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Objective—We investigated whether red cell infiltration of atheromatous lesions promotes the later stages of atherosclerosis.

Methods and Results—We find that oxidation of ferro (FeII) hemoglobin in ruptured advanced lesions occurs generating ferri (FeIII) hemoglobin and via more extensive oxidation ferrylhemoglobin (FeIII/FeIV=O). The protein oxidation marker dityrosine accumulates in complicated lesions, accompanied by the formation of cross-linked hemoglobin, a hallmark of ferrylhemoglobin. Exposure of normal red cells to lipids derived from atheromatous lesions causes hemolysis and oxidation of liberated hemoglobin. In the interactions between hemoglobin and atheroma lipids, hemoglobin and heme promote further lipid oxidation and subsequently endothelial reactions such as upregulation of heme oxygenase-1 and cytotoxicity to endothelium. Oxidative scission of heme leads to release of iron and a feed-forward process of iron-driven plaque lipid oxidation. The inhibition of heme release from globin by haptoglobin and sequestration of heme by hemopexin suppress hemoglobin-mediated oxidation of lipids of atheromatous lesions and attenuate endothelial cytotoxicity.

Conclusion—The interior of advanced atheromatous lesions is a prooxidant environment in which erythrocytes lyse, hemoglobin is oxidized to ferri- and ferrylhemoglobin, and released heme and iron promote further oxidation of lipids. These events amplify the endothelial cell cytotoxicity of plaque components. (*Arterioscler Thromb Vasc Biol*. 2010; 30:1347-1353.)

Key Words: atherosclerosis ■ hemoglobin ■ oxidized lipids ■ reactive oxygen species ■ heme ■ heme-oxygenase-1

Hematomas are formed either by fissures at the atherosclerotic lesion surface[1] or within the lesions as hemorrhages from neovasculature that sprout from the vasa vasorum.[2,3]

Oxysterols and oxidation products of polyunsaturated fatty acids are present in human atheromatous lesions.[4,5] Atherosclerotic lesions are hazardous regions for nucleated cells, both endothelial cells and, quite probably, incoming macrophages.[6] The major cytotoxic species may be oxidation products of lipids, particularly lipid hydroperoxides (LOOHs), aldehydes, and carbonyls.[6,7]

In artificial systems, oxidation of polyunsaturated fatty acids requires reactive transition metals such as iron and copper. Based on our earlier work,[6,8,9] the metal in atheromatous lesions might be iron derived from heme. Nonprotein-bound heme is a particularly deleterious species inasmuch as it is hydrophobic and easily able to enter cell membranes.[10]

In previous studies, we found that endothelial cells exposed to oxidized low-density lipoprotein (LDL) upregulated both heme oxygenase-1 (HO-1) and ferritin,[8,9] presumably as a defense mechanism.[6,11–14] Upregulation of HO-1[15] and ferritin H chain[16] in endothelial cells has been reported in the early phase of progression of atherosclerotic lesions. Expression of HO-1 provides protection against atherosclerosis in several experimental models,[17,18] and HO-1 deficiency in humans has been associated with the appearance of vasculature fatty streaks and atheromatous plaques at the age of 6.[19]

We tested the hypothesis that heme-iron may accumulate in atherosclerotic lesions by intrusion and lysis of erythrocytes. Liberated hemoglobin is oxidized, and released heme-iron–dependent oxidation of lipids is strongly favored, contributing to further plaque development.

Methods

Tissue Samples

Specimens of human atherosclerotic lesions were obtained from aorta or its primary branches of beating-heart donors for organ transplantation, approved by the Scientific and Research Ethic Committee of Scientific Council of Health of the Hungarian government. Tissue samples were washed with saline, dried, weighed, frozen in liquid nitrogen, and stored at −70°C until assay. Samples showing no macroscopic evidence of atherosclerosis were designated as controls. Samples of lesions exhibiting thickened intima and large

lipid cores with no sign of disruption represented atheromatous lesions. Disrupted plaques with hematomas were designated as complicated lesions. For histopathologic examination, tissues were fixed in 10% formalin and embedded in paraffin. Five-micrometer sections were deparaffined with xylol for 8 minutes and rehydrated in a descending series of isopropyl-alcohol. Hematoxylin/eosin staining was performed (hematoxylin for 6 minutes, followed by a wash in distilled water for 8 minutes, staining with eosin for 2 minutes, dehydrating, and mounting on a coverslip). Stained slides were scanned with a Mirax Midi scanner (3D Histech, Budapest, Hungary) for digital documentation.

Lipids of vessel samples were extracted from tissue by chloroform-methanol (2:1, vol/vol).[20] The organic phase was evaporated under N_2, and the weight of the lipid extract was measured. The extract was redissolved in a small volume of chloroform and suspended in Hanks' balanced salt solution to produce a suspension of 2 mg lipid/mL solvent. Lipid aggregates were dispersed by the evaporation of chloroform coupled with vