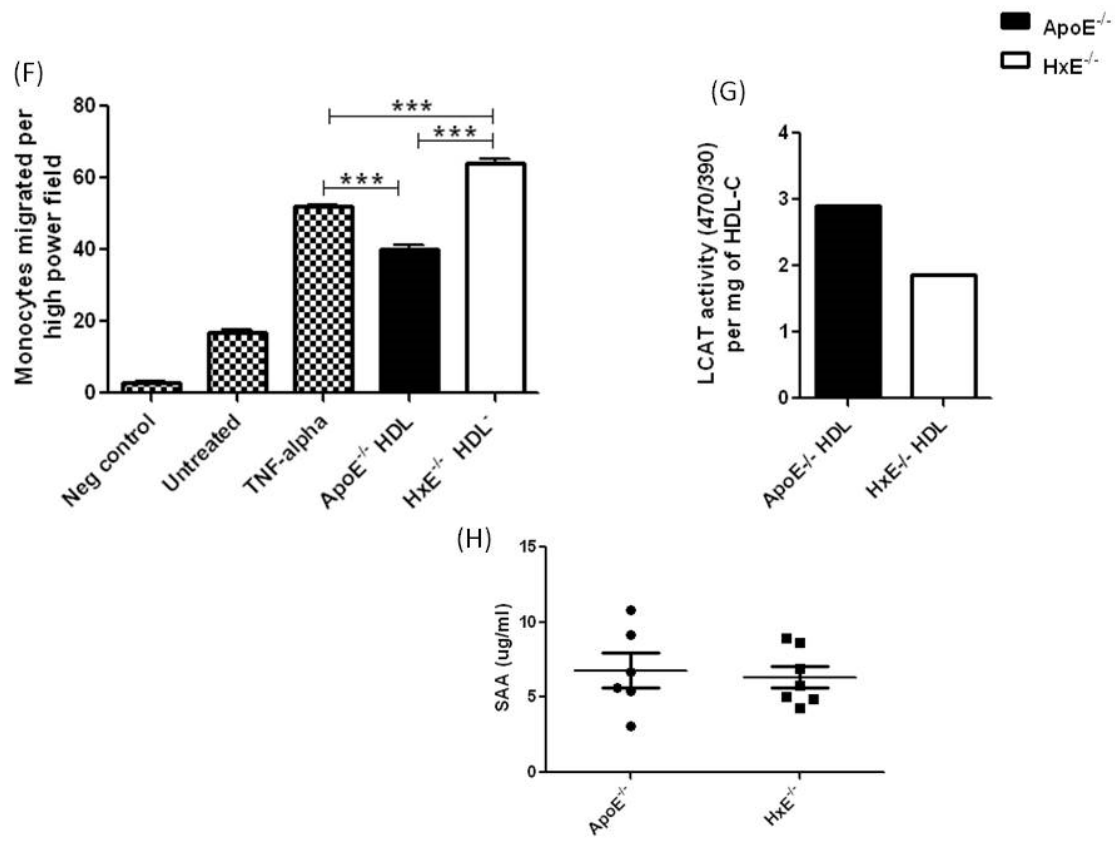
synthesis, accelerated HDL catabolism and apoA-I replacement by serum amyloid A (131, 132). SAA is an acute-phase protein and is mainly of hepatic origin, and circulating levels can be induced to increase up to 1,000-fold in the presence of inflammation (133). Serum amyloid A can displace apoA-I and other HDL apolipoproteins from the surface of HDL particles upon induction of the acute phase; small HDL3 is a major plasma carrier of apoA-I (133) Like C- reactive protein, elevated plasma levels of serum amyloid A represent an important, although weaker, cardiovascular risk factor (134). The proatherogenic properties of serum amyloid A might result from its capacity to enhance HDL binding to proteoglycans, to promote HDL immobilization in the arterial wall, to accelerate HDL clearance from the circulation, or a combination of these (133, 135).

To study the effect of higher heme and ROS levels on HDL function, we performed the following experiments.

Results II

HDL function can be measured *ex vivo* by a monocyte chemotactic assay as described in the Materials and Methods. We observed a significantly higher degree of monocyte migration from the trans-wells treated with the conditioned media collected from human aortic endothelial cells treated with HDL from HxE^{-/-} mice compared to HDL from apoE^{-/-} mice (Figure 4F). Additionally, serum was pooled from the apoE^{-/-} and HxE^{-/-} mice (n=6-8) and lipoproteins were separated using FPLC. HDL fractions were concentrated as described in the Materials and Methods and used to measure LCAT activity. Fluorometric estimation of acyltransferase activity of LCAT demonstrated a trend of

lower LCAT activity in HxE^{-/-} HDL samples compared to apoE^{-/-} HDL samples (Figure 4G). This demonstrated that HDL from HxE^{-/-} mice was more proinflammatory compared to the HDL from apoE^{-/-} mice. However, Serum Amyloid A (SAA) levels measured in the serum by ELISA were unchanged between the two groups (Figure 4H). Although, hepatic PON1 gene expression levels were trending lower in HxE^{-/-} mice compared to apoE^{-/-} mice (Figure 4I), surprisingly, PON1 activity on serum and HDL samples were significantly higher in HxE^{-/-} mice compared to apoE^{-/-} controls (Figure 4J and K). In addition, serum arylesterase (Figure 4L) and lactonase activity (Figure 4M) were significantly higher in the HxE^{-/-} mice compared to the control apoE^{-/-} mice. Therefore, these results showed that Hx deficiency in apoE^{-/-} mice leads to accumulation of proinflammatory HDL and higher oxidative stress systemically although unchanged SAA and higher PON1, arylesterase and lactonase levels in HxE^{-/-} mice could not be explained.



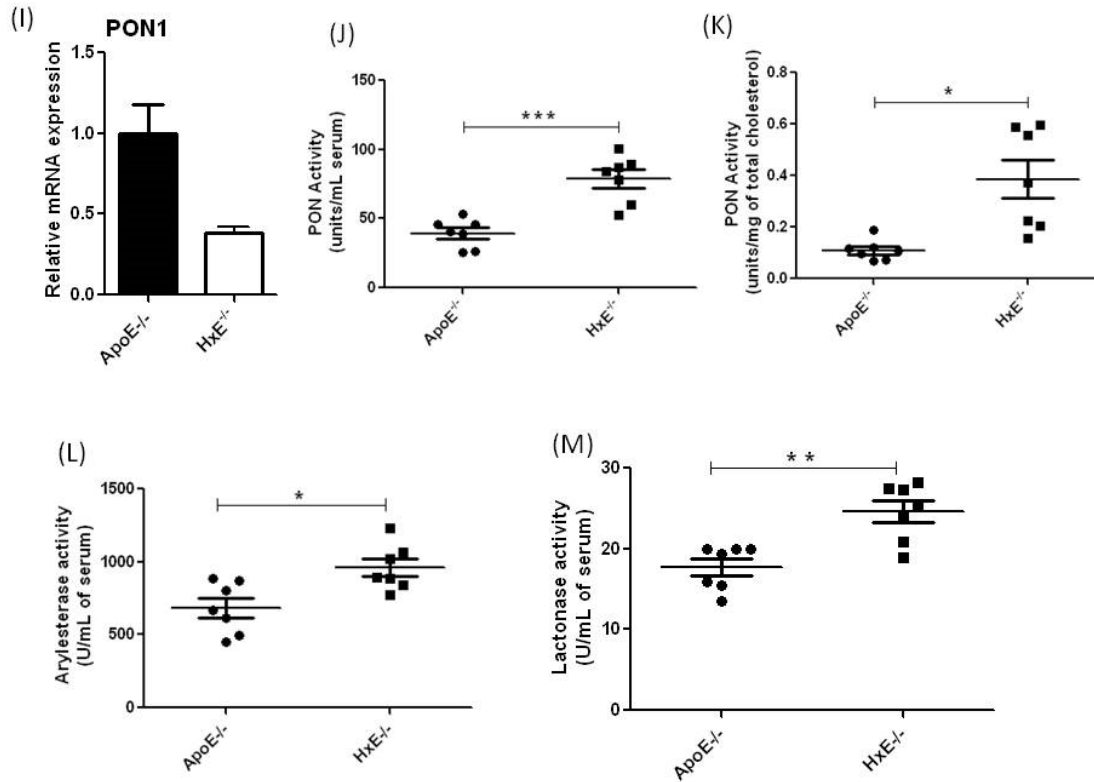


Figure 4: HDL from apoE^{-/-} mice lacking hemopexin is dysfunctional: (F) 50µg/dL of HDL cholesterol from apoE^{-/-} and HxEx^{-/-} mice (n = 3) was used to perform a monocyte migration assay as described in Materials and Methods. (G) LCAT activity was measured on pooled HDL samples from apoE^{-/-} and HxEx^{-/-} mice (n = 6 – 8) as described in Materials and Methods. (FH SAA levels were determined on apoE^{-/-} and HxEx^{-/-} serum samples (n = 7) by ELISA. (I) Hepatic gene expression was determined by RT-qPCR for apoE^{-/-} and HxEx^{-/-} mice (n = 4) and normalized to GAPDH. (J and K) PON1 activity was measured in serum and HDL samples respectively (n = 7) using paraoxon as described in Materials and Methods. (L) Arylesterase activity and (M)) Lactonase activity was measured on apoE^{-/-} and HxEx^{-/-} serum samples (n = 7) as described in the Materials and Methods. The mean value for each group of mice is indicated by the horizontal bars.

*The data shown are mean $\pm$ SEM. *** Significance: $p < 0.001$; ** Significance: $p < 0.01$; * Significance: $p < 0.05$.*

Discussion

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 HxE^{-/-} mice compared to the apoE^{-/-} controls (Figure 3). 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 (13, 14). 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 HxE^{-/-} mice had increased levels of malondialdehyde in their livers compared to apoE^{-/-} mice demonstrating that increased heme and ROS can lead to increased lipid peroxidation. Watanabe *et al.* have shown that in patients with cardiovascular disease, Hb and its scavenger proteins: Hp and Hx can associate with HDL and influence the inflammatory properties of HDL (4). This study also reported that absence of Hx could convert HDL from anti- to a proinflammatory HDL in C57BL/6J mice. Our results confirm their findings in apoE^{-/-} mice lacking Hx. In a monocyte chemotactic assay, the HDL from HxE^{-/-} mice was more proinflammatory, causing higher monocyte migration in an arterial wall co-culture system. Our results suggest that Hx confers protection against heme and ROS driven HDL dysfunction and oxidative stress in apoE^{-/-} mice.

Methods

Cell free assay for ROS estimation- ROS content in serum and lipoproteins was determined with 2,7,7'-dichlorofluorescein diacetate (DCFH-DA, Invitrogen (Thermo Scientific), Waltham, MA, USA). Serum or HDL from each mouse was incubated with DCFH-DA (10 µg/mL) in methanol for 30 min at 37°C) in black, flat bottom polystyrene micro-titer plates. The presence of ROS was detected by measuring fluorescence intensity on a plate reader (Spectra Max, Gemini XS; Molecular Devices) at an excitation wavelength of 485 nm, emission wavelength of 530 nm, and cut-off of 515 nm with photomultiplier sensitivity set at medium and expressed in relative fluorescence units (RFUs).

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 chromogenic reagent which reacts with the lipid peroxidation products malondialdehyde and 4-hydroxynonenaes at 45°C, yielding a stable chromophore with maximum absorbance at 586 nm.

Serum metabolite estimation- Refer to Chapter 3

Monocyte Chemotactic Assay- Monocyte chemotaxis assay was performed as previously described (II). This assay is a measure of the ability of test HDL to activate human aortic endothelial cells in an arterial wall co-culture system and cause a

monocyte migration using chemotaxis. In general, human aortic endothelial cells were untreated or treated with (Tumor Necrosis Factor) 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 (50 μ g/ml) or TNF- α (10ng/ml) in phosphate-buffered saline were diluted with culture medium 199 (M199, Life Technologies (Thermo Scientific), Waltham, MA, USA) 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 of 12 standardized high power fields counted in quadruple wells.

LCAT activity assay- 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 μ l samples were incubated with a fluorescently labeled substrate for 2.5 h at 37 °C. The conversion of cholesterol (Em. 470 nm) to cholesteryl ester (Em. 390 nm) at 340 nm excitation 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.

SAA levels- SAA levels were determined using a non-species-specific SAA ELISA kit (Tridelta Development Limited; obtained through Tri-Delta Diagnostics, Inc). Briefly, serum samples, including standards of known SAA content, were added to pre-coated microwells containing a captured monoclonal antibody specific for SAA, together with biotinylated anti-SAA monoclonal antibody. SAA present in the well was both captured on the plate by the immobilized antibody and labeled with the conjugate antibody in a one-step procedure. After washing to remove all of the unbound material, streptavidin-horse radish peroxidase conjugate was added to the wells and incubated. Following the second incubation, tetramethyl benzidine substrate solution was added for 30 min at room temperature, at which time an acidic stop-solution provided by the manufacturer was added, and the intensity of the resulting color was determined in a spectrophotometer at 450 nm.

Gene expression analysis – Refer to Chapter 3

PON1 activity assay- PON1 activity was measured using paraoxon as the substrate and measuring the increase in the absorbance at 405 nm due to the formation of 4-*p*-nitrophenol over a period of 12 minutes (at 20-second intervals). Paraoxon was purchased from Sigma and was further purified using chloroform extraction. A unit of PON1 activity was defined as the formation of 1 nmole of 4-*p*-nitrophenol per minute per ml of sample used. Enzyme activity was measured with 40-fold–diluted plasma or HDL (final concentration) in a reaction mixture containing 4 mM paraoxon working solution,

500 mM glycine/10 mM CaCl₂ buffer at pH 10.5, and 155 mM NaCl/3 mM NaN₃ buffer at pH 8.2.

Arylesterase activity assay- For the arylesterase assay, serum was diluted 1:50 with assay buffer and incubated with phenyl acetate. Arylesterase activity was measured by kinetic reading with absorbance at 270 nm (A₂₇₀ nm) for 2 minutes. The kinetic rate at A₂₇₀ nm was multiplied by the molecular extinction coefficient of 0.7633 and dilution factor of 2.5 to represent a unit of arylesterase activity per ml of plasma.

Lactonase activity assay- For the lactonase assay, serum was diluted 1:10 with reaction buffer and incubated with 2 mM dihydrocoumarin. Lactonase activity was measured by kinetic reading with absorbance at 270 nm (A₂₇₀ nm) for 10 minutes every 15 seconds. Self-hydrolysis of dihydrocoumarin was measured in a separate well. The extinction coefficient is: $E_{270} = 1295/\text{M}/\text{cm}$. To calculate lactonase activity this formula was used: $[(\text{Mean absorbance of sample} - \text{Mean absorbance of self-hydrolysis}) \times 5 \times \text{dilution factor}]/700 = \text{units of lactonase activity per ml of serum}$.

Statistical Analysis- Refer to Chapter 3

- II) Navab, M., S. Hama, J. Cooke, G. M. Anantharamaiah, M. Chaddha, L. Jin, G. Subbanagounder, K. F. Faull, S. T. Reddy, N. E. Miller, and A. M. Fogelman. Normal HDL inhibits three steps in the formation of mildly oxidized LDL—step 1. *J. Lipid Res.* 2000: 41:1481–1494.

Chapter 5

Hemopexin ablation in apoE^{-/-} mice causes increased atherosclerosis

Introduction

Heme in the cardiovascular system: In the cardiovascular system, heme plays a major role in gas exchange, mitochondrial energy production, antioxidant defense, and signal transduction. Recently, a heme-containing mitochondrial respiratory complex, succinate dehydrogenase, has been implicated in the generation of ROS during ischemia–reperfusion injury in different organs, including the heart (27). Heme also plays a role in the function of many cardiac enzymes, including mitochondrial electron transport chain complexes and heme-containing antioxidant proteins, such as peroxidase and catalase. Heme containing proteins have also been implicated as signaling molecules (guanylate cyclase), as enzymes (cyclooxygenase, nitric oxide [NO] synthase), and in the synthesis of hormones (hydroxylases) in the cardiovascular system.

Heme's role in endothelial and smooth muscle cell dysfunction: Free heme is also a powerful proinflammatory molecule that is recognized by the innate immune system and causes inflammation in both sterile and infectious conditions. Heme is involved in normal function of various cell types in the cardiovascular system. In addition to cardiomyocytes, heme also modulates the function of smooth muscle and endothelial cells. Cell-free heme promotes the generation of ROS (28, 29), which also leads to endothelial activation. The changes in adhesion molecule expression on the surface of endothelial cells promote platelet adhesion and aggregation.

In addition to inducing endothelial dysfunction and platelet activation, excess heme can induce smooth muscle proliferation and contribute to cardiovascular pathology.

Turbulent blood flow may cause hemolysis and release free heme (30). Free heme activates NADPH oxidase, a major source of ROS in the cytoplasm, and ROS from heme has been shown to induce smooth muscle proliferation, a pathological feature of atherosclerosis and hypertension (31-33). Therefore, effective modulation of free heme levels can be another approach against smooth muscle proliferation in diseases such as atherosclerosis or hemolytic anemia.

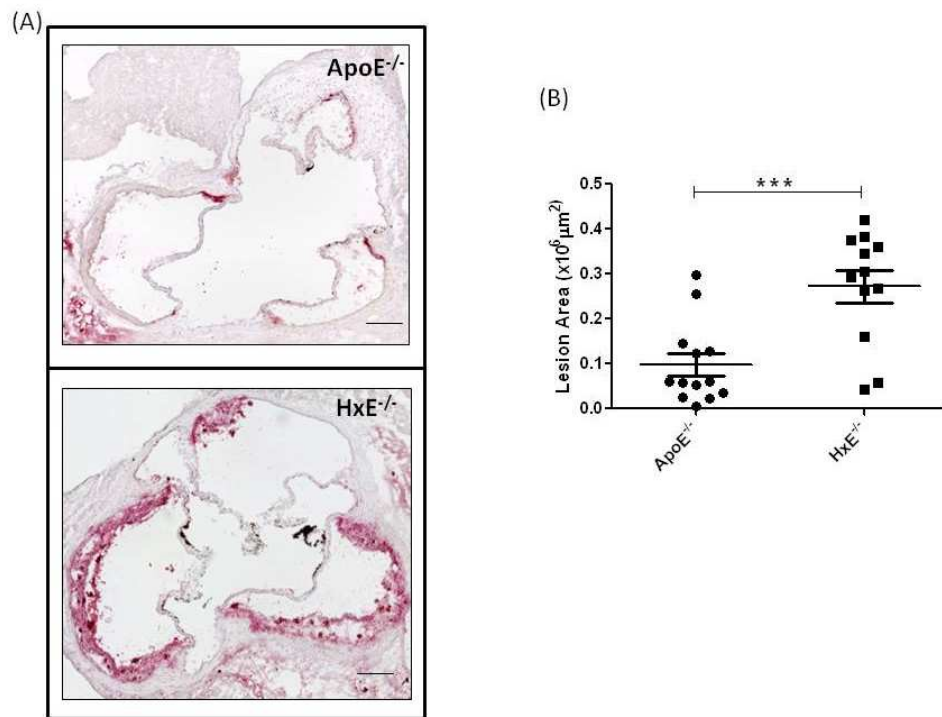
In light of the previous results described in chapters 2 and 3 and, above mentioned functions of heme in the cardiovascular system, we performed the following experiments to study the role of excess heme and ROS due to Hx deletion in the development of atherosclerosis in apoE^{-/-} mice.

Results

We investigated the effect of Hx ablation on the extent of atherosclerosis in apoE^{-/-} mice. Six-month-old female mice (n = 13 apoE^{-/-}, n = 12 HxE^{-/-} mice) maintained on a chow diet were euthanized after overnight fasting. The HxE^{-/-} mice showed a higher Oil-Red-O staining of the sinus, indicative of an increase in the plaque area in the heart, compared to the control apoE^{-/-} mice (Figure 5A). Quantitative analysis showed a significant increase in plaque area in the HxE^{-/-} mice compared to the apoE^{-/-} mice. On average, a three-fold increase in the area of plaque was observed in the double knockout HxE^{-/-} mice compared to its respective control (Figure 5B). To examine the extent of atherosclerotic lesions in the proximal aortic arch, *en face* analysis was performed on the para-formaldehyde fixed aortas from the two groups of mice (n = 24 apoE^{-/-}, n = 25 HxE^{-/-} mice). Figure 5C show representative images of the whole aorta

stained for lesions using Sudan IV. Quantitative analysis expressed as a percentage lesion of the entire aortic surface area was determined. There was a significant increase in percent lesion on the aorta in the HxE^{-/-} mice compared to the apoE^{-/-} mice (Figure 5D). These results confirmed that Hx deficiency in apoE^{-/-} mice causes increased atherosclerosis. Further, macrophage infiltration is a hallmark for the onset of atherosclerosis. Immunohistochemistry using the macrophage marker CD68 was performed on the sections of the heart to examine the extent of macrophage content in the lesions (n = 11 apoE^{-/-}, n = 15 HxE^{-/-} mice). Morphometric analysis for antibody stained area revealed an augmentation of macrophage accumulation in the double knockout mice compared to control mice (Figure 5E). A two-fold increase in macrophage content in the plaque area was observed in the HxE^{-/-} mice compared to apoE^{-/-} mice (Figure 5F). These data confirm that HxE^{-/-} mice with higher oxidative stress and dysfunctional HDL also have augmented atherosclerosis compared to apoE^{-/-} mice.

Figure 5



ApoE^{-/-}

Hx/E^{-/-}



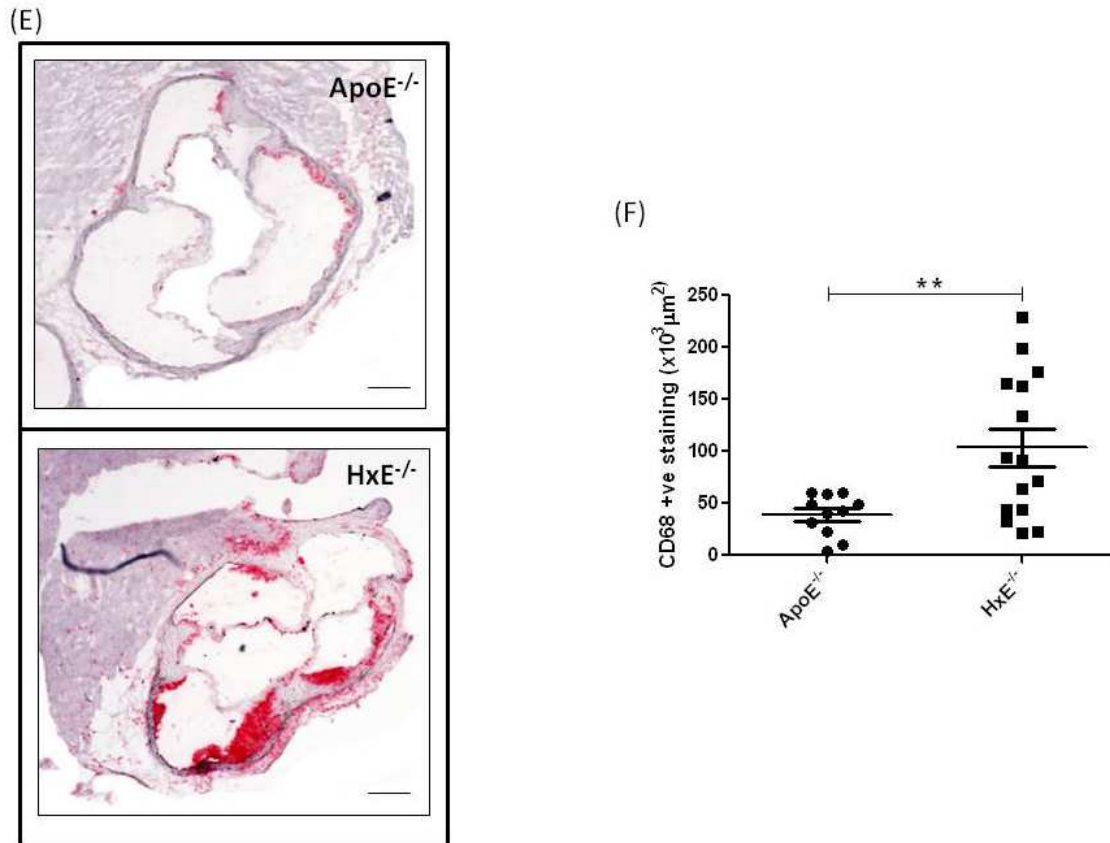


Figure 5: ApoE^{-/-} mice lacking hemopexin develop increased atherosclerosis: (A) Atherosclerotic plaque area was determined in the aortic sinus of the heart via Oil-Red-O staining of the lesions (scale bar = 500μm). (B) Quantitative analysis was performed of the plaque area in HxE^{-/-} (n = 12) and apoE^{-/-} (n = 13) mice. Each point represents the mean (8 - 10 sections per mouse) atherosclerotic plaque area per mouse. (C) Whole aorta lesions were determined using Sudan IV staining as described under Materials and Methods (Scale bar = 0.5cm). (D) Quantitative analysis of the extent of atherosclerosis was expressed as percentage of aortic surface area covered by atherosclerotic lesions in HxE^{-/-} (n = 25) and apoE^{-/-} mice (n = 24). Each point represents the mean lesion area per mouse. (E) CD68 positive staining was performed

in the aortic sinus for macrophage content (scale bar = 500 μ m). (F) Quantitative analysis of the macrophage content in the HxE^{-/-} (n = 15) and apoE^{-/-} (n = 11) mice was performed. Each point represents the mean macrophage laden area per mouse (3 sections per mouse). The mean area for each group of mice is indicated by the horizontal bars. The data shown are mean $\pm$ SEM. *** Significance: $p < 0.001$; ** Significance: $p < 0.01$; * Significance: $p < 0.05$.

Table 1

	ApoE ^{-/-}	HxE ^{-/-}	<i>p</i>
Triglycerides (mg/dL)	49.3 $\pm$ 35.5	43.7 $\pm$ 23.31	NS
Total cholesterol (mg/dL)	318.3 $\pm$ 153.8	345.3 $\pm$ 178.0	NS
HDL cholesterol (mg/dL)	30.4 $\pm$ 12.5	25.85 $\pm$ 11.5	NS
LDL cholesterol (mg/dL)	278.1 $\pm$ 161.4	310.7 $\pm$ 186.0	NS
Unesterified cholesterol (mg/dL)	95.5 $\pm$ 40.2	106.47 $\pm$ 44.5	NS
Free Fatty Acids (mg/dL)	25.7 $\pm$ 6.8	32.8 $\pm$ 7.1	0.001
Glucose (mg/dL)	107.8 $\pm$ 29.2	92.1 $\pm$ 18.53	0.037

Table 1: Serum lipoproteins: Higher levels of total, unesterified and LDL-Cholesterol were recorded in HxE^{-/-} mice compared to apoE^{-/-} mice. The data shown are mean $\pm$ SD. NS: Not significant.

Discussion

Excess heme and ROS are implicated in various aspects of cardiovascular pathology. Increased free radicals in the circulation cause endothelial dysfunction, platelet activation and induces smooth muscle proliferation (33-37), which are features of

atherosclerosis and hypertension (38-40). HxE^{-/-} mice on a chow diet developed increased atherosclerotic lesions in the aortic sinus and the aorta compared to apoE^{-/-} mice. Macrophage content in the aortic sinus was also significantly higher in HxE^{-/-} compared to apoE^{-/-} mice as seen in Figure 5 of this study. In addition, total and LDL-C levels were elevated in the HxE^{-/-} mice compared to apoE^{-/-} mice and HDL-C levels were lower (Table 1). Collectively, these results demonstrated that Hx ablation in apoE^{-/-} mice causes aggravated plaque formation and macrophage infiltration leading to increased atherosclerosis. Based on the evidence so far, the molecular mechanisms behind increased atherosclerosis in HxE^{-/-} mice are likely multifactorial, including proinflammatory HDL and increased oxidative stress.

Methods

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 Bioevo BZ-9000 series (Keyence Corp., Itasca, IL, USA) 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, Rockville, MD, USA)

in a blinded fashion. The area covered by atherosclerotic lesions divided by the area of the entire aorta was calculated and compared.

CD68 positive staining - Fresh-frozen aortic root cryosections (10 μ m-thick) were stained for CD68, antibody for macrophage marker. Briefly, after fixation in ice-cold acetone, sections were blocked in 4% bovine serum albumin plus 10% goat serum for 3 hours at room temperature. Rat antimouse CD68, (1:100; Serotec, Raleigh, NC, USA), was used with an overnight incubation at 4°C. Goat anti-rat alkaline phosphatase secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) was used at 1:200 with 1-hour incubation at room temperature. Immunostaining was visualized using Vector Red substrate plus levamisole to inhibit endogenous alkaline phosphatase activity (Vector Laboratories, Burlingame, CA). Immunostaining was quantified in a blinded fashion on the Bioevo BZ-9000 series (Keyence Corp., Itasca, IL, USA) microscope.

Statistical Analysis- Refer to Chapter 3

Methods for Table 1

Serum Analysis – Lipoprotein analyses were performed using serum isolated from blood at the Lipid Core laboratory in the Atherosclerosis Research Unit at the University of California at Los Angeles.

Statistical Analysis: Numerical data are expressed as means ($\pm$ SD) as indicated in the figure legends. Differences between experimental groups were evaluated for statistical significance using unpaired two-tail Student *t* -test (non-parametric) using GraphPad

Prism version 5.01 (GraphPad Software, San Diego, CA). Significance was determined as indicated in the figure legends.

Chapter 6

HxE^{-/-} bone marrow-derived macrophages have impaired/reduced cholesterol efflux capacity through the ABCA1 receptor.

Introduction

Heme is proinflammatory: Free heme is also a powerful proinflammatory molecule that is recognized by the innate immune system and causes inflammation in both sterile and infectious conditions. TLRs are membrane-spanning receptors that play a key role in innate immunity by recognizing pathogen-associated molecular patterns derived from microbes (23). Excess free ferric heme in plasma has recently been demonstrated to activate TLR4, which mediates the innate immune response to bacterial lipopolysaccharide (LPS) (24). Heme is capable of binding to TLR4 and inducing TLR4-dependent production of TNF. Heme also potentiates microglial activation via TLR4, which in turn induces nuclear factor- κ B activation by the MyD88/TRIF signaling pathway and ultimately increases inflammation (25). Heme-induced TLR4 signaling may have both proinflammatory and procytotoxic effects (26). Heme is also found to be a potent inducer of interleukin-1 β processing through the activation of the NLRP3 inflammasome in macrophages primed by LPS or TNF. Heme has also been shown to activate neutrophils by promoting the release of neutrophil extracellular traps. Hence free heme in circulation is involved in causing inflammatory conditions.

HO1 and LXR- α co-induction- Heme induces HO-1 through activating transcription factor 1 (ATF-1) (19). Moreover, ATF-1 couples the induction of HO-1 to that of Liver X receptors (LXRs), thereby preventing foam cell formation and promoting the export of cholesterol to HDL (19). This represents the first known example of coordination of iron and lipid metabolism. Together, these coordinate the safe handling of iron in parallel with lipid export from the cell. The segregation of lipid storage in foamy macrophages

versus iron storage in hemosiderin-laden macrophages makes functional sense as a strategy to help prevent iron-mediated lipid peroxidation (19).

LXR- α and ABCA1 in the context of macrophage function: Extensive work *in vitro* and *in vivo* has focused on how sterol-regulated transcription factors, LXR- α and LXR β , induce ATP-binding cassette, sub-family A, member: ABCA1 and ABCG1 expression and promote regression of foam cell lesions through this and other mechanisms (177). Free cholesterol (FC) within macrophages has recently been proposed as an initiator of a proinflammatory signaling response in developing atherosclerotic lesions (178). Oxysterols like minimally modified-(mm-LDL) and ox-LDL, from FC phagocytosis, are LXR agonists and increase reverse cholesterol transport (RCT) from macrophages by increasing expression of macrophage cholesterol efflux transporters ABCA1 and ABCG1, inhibiting NF- κ B signaling (179) and inducing downstream inflammatory cytokines, chemokines (CCL2), inducible nitric oxide synthase (iNOS) (179, 180) and arginase II (181). The mechanisms and exact route of cholesterol transport to the plasma membrane is through the Golgi-to-plasma membrane vesicular transport system (182). Once at the plasma membrane, cholesterol is transferred to the outer leaflet, where it is removed from cells by ABCA1- and ABCG1-mediated transport to apolipoprotein A1 and HDL, respectively, or by “passive diffusion” to cholesterol-poor HDL (183). Because accumulation of FC within macrophages at sites of atherosclerotic lesions converts them into foam cells (184); by stimulating RCT, LXR reduces foam cell formation and lesion cholesterol content directly. This is likely an important part of the mechanism for LXR-dependent protection from atherosclerosis because these effects are not observed in LXR knockout mice (185). As predicted, genetic deficiencies of

ABCA or ABCG account for enhanced inflammation in atherosclerosis, especially after treatment with TLR ligands (186) and result in foam cell formation and further acceleration of atherosclerosis (187). Although both mmLDL and oxLDL are seen as ligands to TLR4, the pathways by which recognition occurs differ. The recognition of mmLDL is similar to that of LPS, as LPS is also a TLR4 ligand (188).

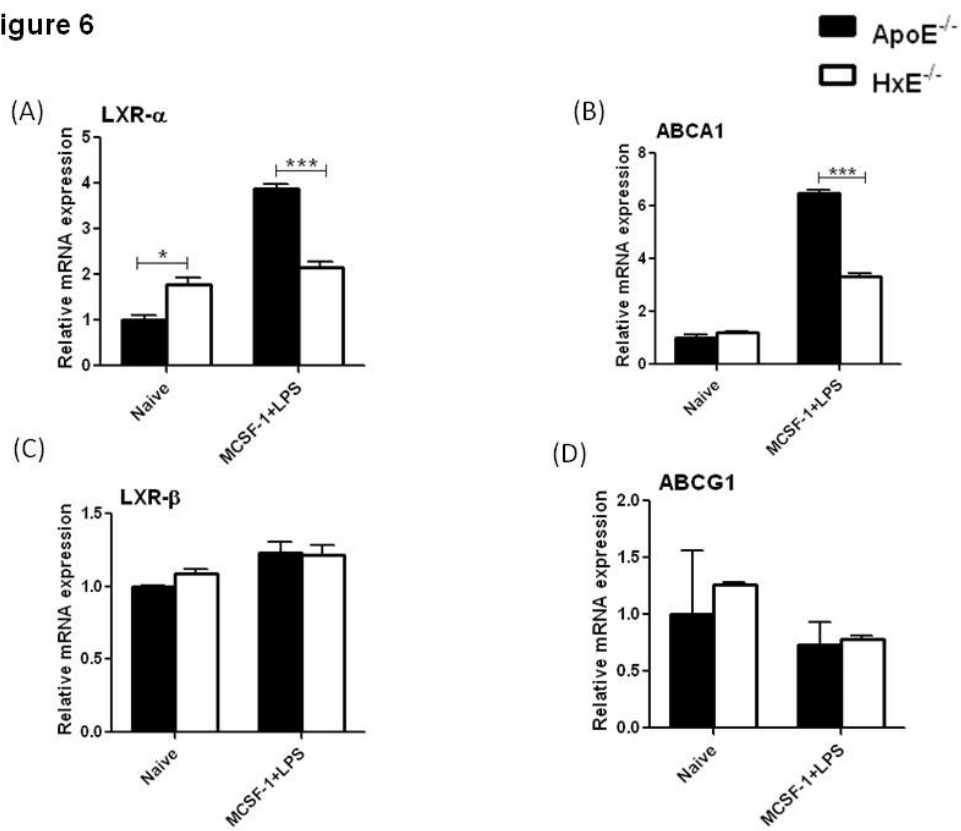
To study the effect of Hx deletion and excess free circulating heme on the expression of LXR genes and its downstream targets in HxE^{-/-} macrophages, we performed the following experiments.

Results

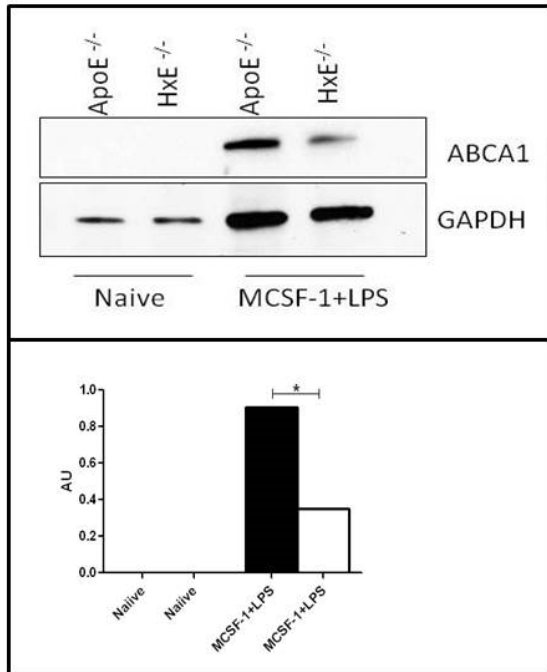
Hx along with macrophages plays an important role in the clearance of cell-free heme from the circulation, thereby mitigating oxidative damage caused by heme. Excess free ferric heme in plasma activates TLR4, which is also a receptor for LPS stimulation (24). In addition, Boyle *et al.* recently demonstrated that heme can transcriptionally co-activate HO-1 and LXR genes in macrophages (19). In light of this information we used naïve and differentiated ((macrophage colony stimulating factor-1) MCSF-1(10ng/ml)) + LPS (10ng/ml) bone marrow cells (BMDMs) from 8-12 week old apoE^{-/-} and HxE^{-/-} mice (n=3-4) to determine the expression of the LXR family genes and its downstream targets using RT-qPCR gene expression analysis. Interestingly, levels of LXR-α but not LXR-β were significantly down regulated in BMDMs from HxE^{-/-} mice compared to apoE^{-/-} mice (Figure 6A and C). ABCA1, a downstream target of LXR-α was also significantly down regulated in HxE^{-/-} BMDMs, however, there was no change in the expression of ABCG1 (Figure 6B and D) between the two groups of mice. Additionally, we confirmed

the significant down regulation of ABCA1 protein expression by western blot analysis (Figure 6E). ABCA1 is an important cell surface cholesterol efflux transporter, important to maintain cholesterol homeostasis in the macrophage and prevent foam cell formation. We determined levels of ABCA1 on the cell surface of the BMDMs by flow cytometry analysis as described in the Materials and Methods. We observed a significant reduction in expression of cell surface ABCA1 in HxE^{-/-} BMDMs compared to apoE^{-/-} BMDMs (Figure 6F). Since ABCA1 is a key cholesterol efflux transporter we performed a cholesterol efflux assay with the BMDMs loaded with Ac-LDL (50µg/mL) and measured the apoA-I (10µg/mL) mediated cholesterol efflux. Net percent cholesterol efflux was measured as a ratio of counts in media divided by counts in media plus cells. BMDMs differentiated with LPS from HxE^{-/-} mice showed a significant reduction in cholesterol efflux capacity compared to apoE^{-/-} BMDMs (Figure 6G). LXR-α agonist treated cells showed an increase in efflux capacity but there was no significant difference between the two groups of mice. LPS and LXR-α agonist treated BMDMs also showed a slightly reduced efflux capacity compared to LXR-α agonist only or higher than LPS treatment only but not significant between the two groups of mice (Figure 6G). Since the efflux was apoA-I mediated we can propose that the efflux was via the ABCA1 transporter. Ac-LDL cholesterol uptake was similar in BMDMs from both the groups of mice (Figure 6H) and serum apoA-I levels estimated by ELISA were not different between apoE^{-/-} and HxE^{-/-} mice (Figure 6I). These results showed that absence of Hx may inhibit macrophage-heme driven atheroprotective effects via the LXR and ABCA1 pathway and may cause, in part, the increased atherosclerosis observed in the HxE^{-/-} mice due to reduced cholesterol efflux capacity.

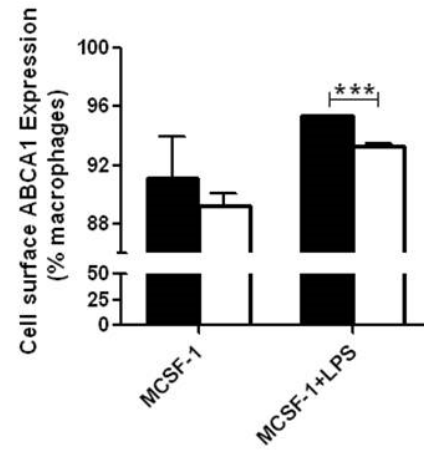
Figure 6



(E)



(F)



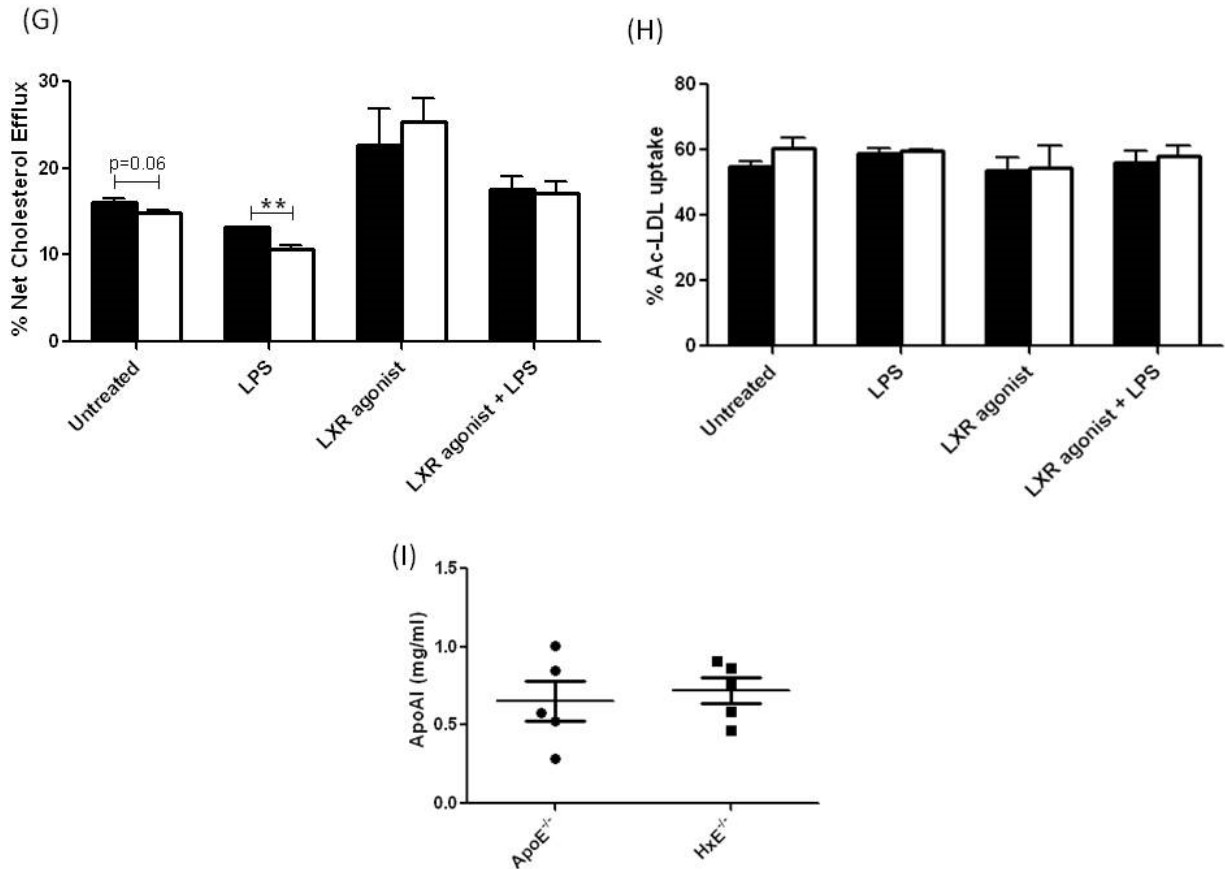


Figure 6: *HxE*^{-/-} macrophages have reduced cholesterol efflux capacity through the ABCA1 transporter: (A-D) RT-qPCR analysis was performed for naïve and LPS (10ng/ml) treated bone marrow cells isolated from *apoE*^{-/-} and *HxE*^{-/-} mice (n = 3 - 4). MCSF-1(10ng/ml) was used to differentiate the bone marrow cells. (E) Western blot analysis for ABCA1 was performed using 60µg of whole cell lysates from BMDMs as described in Materials and Methods. Densitometric analysis of ABCA1 protein expression was performed. (F) Cell surface expression analysis of ABCA1 was performed using flow cytometry. (G) ApoA-I (10µg/ml) mediated cholesterol efflux assay was performed using tritiated Ac-LDL (50µg/ml) loaded BMDMs from *apoE*^{-/-} and *HxE*^{-/-} mice (n = 3 - 4). All treatments were done in the presence of MCSF-1 (10ng/ml). (H)

*Ac-LDL uptake assay was performed using tritiated Ac-LDL (50µg/ml) loaded BMDMs from apoE^{-/-} and HxE^{-/-} mice (n = 3 – 4). (I) Serum apoA-I levels in apoE^{-/-} and HxE^{-/-} mice (n = 5) were determined using ELISA as described under Materials and Methods. The data shown are mean ± SEM. *** Significance: p<0.001. ** Significance: p<0.01; * Significance: p<0.05.*

Discussion

Monocytes originate from bone marrow-derived progenitor cells, and at early stages of monocyte development may 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 due to deficiency of ABCA1 and ABCG1 transporters, showed an increase in atherosclerosis (41). We found a significant reduction in ABCA1 gene and protein expression in the HxE^{-/-} BMDMs compared to apoE^{-/-} BMDMs (Figure 5). PPAR 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 (42-46). LXR-α, a well-established transcriptional regulator for ABCA1, was also significantly down-regulated 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 5). 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 (19, 47). 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, which may contribute to increased foam cell formation due to reduced cholesterol efflux capacity, and which may play a role in the increased atherosclerosis observed in our studies.

Methods

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.

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; Enzo Life Sciences # ALX-581-008-L002) and MCSF-1 (10ng/ml; Gemini Bio-products #300-847P) in DMEM complete media for 3 days. 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 -80°C if RNA was not isolated immediately. RNA was isolated using the manufacturer's protocol (RNEasy Mini kit, Qiagen). 40µl 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 10 µl of Master Mix SYBR Green in a final volume of 20 µl. Samples underwent the standard PCR protocol. 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. Cell lysates for protein were collected with 50 µl RIPA lysis buffer in order to concentrate the cell lysate. Cell lysates were immediately frozen at -80°C if not used for Western Blot immediately.

Western blotting – Serum or BMDM cell lysate samples were fractionated on 4-20% pre-cast SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes (Bio-Rad, Hercules, California) using a wet transfer apparatus (Bio-Rad, Hercules, California). 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 ABCA1 antibody at 1:1000 Novus Biologicals (#NB400-105, Littleton, CO, USA) and GAPDH antibody at 1:5000 Santa Cruz Biotechnology (sc-25778, Dallas, Texas, USA) 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 phosphatebuffered 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).

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 as 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 FACS Aria-II high-speed cell sorter and data were analyzed with FlowJo software (TreeStar).

Cholesterol Efflux in BMDMs- Cellular cholesterol efflux was performed as described previously with minor modifications (1). 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) for 3 days. 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 ³H-cholesterol (1μCi/mL), 25μg/ml acetylated-LDL 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 TO-901317 (1uM; 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.

ApolipoproteinA-I levels (ApoA-I) – ApoA-I levels were determined using a mouse specific apoA-I ELISA kit (Uscn Life Science Inc. Wuhan, Hubei, PRC) Briefly, serum samples, including standards of known apoA-I content, were added to pre-coated micro wells containing a captured monoclonal antibody specific for apoA-I for 2 hours at 37°C. Added Detection Reagent A and incubated 1 hour at 37°C. ApoA-I present in the well was both captured on the plate by the immobilized antibody and labeled with the conjugate antibody. After washing to remove all of the unbound material, Detection Reagent B was added to the wells and incubated for 30 minutes at 37°C. Following another wash step substrate solution was added for 15-25 minutes at 37°C. Subsequently, an acidic stop-solution provided by the manufacturer was added, and the intensity of the resulting color was determined in a spectrophotometer at 450 nm.

Statistical Analysis- Refer to Chapter 3

- III) Navab M, Anantharamaiah GM, Reddy ST, Hama S, Hough G, Grijalva VR, Wagner AC, Frank JS, Datta G, Garber D, Fogelman AM. Oral D-4F causes formation of pre-beta high-density lipoprotein and improves high-density lipoprotein-mediated cholesterol efflux and reverse cholesterol transport from macrophages in apolipoprotein E-null mice. *Circulation*. 2004;109:3215-3220.

Chapter 7

Bone marrow-derived macrophages from the HxE^{-/-} mice are proinflammatory

Introduction

Macrophages play a role in atherosclerosis: CAD is a leading cause of mortality in developed countries and is mainly caused by atherosclerosis, a chronic inflammatory disease. Macrophages are the first inflammatory cells, which differentiate from monocytes that are recruited from the blood and account for the majority of leukocytes in atherosclerotic plaques (136). Monocytes originate from bone marrow-derived progenitor cells and do not proliferate in the blood (137). In both mice and humans, monocytes comprise 5 to 10% of peripheral blood leukocytes (138). Macrophages are innate immune system cells therefore they exhibit great plasticity. Different stimuli and environments can lead to diverse phenotypes. Their functions comprise inflammatory responses, antimicrobial activity, tissue remodeling and iron recycling.

Recent studies have highlighted significant heterogeneity in macrophage behaviour and activation within atherosclerotic plaque. This heterogeneity is derived both from the heterogeneity of originating monocytes, and the inflammatory and lipidic stimuli available in the plaque.

In mice, monocytes can be identified from other circulating cells by the differential expression of chemokine C-C motif receptors 2 (CCR2), chemokine C-X3-C motif receptor 1 (CX3CR1), and Ly6C antigen, which is monocyte/ macrophage cell differentiation antigen regulated by interferon gamma (137). $\text{Ly6C}^{\text{high}}\text{CCR2}^+\text{CX3CR1}^{\text{Low}}$ monocytes are termed 'inflammatory' monocytes, and $\text{Ly6C}^{\text{low}}\text{CCR2}^-\text{CX3CR1}^{\text{high}}$ are referred to as 'resident' monocytes (142, 143, 144). $\text{Ly6C}^{\text{high}}\text{CCR2}^+\text{CX3CR1}^{\text{Low}}$ monocytes, which are precursors of inflammatory macrophages, have been observed to

adhere to activated endothelium in apoE^{-/-} mice (a model system for atherosclerosis) (145). Chemokine receptors are necessary for monocytes to traverse the endothelium (146, 147). Ly6C⁺ monocytes are recruited to mouse atherosclerotic plaque via CCR2, CCR5 and CX3CR1 (148).

Early work relating to the effect of the colony stimulating factors (CSFs) on macrophage phenotype was undertaken by Hamilton and colleagues (149, 150). Driven by macrophage colony-stimulating factor (MCSF) and other differentiation factors, monocytes differentiate into two major types of macrophages: M1 and M2 (151, 152). M1 and M2 macrophages play opposite roles during inflammation, although both are present in atherosclerotic lesions. This nomenclature mirrors the Th1 and Th2 polarization system (153).

M1 macrophages, which are differentiated from Ly6C^{high} monocytes and promote inflammation, are classically activated by LPS through the toll like TLRs in the presence of IFN- γ , leading to the production of high levels of IL-2, IL-23, IL-6, IL-1, and TNF- α . TLRs may be found on the cell surface, as in the case of TLR4 or reside intracellularly (154, 155). TLRs are key activators of monocytes and macrophages. The most characterized recognition system is the one sensing LPS and it is known that LPS activates downstream targets through the TLR4. In mouse atherosclerosis, TLR4 expression is exclusively by macrophages (156) and TLR4 ligands promote lipid uptake by macrophages and, hence cause foam cell formation (157-160). Differentiated macrophages exhibit macropinocytosis (fluid phase uptake of lipids) which is dependent upon TLR4 (161). However, the effect of TLR signaling are not limited to foam cell formation but have a direct effect on inflammation and matrix degradation also.

In contrast, alternatively activated M2 macrophages, which are differentiated from Ly6C^{low} monocytes and promote resolution of inflammation, have been originally characterized in the context of Th2-type immune responses (162). They differentiate in the presence of IL-4, IL-13, IL-1, or vitamin D3 and tend to produce a large amount of IL-10 and express scavenger receptors, mannose receptors, and arginase (163). M2 activated macrophages come in different flavors depending on the eliciting signals. Subsets of M2-like macrophages have been found to contribute to wound healing and regulation of inflammatory processes (164).

Listed below are some of the markers to identify M1 vs. M2 macrophage phenotype:

Chitinase 3-like 3 lectin (Ym1): Ym1 is the transcription factor found in inflammatory zone 1 (FIZZ1), and arginase 1 (ARG1) genes (165) and is upregulated in M2 macrophages.

Inflammatory zone 1 (FIZZ1): FIZZ1 is known to be upregulated in M2 macrophages due to the induction of its transcription factor Ym1 (165).

Arginase I (Arg I): IL4 is an early innate signal released during tissue injury, stimulating macrophage arginase to convert arginine to ornithine which is a step in extra-cellular matrix collagen production (166) in M2 macrophages. This ornithine is a precursor for polyamines which have an effect on cytokine production, affording wound healing macrophages regulatory capabilities (167). In an apoE^{-/-} murine model of atherosclerosis, early lesions were seen to be infiltrated by M2 (arginase I+) macrophages (168). As lesions progressed a phenotypic switch was observed, with an eventual predominance of M1 (arginase II+) macrophages.

Tumour Necrosis Factor- α (TNF- α): TNF- α regulates a number of critical cell functions including cell proliferation, survival, differentiation, and apoptosis. Macrophages produce TNF- α induced by TLRs through LPS activation and are also highly responsive to TNF- α through the TNF receptor (169, 170). One of the various functions is a pivotal role in orchestrating the production of a proinflammatory cytokine cascade. TNF- α is thus considered to be a “master regulator” of proinflammatory cytokine production (171). TNF- α -deficient apoE^{-/-} mice show a reduction in lesion formation, with a concomitant decrease in VCAM-1 and ICAM-1 expression, which are important for monocyte rolling on endothelial cells (172). In contrast, mice deficient in the TNF- α receptor develop larger lesions than control mice. In addition to these roles, Witsell and Schook (173) demonstrated that TNF- α has macrophage differentiation capabilities. TNF- α gene transcripts are expressed during differentiation of BMDMs. TNF- α affects the development of atherosclerosis at the fatty streak stage, and cleavage of TNF is an important step in activating the proatherogenic properties of TNF- α (174).

Chemokine C-C motif receptors 2 (CCR2): Monocytes can be identified from other circulating cells by the differential expression of CCR2. CCR2⁺ monocytes/macrophages are termed as inflammatory monocytes and are characteristic markers for M1 macrophages (137). It is also the receptor for the proinflammatory chemokine CCL2 (Chemokine C-C motif ligand2), also known as MCP-1 (monocyte chemoattractant protein-1).

Interleukin-10 (IL-10): IL-10, which is derived from monocytes and macrophages, is an important anti-inflammatory regulator for the development of advanced atherosclerosis. As expected, IL-10-deficient mice showed a decreased amount of collagen, induced by

IFN- γ production in the atherosclerotic vessels (175). Studies with IL-10^{-/-}/apoE^{-/-} mice confirmed the atheroprotective properties of IL-10 in early stage atherosclerosis and showed that IL-10 promotes the stability of advanced plaques (176).

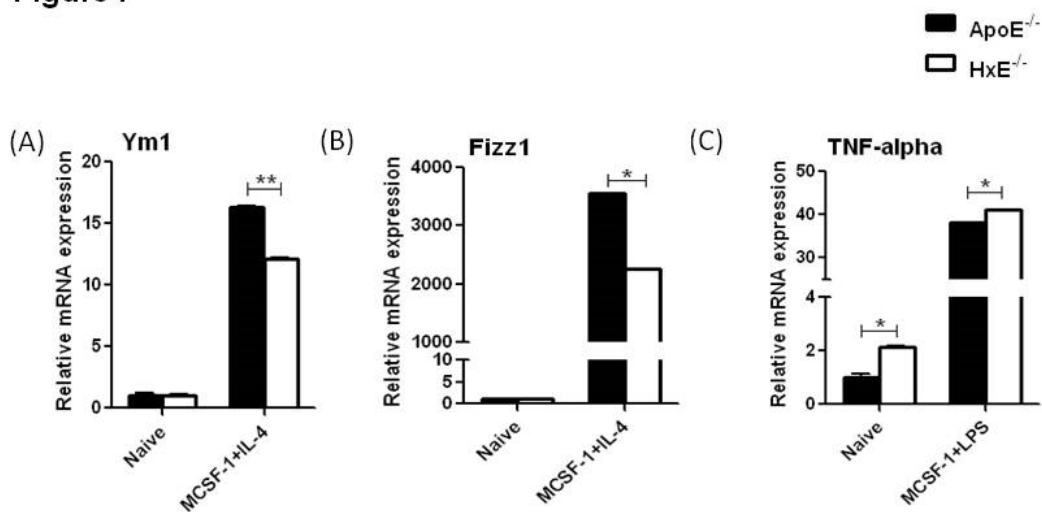
To characterize the BMDMs from HxE^{-/-} and apoE^{-/-} mice we performed the following experiments.

Results

Oikawa *et al.* summarized the effect of diabetes induced bone marrow microenvironment defects through oxidative stress and disruption of the normal redox balance (25). In light of this information and our current results, we investigated the macrophage phenotype of the BMDMs from HxE^{-/-} and apoE^{-/-} mice. We used bone marrow from 8-12 week old apoE^{-/-} and HxE^{-/-} mice (n=3-4) to determine the macrophage polarization of M1 (classically activated) vs. M2 (alternatively activated) type. To evaluate the macrophage population in our mice, we used naïve bone marrow cells or differentiated bone marrow cells from apoE^{-/-} and HxE^{-/-} mice in the presence of MCSF-1(10ng/ml) + IL-4(10ng/ml) and MCSF-1(10ng/ml) + LPS (10ng/ml) as described in Materials and Methods. RNA was isolated and analyzed for gene expression analysis using RT-qPCR. Macrophages from HxE^{-/-} mice showed a significant lower expression of FIZZ1 and Ym1 genes compared to the control apoE^{-/-} mice after IL-4 stimulation (Figure 7A and B). The expression of the downstream target of LPS stimulation, TNF- α was significantly upregulated in naïve as well as differentiated state in the HxE^{-/-} macrophages compared to apoE^{-/-} macrophages (Figure 7C). Chemokine Receptor-2 (CCR-2), a well-established M1 macrophage marker was significantly increased in the

BMDMs stimulated with LPS from the HxE^{-/-} mice compared to the control (Figure 7D). The HxE^{-/-} macrophages had a significantly lower expression of F4/80, Arg-1 and anti-inflammatory IL-10 (Figure 7E - G) compared to their apoE^{-/-} controls, indicating that the macrophages are unable to suppress proinflammatory responses. Together, the different gene expression analyses and reduced ABCA1 gene expression (Figure 6B) reveal that the macrophages from HxE^{-/-} mice are more M1-like (more proinflammatory).

Figure 7



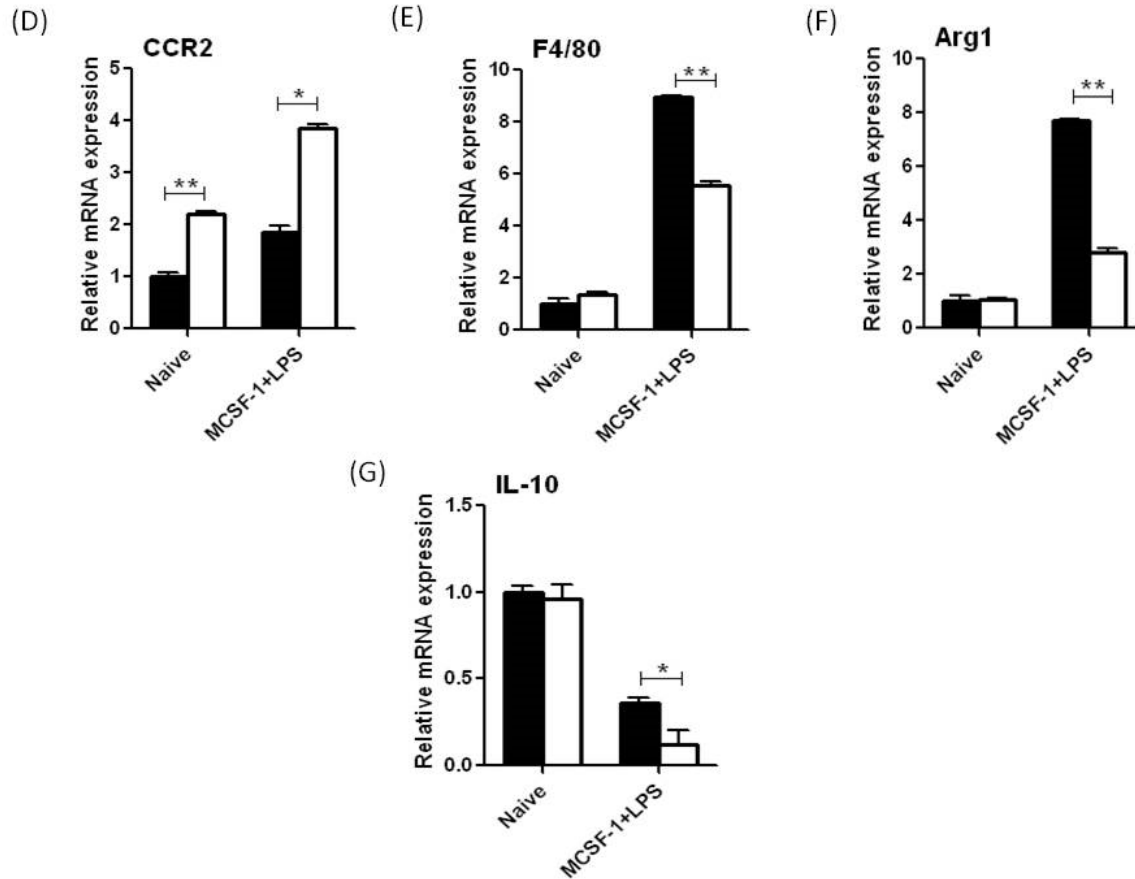


Figure 7: Bone marrow-derived macrophages from the $Hx E^{-/-}$ mice are proinflammatory: RT-qPCR analysis was performed using (A-B) naïve and IL-4 (10ng/ml) treated bone marrow cells (C-G) naïve and LPS (10ng/ml) treated bone marrow cells isolated from $Hx E^{-/-}$ and $apo E^{-/-}$ mice ($n = 3 - 4$) as described under Materials and Methods. MCSF-1(10ng/ml) was used to differentiate the bone marrow cells. The data shown are mean $\pm$ SEM. ** Significance: $p < 0.01$; * Significance: $p < 0.05$.

Table 2

Primer #	Gene Symbol	Forward primer	Reverse primer
1	YM1	5'- ACT TTG ATG GCC TCA ACC TGG ACT -3'	5'- TGG AAG TGA GTA GCA GCC TTG GAA-3'
2	CCR2	5'- GGT CTC GGT TGG GTT GTA AA-3'	5'- AAG AGG CTC CTC ATA GGG ATA G-3'
3	Arg1	5'- ACCTGG CCT TTG TTG ATG TCC CTA-3'	5'- AGA GAT GCT TCC AAC TGC CAG ACT-3'
4	FIZZ1	5'-CTT GTG GCT TTG CCT GTG GAT CTT-3'	5'-TGG TCC AGT CAA CGA GTA AGC ACA-3'
5	IL-10	5'- GGG TTG CGA AGC CTT ATC GGA AAT-3'	5'- TCT TCA GCT TCT CAC CCA GGG AAT-3'
6	ABCA1	5'-CCCTGCTTCCGTTATCCAAC-3'	5'-GACCTTGTGCATGTCTTAATGC-3'
7	ABCG1	5'-AGACTCTCCAACCCAACCT-3'	5'-CCAACCGTCCTACATCTTCC-3'
8	LXR- α	5'-TGTGCGCTCAGCTCTTGT-3'	5'-CCCTGGACATTACCAAGACAC-3'
9	LXR-B	5'-CAGGAGATTGTGGACTTTGC-3'	5'-TTGTAGCGTCTGGCTGTTTC-3'
10	F4/80	5'-CTTGGCTATGGGCTTCCAGTC-3'	5'-GCAAGGAGGACAGAGTTTATCGTG-3'
11	TNF- α	5'- TACTGAACTTCGGGGTGATTGGTCC -3'	5'- CAGCCTTGTCCCTGAAGAGAACC -3'

Table 2: RT-qPCR primer list: Chitinase 3-like 3 lectin (Ym1), Chemokine C-C motif receptors 2 (CCR2), Arginase I (Arg I), Inflammatory zone 1 (FIZZ1), Interleukin-10 (IL-10), ATP-binding cassette, sub-family A, member1 (ABCA1), ATP-binding cassette, sub-family G, member1 (ABCG1). Liver X Receptor- α (LXR- α), Liver X Receptor- β (LXR- β), EGF-like module-containing mucin-like hormone receptor-like 1 (F4/80), Tumour Necrosis Factor- α (TNF- α),

Discussion

In pathological conditions such as aging, atherosclerosis, and diabetes, excess amounts of ROS in the bone marrow microenvironment may impair stem and progenitor cell function, and cause hematopoietic dysfunction (48, 49). 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 to 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; these macrophages are thought to be more proinflammatory (26, 27). In addition, proinflammatory M1 macrophages have been shown to exhibit reduced expression of ABCA1 and other markers (28). Cytokines such as IL-4 can antagonize classical macrophage activation and induce the development of alternatively activated or anti-inflammatory M2 macrophages (29). It is well established that the expression of FIZZ1 and Ym1 is induced in alternatively activated macrophages as compared with classically activated macrophages while TNF- α and CCR2 are highly expressed in M1 macrophages (50, 51-53). BMDMs from HxE^{-/-} mice showed significantly reduced expression of Ym1 and FIZZ1 and significantly increased expression of TNF- α and CCR2, when compared to apoE^{-/-} mice. Myeloid-derived suppressor cells (MDSCs) are considered to be an immature population of myeloid cells associated with infections or tumors that are cable of suppressing proinflammatory responses. Mouse MDSCs have been found to express F4/80, IL-4R α , Arg1, IL-10, and TGF- β amongst many others (53). BMDMs from HxE^{-/-} mice showed a significant down regulation of F4/80, Arg1 and

IL-10 compared to apoE^{-/-} BMDMs. Our data suggests that BMDMs from HxE^{-/-} mice promote proinflammatory responses compared to BMDMs from apoE^{-/-} mice. In addition, it has been shown that M1 macrophages have reduced expression of ABCA1, which has important implications in the development of atherosclerosis. Taking into account the different gene expression profiles of BMDMs from HxE^{-/-} and apoE^{-/-} mice, we suggest that the lack of Hx from birth in apoE^{-/-} mice, influences the bone marrow microenvironment and makes their macrophages more proinflammatory or M1-like.

Methods

Isolation of bone marrow cells- Refer to Chapter 6

RNA Extraction, Reverse Transcription, and Quantitative-PCR- Refer to Chapter 6

Statistical Analysis- Refer to Chapter 3

Chapter 8

Hepatic microarray analysis show mice lacking Hx from birth have defects in fertility in addition to increased inflammation

Introduction

We had observed differences in expression of some genes in the liver and bone marrow derived macrophages between the HxE^{-/-} and apoE^{-/-} mice (Chapter 3, 6 and 7). We wanted to study further the liver gene expression profile as Hx is an acute phase protein, synthesized mainly in the liver. In addition, Hx scavenges free heme, and the resultant heme–Hx complex is taken up by the liver through receptor-mediated endocytosis to avoid heme mediated oxidative damage to other tissues (40). In order to investigate further the differential expression of genes in the liver of apoE^{-/-} mice with and without Hx from birth, we performed a microarray experiment.

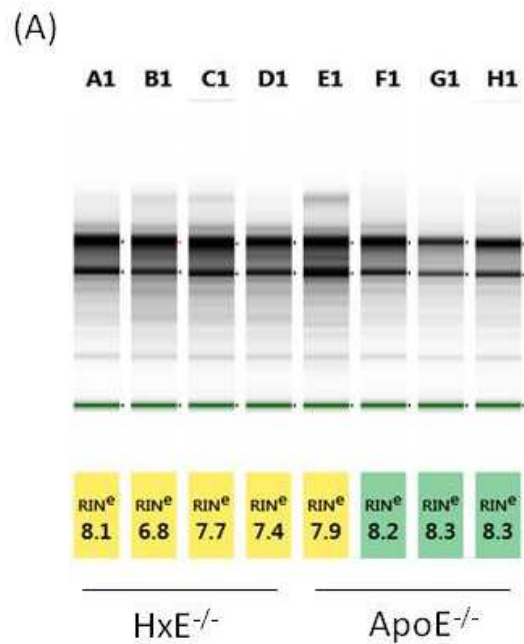
Results

To perform a microarray on liver RNA samples, we estimated the quality of the RNA by running an electrophoresis experiment using the Bioanalyzer 2100. As shown in Figure 8A, eight RNA samples were used (apoE^{-/-} = 4 and HxE^{-/-} = 4). Some of the RNA samples displayed some gDNA contamination; however the RIN number was above 7 which indicated good RNA quality. A DNA digestion step was performed using Buffer RDD and Proteinase K in order to eliminate gDNA contamination.

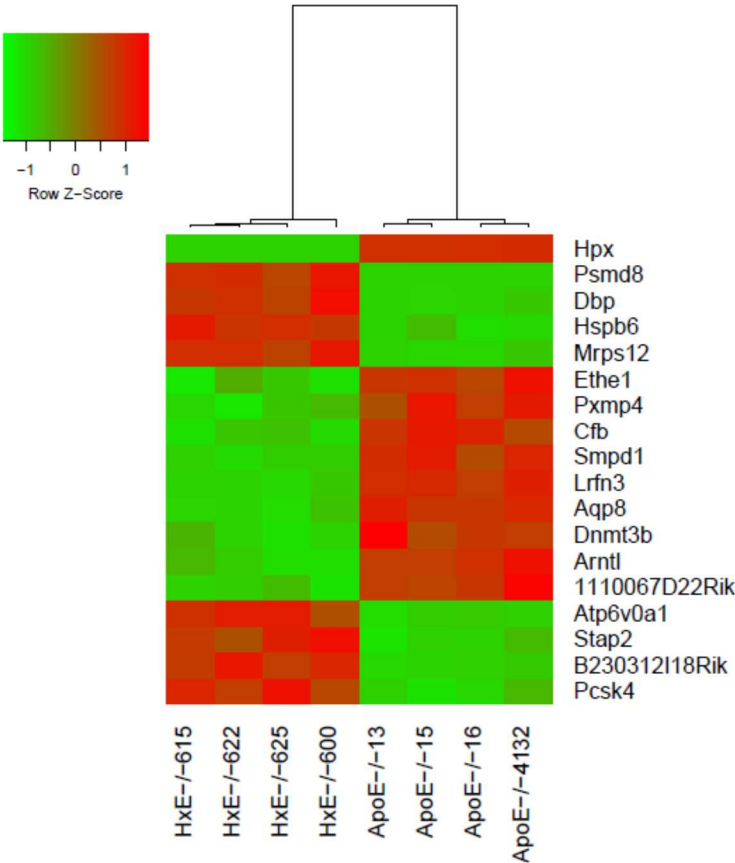
Hepatic microarray analysis was performed using the mouse re8 v2.0 expression chip (Illumina). The analysis revealed several genes including the expression of Hx (Figure 8B and C) to be dysregulated in the HxE^{-/-} mice compared to apoE^{-/-} mice. Most genes that were differentially regulated such as Arntl, PCSK4 and Aqp8 were involved in problems with fertility, lipogenesis and ectopic fat formation. Some of the genes such as Hspb6 and Stap2 which are involved in ER stress, liver damage and inflammation were significantly higher in the HxE^{-/-} mice while some genes whose loss of function involved

profound brain damage, premature death and loss of viability such as *Dnmt3b*, *Smpd1* and *Ethe1* were significantly lower in the *HxE^{-/-}* mice. Genes such as *Pxmp4* whose silencing can be involved in carcinogenesis was significantly lower in *HxE^{-/-}* mice compared to their wildtype counterparts. The microarray analysis showed that *HxE^{-/-}* mice were under more stress and inflammation compared to the *apoE^{-/-}* mice.

Figure 8



(B)



(C)

Gene symbols	Gene names	HxE-/-	ApoE-/-	Gene function
Hpx	Mus musculus hemopexin (Hpx), mRNA.	low	high	Heme scavenger, acute phase protein
Psm8	Mus musculus proteasome (prosome, macropain) 26S subunit, non-ATPase, 8 (Psm8), mRNA. XM_001002011	high	low	An essential function is the processing of class I MHC peptides. This gene encodes a non-ATPase subunit of the 19S regulator
DBP	Mus musculus D site albumin promoter binding protein (Dbp), mRNA.	high	low	This gene governs circadian transcription of a number of hepatic detoxification and metabolic enzymes prior to the activity phase and subsequent food intake of mice.
Hspb6	Mus musculus heat shock protein, alpha-crystallin-related, B6 (Hspb6), mRNA.	high	low	Hsp gets elevated during ER stress in the livers. It is Nrf-2 regulated
Mrps12	Mus musculus mitochondrial ribosomal protein S12 (Mrps12), mRNA.	high	low	This gene encodes a 28S subunit protein that belongs to the ribosomal protein S12P family. The encoded protein is a component of the ribosomal small subunit and controls the decoding fidelity and susceptibility to aminoglycoside antibiotics.
Ethe1	Mus musculus ethylmalonic encephalopathy 1 (Ethe1), mRNA.	low	high	This gene can suppress p53-induced apoptosis. Defects in ETHE1 are a cause of ethylmalonic encephalopathy (EE). Mice homozygous for a null mutation display premature death with elevated levels of hydrogen sulfide and thiosulfates.
Pxmp4	Mus musculus peroxisomal membrane protein 4 (Pxmp4), mRNA.	low	high	Hypermethylation-mediated silencing of PXMP4 may be involved in prostate carcinogenesis.
Cfb	Mus musculus complement factor B (Cfb), mRNA.	low	high	Homozygotes for targeted null mutations lack the alternative complement pathway, and have reduced overall complement activity
Smpd1	Mus musculus sphingomyelin phosphodiesterase 1, acid lysosomal (Smpd1), mRNA.	low	high	Defects in this gene causes diseases characterized by jaundice, an enlarged liver, failure to thrive, progressive deterioration of the nervous system and profound brain damage.
Lrfr3	Mus musculus leucine rich repeat and fibronectin type III domain containing 3 (Lrfr3), mRNA.	low	high	This is a transmembran glycoprotein
Aqp8	Mus musculus aquaporin 8 (Aqp8), mRNA.	low	high	Disruption in this gene results in enlarged testes and a reduction in water permeability in plasma membrane of testes.
Dnmt3b	Mus musculus DNA methyltransferase 3B (Dnmt3b), transcript variant 4, mRNA.	low	high	This gene encodes a DNA methyltransferase which is thought to function in de novo methylation. Loss of function results in severe developmental defects and loss of viability.
Arntl	Mus musculus aryl hydrocarbon receptor nuclear translocator-like (Arntl), mRNA.	low	high	Defects in this gene have been linked to infertility, problems with gluconeogenesis and lipogenesis, altered sleep patterns, ectopic fat formation
1110067D22Rik	Mus musculus RIKEN cDNA 1110067D22 gene (1110067D22Rik), mRNA.	low	high	No phenotype has yet been reported
Atp6v0a1	Mus musculus ATPase, H ⁺ transporting, lysosomal V0 subunit A1 (Atp6v0a1), mRNA.	high	low	These transporters play an important role in processes such as receptor-mediated endocytosis, protein degradation and coupled transport.
Stap2	Mus musculus signal transducing adaptor family member 2 (Stap2), mRNA.	high	low	STAP-2 plays a regulatory role in the Acute Phase response in systemic inflammation.
30312118Rik	PREDICTED: Mus musculus RIKEN cDNA B230312118 gene, transcript variant 3 (B230312118Rik), mRNA.	high	low	No phenotype has yet been reported
PCSK4	Mus musculus proprotein convertase subtilisin/kexin type 4 (Pcsk4), mRNA.	high	low	PCSK4 null mice are viable but have reduced fertility

Figure 8: Microarray analysis: (A) RNA electrophoresis on Bioanalyzer 2100. (B) Heat map of the dysregulated genes obtained from the microarray data analysis. (C) Summary of the functions of the dysregulated genes in apoE^{-/-} and HxE^{-/-} mice (n = 4). Significance was based on $p < 0.05$.

Discussion

Malondialdehyde levels are a measure of hepatic lipid peroxidation. Levels of malondialdehyde can be used as a biomarker to estimate oxidative stress *in vivo*. Our data showed that HxE^{-/-} mice had increased levels of malondialdehyde in their livers compared to apoE^{-/-} mice (Figure 4C) demonstrating that increased heme and ROS due to Hx deletion can lead to increased lipid peroxidation. In addition, the microarray data revealed some genes such as Hspb6 and Stap2 (Figure 8B and C) involved in acute phase responses and ER stress in the livers to be elevated in HxE^{-/-} mice compared to the control apoE^{-/-} mice. Surprisingly, genes involved in the circadian rhythm were dysregulated. However, we cannot explain this dysregulation in gene expression of the circadian rhythm. Interestingly, genes linked to infertility were dysregulated in the mice. Defects in genes such as Arntl (aryl hydrocarbon receptor nuclear translocator-like), whose function is linked to infertility, problems with gluconeogenesis and lipogenesis, altered sleep patterns, ectopic fat accumulation was significantly down regulated in HxE^{-/-} mice compared to apoE^{-/-} mice indicating, that Hx deletion can lead to infertility in the apoE^{-/-} mice.

Methods

RNA quality check by Bioanalyzer: ApoE^{-/-} and HxE^{-/-} mouse liver RNA was isolated using the manufacturer's protocol (RNEasy Mini kit, Qiagen). 40 µl of RNA was eluted

and quantified using Nanodrop. The quality of RNA was determined using the Bioanalyzer 2100 (Agilent) machine and the High Sensitivity RNA ScreenTape Assay system (Agilent) which generated a RIN value. RNA samples with a RIN value of 7 or greater were used for microarray analysis.

Microarray: The Semel Institute UCLA Neurosciences Genomics Core performed the microarray. They used the mouse re8 v2.0 expression chip (Illumina) to determine the gene expression levels in apoE^{-/-} and Hx^{E-/-} mouse liver RNA submitted.

Microarray data analysis: The QCB Collaboratory at UCLA performed the microarray analysis. R package limma was used for the microarray data analysis. Background correction and normalization was performed using control probes. Probes defined as “No match” and “Bad” in illuminaMousev2 database were removed from analysis. Limma for differential expression analysis were performed based on the log2 probe intensity values with the arrayWeights function applied.

Gene functional analysis: The Ingenuity pathway analysis was performed for genes expressed significantly different between apoE^{-/-} and Hx^{E-/-} mice. Genes with adjusted $p < 0.05$ were analyzed using the MouseRef-8 v2.0 as the reference.

Chapter 9

hHx injections reduce serum heme content and apoB-containing lipoprotein cholesterol levels in HxE^{-/-} mice.

Introduction

Role of Hx in the CNS: The expression of Hx in central nervous system (CNS) has been gradually established. The first analysis in rats failed to detect Hx in the CNS (65). Conversely, one work on the human brain showed immunoreactivity for Hx in neurons and a few scattered glial cells, whereas oligodendrocytes and epithelial cells of choroid plexi were negative (66). Human Hx promoter activity has been reported in several brain regions in mice, including cortex, hippocampus, thalamus, cerebellum, and brainstem nuclei (67, 68). Finally, Western blotting analysis of brain homogenates confirmed Hx expression (69), and a proteomic study identified the protein in human cerebrospinal fluid (CSF) (70). Data demonstrate that Hx is expressed at different levels by ependymal cells and cell populations within the brain.

Little is known about the function of Hx in the CNS, the only data coming from the analysis of the brain of Hx-null mice. Hx-null mice have shown a strong increase in the number of iron-loaded oligodendrocytes in the basal ganglia and thalamus compared with wild-type controls, not associated with an increase in H- and L-ferritin expression in these regions. Consistent with increased iron deposits and inadequate ferritin expression, oxidative stress markers were higher in the brains of Hx-null mice than in those of wild-type controls (69). These data suggest a role for Hx in controlling heme–iron recovery within the brain. Other than in the CNS, Hx expression has been demonstrated in the peripheral nervous system (PNS).

Hx in the retina: Hx expression has been demonstrated also in the retina, which is separated from the circulation by the blood–retinal barrier. Chen and co-workers (71)

showed that Hx is expressed in neural retina by photoreceptors and ganglion cells. Moreover, retinal pigment epithelial cells are able to bind the heme–Hx complex and, after its internalization, to induce the expression of HO-1, MT1, and ferritin (72, 73). At this level, Hx-mediated heme scavenging might be important in preventing oxidative damage after traumas and hemorrhages.

Hx levels predict Hemolytic diseases and Septic shock: Heme is an essential molecule for living aerobic organisms and is involved in a remarkable array of diverse biological processes and disease conditions. Hemolytic diseases such as sickle cell disease and β -thalassemia are characterized by enhanced intravascular hemolysis resulting in heme-catalyzed ROS generation, which leads to endothelial dysfunction and oxidative damage (78). In addition, low serum Hx levels are related to sepsis severity and can indicate poor prognosis for septic shock patients (79). Jung JY et al. (79) measured the concentrations of Hx in a rat model featuring distinct grades of severity of endotoxemia and in patients who survived and did not survive septic shock. Jung JY et al. (79) showed that the levels of Hx are substantially lower during severe endotoxemia than during mild endotoxemia in the rat model. In addition, serum Hx levels are dissimilar in patients under septic conditions of distinct severity and, the initial serum Hx concentration in non-survivors is significantly lower than in survivors. Jung JY et al. (79) concluded that serum Hx concentration can help predict the 28-day mortality among septic shock patients, and suggested that a diminished level of Hx might be associated with poor prognosis in severe sepsis validating previous reports by Larsen R, et al. (80). Moreover, previous studies also reported that Hx can potently suppress the synergistic

effect exerted by heme and Hb on sterile and septic inflammatory stimuli such as HMGB1 and LPS (81-83).

Hx administration protects cerebral ischemic injury: Free heme is released from methemoglobin following cerebral ischemia-reperfusion and represents a toxic component in the peripheral blood. Hx, due to its high binding affinity to heme and an efficient scavenger of heme in overloaded peripheral blood was suggested to be neuroprotective. Studies by Li RC et al. (84) reported that Hx-null mice sustain greater infarct volumes and behavioral deficits than their wild-type counterparts in a model of transient middle cerebral artery occlusion, indicating its neuroprotective role against stroke-related damage (84). However, it has not been clear how Hx exerted its neuroprotective role until a recent study by Dong B, et al. (85) who investigated the expression of Hx in the normal rat brain by immunofluorescent staining. Dong B, et al. (85) showed that Hx is mainly expressed in the neurons and a small portion of astrocytes after a short-term middle cerebral artery occlusion followed by 24-hour reperfusion in the rat brain. Dong B, et al. (85) further demonstrated that administration of exogenous Hx at the onset of reperfusion dose-dependently, reduced the infarct volumes and improved the outcome of cerebral ischemia-reperfusion injury. These studies (85) confirmed the neuroprotective effects of Hx and provide new insights into the potential therapeutic role of Hx in cerebral ischemia.

Hx and heme-iron recycling: Hemopexin limits iron access to microorganisms mediating heme-iron recycling. Fiorito *et al.* (86) recently demonstrated increased iron levels in the duodenum of Hx-null mice due to increased iron uptake by enterocytes and storage in ferritins. The study reported that Hx deficiency resulted in enhanced heme

catabolism in the duodenum by inducing HO-1 activity. The transfer of iron from the enterocytes to the liver was unaffected indicative of abnormal levels of iron deposits in the duodenal mucosa. In addition, they showed that absence of Hx did not affect the expression of iron transporters in the duodenum cells despite increased iron accumulation. This provides new insights to the understanding of body iron homeostasis and possibly at identifying strategies to increase/reduce iron absorption in the therapy of metabolic disorders of iron deficiency and overload respectively (86).

Hx in autoimmune disorders: Hx levels increases and remains high in experimental autoimmune encephalomyelitis (EAE) mice, the mouse model of multiple sclerosis. EAE induced in Hx knockout mice, developed EAE clinically earlier and exacerbated compared with mice having Hx due to a large amount of CD4⁺-infiltrating T cells. Hx-null mice developed severe EAE due to a greater number of infiltrating and circulating Th17 cells. Hx therapy in Hx-null mice before EAE induction reduced the Th17 expansion, as well as disease severity, and was comparable with those of wild-type mice. Taken together, these data indicate that Hx has a negative regulatory effect in Th17-mediated inflammation and propose Hx's therapy to limit the expansion of this cell subset in inflammatory and autoimmune diseases (87).

Hx administration improves cardiovascular function: Circulating ferrous heme (on Hb) is considered as one of the important pathogenic factors in CAD. Increased circulating ferrous heme containing Hb i) scavenges NO, a potent vasodilator (88) and ii) promotes the generation of ROS, which leads to endothelial activation (89), and iii) induces smooth muscle proliferation (90), contributing further to cardiovascular pathology (91).

To study the role of Hx, using a model of heme overload in Hx-null mice, Vinchi *et al.* (78) demonstrated that when excess heme is bound by Hx, ROS production and adhesion molecule expression are reduced and NO availability is increased. Using B-thalassemia and sickle cell disease mouse models of hemolytic diseases Vinchi *et al.* (78) discovered that Hx therapy preserves cardiovascular function and normalization of blood pressure by promoting heme recovery and hepatic detoxification of heme mainly through the induction of HO-1 activity. The authors suggested that Hx treatment is a promising novel therapy to protect against heme-induced cardiovascular dysfunction in hemolytic diseases (78).

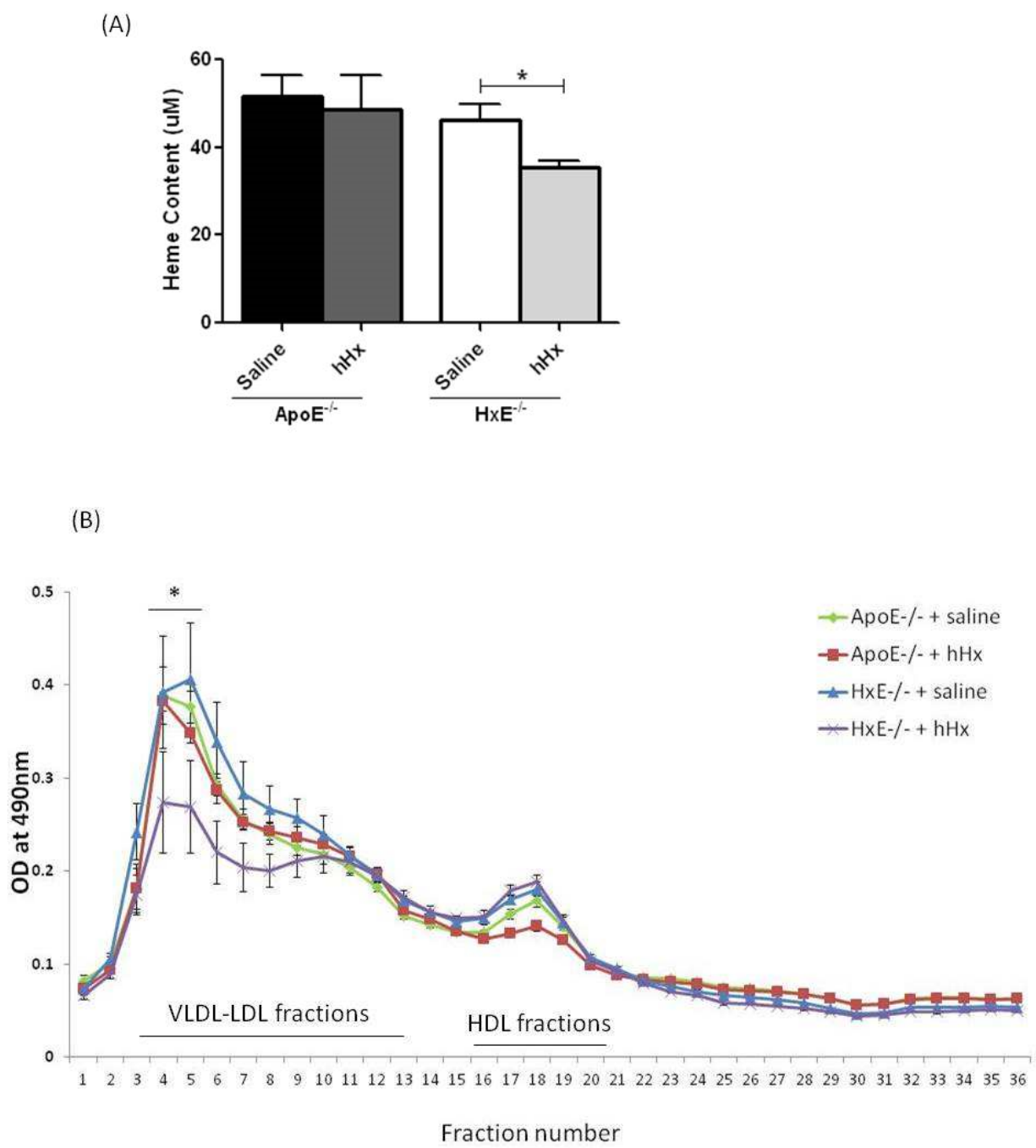
Hence, we learned that Hx is present in the brain, eyes, intestine and the heart, and it has a protective effect in all the organs. Hx protects against the oxidative stress caused by the excess free floating heme in these organs in addition to hemolytic diseases, septic shock and autoimmune diseases like EAE. In light of all the above functions and protective role of Hx, we wanted to study the effect of Hx administration on the apoE^{-/-} mice lacking Hx in our study. In order to determine the role of Hx we performed the following experiments.

Results

Data shown previously showed an increase in serum heme and ROS content in HxE^{-/-} mice compared to apoE^{-/-} mice. We hypothesized that Hx administration might be beneficial to limit heme and ROS based damage by reducing heme content in circulation. To test this hypothesis, HxE^{-/-} and apoE^{-/-} mice (n=3-5) were injected 320 ug purified Human Hx (hHx) or saline intraperitoneally for 24 hours at 4-6 weeks of age. At

the end of the experiment we analyzed the heme content in the serum of the mice. A significant lower level of heme was observed in the HxE^{-/-} mice injected with hHx compared to the HxE^{-/-} mice injected with saline (Figure 9A). No significant change was observed in the apoE^{-/-} mice injected with hHx compared to saline, thus demonstrating that exogenous Hx can scavenge heme in a heme overloaded mouse. FPLC fractionation of serum samples showed a significant reduction of cholesterol content in the fractions #3-13 as measured at 490nm in the HxE^{-/-} mice injected with hHx compared to the saline injected control group (Figure 9B). A significant lower level of total cholesterol was also observed in the HxE^{-/-} mice injected with hHx (Figure 9C). Concentration and quantification of the fractions #3-13 demonstrated significant lower levels of apoB-containing lipoprotein cholesterol levels in the HxE^{-/-} mice injected with hHx (Figure 9D), although, HDL-C levels (fractions 17-20) remained unchanged (Figure 9E). In order to determine if the VLDL/LDL cholesterol was cleared from circulation for hepatic breakdown and excretion, we estimated hepatic and fecal cholesterol levels from the same mice. No significant change was observed in the levels of hepatic or fecal cholesterol levels (Figure 9 F and G). In addition, no significant difference was observed in the levels of hepatic triglycerides between hHx or saline injected apoE^{-/-} or HxE^{-/-} mice (Figure 9H). These results indicated that exogenous hHx can reduce VLDL/LDL-cholesterol levels as well as heme levels in hyperlipidemic mice lacking Hx from birth.

Figure 9



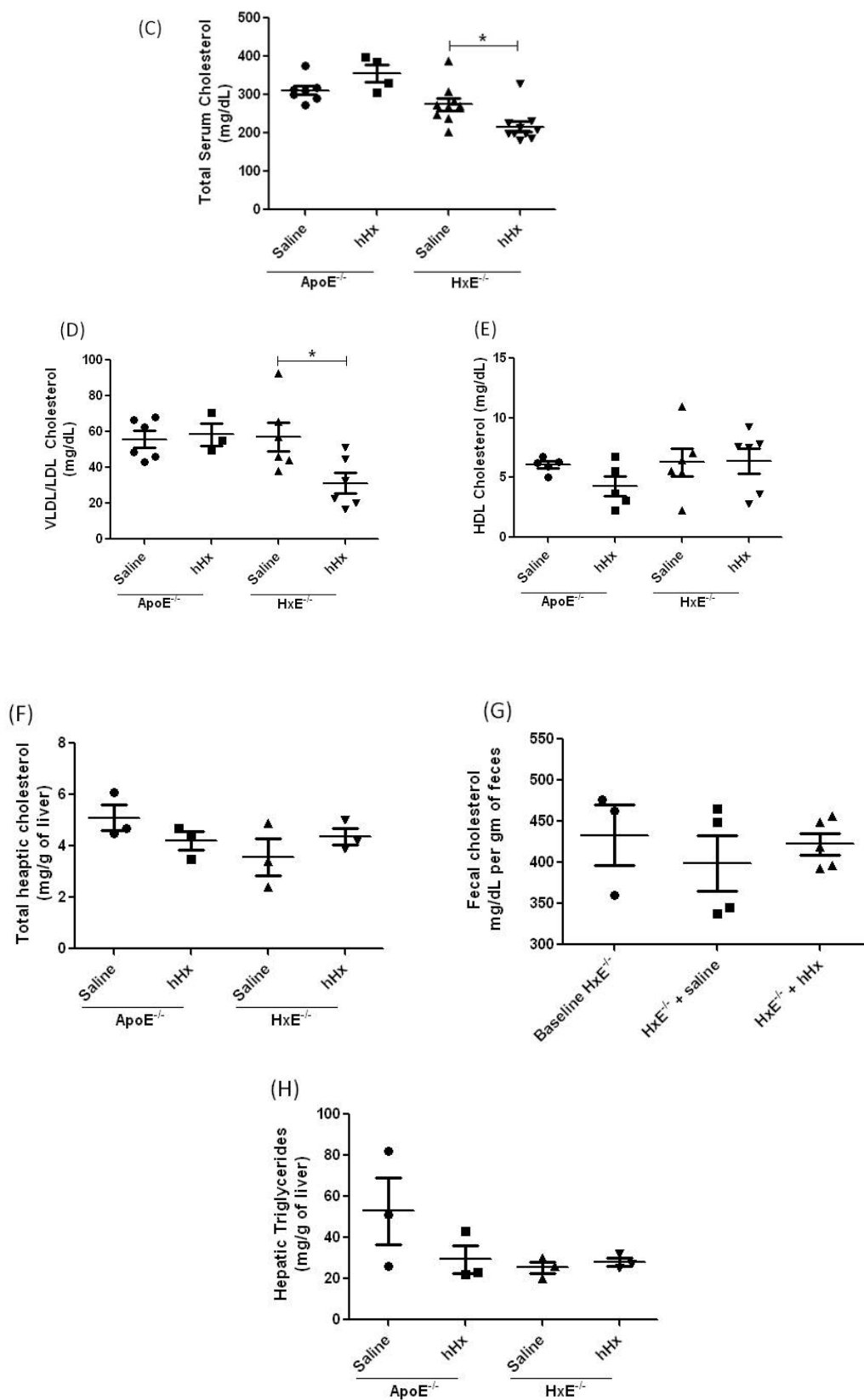


Figure 9: Hemopexin add-back decreases heme content and apoB-containing lipoprotein-cholesterol in Hx^{E-/-} mice: (A) Heme content measured in serum samples from Hx^{E-/-} and apoE^{-/-} mice injected with saline or hHx (n=3-5). (B) FPLC fractionation of serum samples from Hx^{E-/-} and apoE^{-/-} mice injected with saline or hHx (n=3-5). (C) Total cholesterol levels determined in serum and samples from Hx^{E-/-} and apoE^{-/-} mice injected with saline or hHx (n=3-5). (D) VLDL/LDL cholesterol (E) HDL cholesterol levels estimated in lipoprotein fractions separated by FPLC using serum samples from Hx^{E-/-} and apoE^{-/-} mice injected with saline or hHx (n=3-5). (F) Total hepatic cholesterol levels (H) Hepatic triglyceride levels measured in liver samples from Hx^{E-/-} and apoE^{-/-} mice injected with saline or hHx (n=3-5). (G) Fecal cholesterol levels determined in Hx^{E-/-} mice at baseline and after saline or hHx injections (n=3-5). The mean value for each group of mice is indicated by the horizontal bars. The data shown are mean $\pm$ SEM. * Significance: $p < 0.05$.

Discussion

Hx administration has shown to help many diseases such as septic shock, cerebral ischemia and protect cardiovascular function (78-79, 84-85). Hx therapy has been proposed to alleviate the diseases severity of autoimmune disorders such as EAE (87). The expression of Hx in the CNS demonstrates Hx protective role in heme-iron recovery from iron-loaded oligodendrocytes in the basal ganglia and thalamus thereby preventing brain damage (69). The presence of Hx in the retina and induction of heme catabolic enzymes like HO-1 show that Hx-mediated heme scavenging is important in mitigating oxidative damage after traumas and hemorrhages (71-73). These therapies have shown promising results for the protective role of Hx in preventing heme mediated distress and

damage to the body. We show that hHx administration in Hx-null, heme overloaded mice can significantly reduce levels of free floating heme in circulation after 24 hours of administration (Figure 9A). This re-enforces the role of Hx scavenging of free floating excess heme in the HxE^{-/-} mice. In addition, we observed a significant reduction of apoB containing lipoprotein-cholesterol (Figure 9D) in HxE^{-/-} mice indicating hHx administration has an additional protective effect. Free floating heme has shown to transiently bind to albumin, lipoproteins like LDL and HDL and α -microglobulin in circulation before binding to Hx for receptor mediated endocytosis. There could be a simultaneous clearance of heme and VLDL/LDL from circulation which could explain the reduction of VLDL/LDL-C along with heme reduction after injecting Hx exogenously. This short term experiment shows additional promising effects of the protective role of Hx in circulation.

Methods

Hemopexin injections- Hemopexin treatment in HxE^{-/-} and apoE^{-/-} (n=3-5) mice was performed by injecting 320 ug purified human Hx (hHx) (Athens Research) or saline intraperitoneally for 24 hours at 4-6 weeks of age. Blood was collected for serum isolation to measure heme content and lipoprotein levels.

Heme content- Refer to Chapter 2

Lipoprotein isolation and cholesterol estimation - Lipoprotein samples were isolated from individual sera (~100-125 μ l) 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. VLDL/LDL fractions (#3-13) and HDL fractions (#17-20) were pooled and concentrated using filter columns to concentrate (Amicon Centrifugal Units). Concentrated lipoproteins were assayed for cholesterol (Thermo Scientific) according to manufacturer's protocols.

Fecal cholesterol estimation- Mouse fecal pellets were dried at 40°C for 24 hours. 50mg of dried pellet was soaked in 0.88% KCl for 30 mins in glass tubes. Samples were homogenized. 4.5ml of 2:1 Chloroform:Methanol was added and vortexed vigorously. Samples were centrifuged at 1800rpm for 15 mins and the bottom organic layer was collected in a new glass tube. 4.5ml of chloroform was added to the aqueous layer. Samples were vortexed and centrifuged again. The bottom organic layer was collected again and pool with the first extraction. Pooled samples were dried under nitrogen gas. 1ml of 1% triton-x 100 in water was added and incubated for 5mins at 55°C. Samples were vortexed and used for cholesterol estimation (Thermo Scientific) according to manufacturer's protocols.

Hepatic cholesterol estimation- 60mg of mouse liver tissue was homogenized with 3.35ml of methanol. 6.7ml of chloroform was added and vortexed. Homogenized tissue was filtered through sharkskin filter paper. Tubes were washed with 2:1 chloroform:methanol twice and filtered in the same tube through shark skin filter paper. 5ml of 0.043% $MgCl_2$ is added and centrifuged for 15mins at 1800rpm. Aspirated the upper layer including the protein on the interface and evaporated the remaining using nitrogen gas. Dried samples were resuspended in 1% triton-x 100 in chloroform, vortexed and transfered 0.5ml to a new glass tube. Added another 2 ml of 1% triton-x 100 in chloroform and made the final volume 2.5ml. Vortexed and dried the sample

again under nitrogen gas. Dried samples were resuspend in 2ml of 1.8% triton-x 100 in water. Vortexed and incubated for 15 mins at 65°C. Samples were aliquoted and used for cholesterol estimation (Thermo Scientific) according to manufacturer's protocols.

Hepatic triglyceride estimation- 60mg of mouse liver tissue was homogenized with 3.35ml of methanol. 6.7ml of chloroform was added and vortexed. Homogenized tissue was filtered through sharkskin filter paper. Tubes were washed with 2:1 chloroform:methanol twice and filtered in the same tube through shark skin filter paper. 5ml of 0.043% $MgCl_2$ is added and centrifuged for 15mins at 1800rpm. Aspirated the upper layer including the protein on the interface and evaporated the remaining using nitrogen gas. Dried samples were resuspended in 1% triton-x 100 in chloroform, vortexed and transferred 0.5ml to a new glass tube. Added another 2 ml of 1% triton-x 100 in chloroform and made the final volume 2.5ml. Vortexed and dried the sample again under nitrogen gas. Dried samples were resuspend in 2ml of 1.8% triton-x 100 in water. Vortexed and incubated for 15 mins at 65°C. Samples were aliquoted and used for submitted to the Atherosclerosis Research Unit core for triglyceride estimation.

Chapter 10

Conclusion and Future Directions

The results presented here 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 damage mediated atherosclerosis.

Free heme is a potent proinflammatory molecule that is sensed by innate immune receptors and causes inflammation. Heme-mediated synthesis of ROS is implicated in the pathophysiology of atherosclerosis and CAD. . Heme is an essential molecule for living aerobic organisms and is involved in a remarkable array of diverse biological processes and several diseases. As heme overload usually occurs in the bloodstream under pathologic conditions, circulating and endothelial cells are the first cell populations exposed to heme-mediated damage. Heme entraps into red blood cell membranes and shortens the erythrocyte life span, thus enhancing hemolysis (18). The lipophilic nature of heme allows it to intercalate into biologic membranes, perturbing lipid bilayers, promoting the conversion of low-density lipoprotein to cytotoxic oxidized products, and favoring iron to participate in Fenton reaction, a process during which ROS are produced (14, 15, 16, 17, 18, 19).

Heme binds with the highest known affinity ($K_d < \text{pM}$) to Hx in circulation. The concentration of Hx in plasma is 0.5–1.2 mg/ml. This can, however, increase during inflammatory events, because Hx is an acute-phase protein (53). Several studies demonstrated the potent antioxidant role of Hx. Heme binding to Hx has been demonstrated to reduce heme-mediated free radical production from organic peroxides (55). Furthermore, *in vitro* studies by Grinberg et al. (56) demonstrated that Hx strongly decreased the peroxidative and catalytic activity of heme by forming inactive heme–

protein complexes. Interestingly, heme activities were found to be inhibited by 80–90% with Hx. CD91, the receptor involved in the clearance of heme-Hx complex is expressed in most cell types, thus indicating multiple sites of heme-Hx complex recovery. The important function of Hx is heme scavenging at the systemic level, consisting of the transport of heme to the liver, where it is catabolized or used for the synthesis of hemoproteins or exported to bile canaliculi. Much experimental evidence also supports the antioxidant function of Hx *in vivo*. Hx-null mice have been demonstrated to be particularly sensitive to heme overload and more prone to heme-induced oxidative damage and inflammation during hemolytic processes. This is important both in physiologic heme management for heme-iron recycling and in pathologic conditions associated with intravascular hemolysis to prevent the pro-oxidant and proinflammatory effects of heme. Other than scavenging heme, the heme-Hx complex has been shown to be able to activate signaling pathways, thus promoting cell survival and modulate gene expression.

In our study we have shown that absence of Hx can increase heme and ROS levels in serum and HDL of Hx-lacking mice. In addition, heme catabolism is significantly reduced due to the lack of Hx. This is evident by reduced expression of important genes such CD91, HO-1 and HO-2 in HxE^{-/-} mice. In addition, levels of bilirubin are significantly lower in circulation confirming the reduced breakdown of heme (Chapter 3 and 4). Increased heme and ROS as known to be pro-oxidant and proinflammatory have similar effects in the HxE^{-/-} mice in this study. Due to the lack of Hx we have seen an increased level of lipid peroxidation in HxE^{-/-} mice, measure as higher levels of malondialdehyde. In addition, systemic inflammatory conditions have also made the

HDL dysfunctional and proinflammatory in the HxE^{-/-} mice, as seen by a higher monocyte chemotaxis value and lower LCAT activity (Chapter 4).

In order to establish the specific cytokines and chemokines involved in the increased monocyte migration we can check for the levels of cytokines and chemokines in the frozen supernatants from the experiment using ELISA. This will also aid us in identifying the pathways involved in the chemotaxis.

Atherosclerosis is a chronic and systemic vascular inflammatory process that forms the pathological basis of CAD (3). Atherogenic dyslipidemia is a major risk factor for the premature development of CAD. The imbalance between circulating levels of atherogenic lipoproteins (LDL, VLDL and intermediate density lipoprotein) relative to those of HDL is associated with induction of endothelial dysfunction, which is in turn intimately related to inflammation and oxidative stress (5); as a result, dyslipidemia, inflammation and oxidative stress are closely interrelated in the development of atherosclerosis. Preferential retention of LDL in the arterial wall via proteoglycan binding makes this lipoprotein a major substrate for oxidation by arterial wall cells (5, 7). Studies support that HDL is a modulator of systemic inflammation. HDL has the ability to protect against cellular accumulation of cholesterol, oxidative stress and inflammation in the arterial wall. In the absence of inflammation, HDL has a complement of antioxidant enzymes that work to maintain an anti-inflammatory state.

Monocyte recruitment to the intima after inflammatory activation expresses scavenger receptors that permit the uptake of modified LDL particles, such as ox-LDL. Ox-LDL has multiple proatherogenic properties, which include induction of cholesterol accumulation

in monocytes as well as potent proinflammatory, immunogenic, apoptotic and cytotoxic activities (5). Cholesterol loading of monocytes leads to the formation of foam cells, and ultimately leads to the mature lipid-laden macrophages of the plaque's core. HDL antioxidant activity is typically observed as inhibition of LDL oxidation (97). ApoA-I is a major component of the anti-oxidative activity of HDL and can prevent LDL oxidation, delay it, or both, by the removal of oxidized phospholipids from LDL, artery wall cells or both (97). In the presence of such systemic inflammation, the antioxidant enzymes on HDL can be inactivated and HDL can accumulate oxidized lipids and proteins that make it proinflammatory.

Most of the proinflammatory properties of ox-LDL arise from bioactive products of LDL lipid peroxidation. As a result, LDL oxidation further propagates the inflammatory process in the arterial wall, thereby accelerating atherosclerosis (5). Atherosclerosis can be regarded, therefore, as a chronic inflammatory disease of the arterial wall, mediated in part by ox-LDL and other proinflammatory agents, such as ROS. These cells can amplify local inflammation and promote thrombotic complications.

Absence of Hx in apoE^{-/-} mice leads to increased atherosclerosis. This was confirmed by increased lesion area in the aortic sinus of the heart and aorta. As described above, macrophage recruitment to the aortic endothelium is a hallmark of atherosclerosis; we confirmed increased macrophage infiltration in the aortic sinus of HxE^{-/-} mice by staining for foam cell marker, CD68. This further confirmed aggravated atherosclerosis in apoE^{-/-} mice due to the lack of Hx (Chapter 5).

Cholesterol efflux from peripheral tissue and macrophage function is important to prevent atherosclerosis. In apoE^{-/-} mice lacking Hx we have shown macrophages from the bone marrow have impaired cholesterol efflux capacity. Reduced LXR- α and ABCA1 expression support the functional reduction of cholesterol efflux from HxE^{-/-} BMDMs compared to apoE^{-/-} BMDMs. Furthermore, macrophages from HxE^{-/-} mice were M1 type of macrophages. This indicated they were more proinflammatory supporting the increased atherosclerosis phenotype (Chapter 6 and 7).

In order to further investigate the effect of Hx absence on the macrophage population we can characterize the macrophage population in the two groups of mice by studying in the detail the M1-M2 polarization. In addition, we can examine other macrophage-functional differences like phagocytosis which is an important step in the development and progression of atherosclerosis.

The cholesterol efflux assay can also be used to study the dysfunctional nature of HDL from HxE^{-/-} and apoE^{-/-} mice. We can use mouse macrophage cell line like RAW264.7 or J774.2 cells and use mouse test HDL to estimate the percent efflux after loading the macrophages with tritiated Ac-LDL-C.

Lastly, hHx administration confirmed the protective role of Hx towards scavenging free heme. In this study we showed that exogenous hHx can scavenge free heme and clear it from circulation thereby aiding the chelation of heme and mitigating its prooxidant effects (Chapter 9). In addition, we showed that exogenous hHx reduced levels of VLDL/LDL-C. It will be interesting to investigate the mechanism by which VLDL/LDL-C levels are reduced due to hHx administration in HxE^{-/-} mice.

Hence, we can conclude that Hx is important to prevent increased atherosclerosis in apoE^{-/-} mice, and can be used as a heme chelating agent to prevent oxidative damage in diseases mediated by heme and ROS.

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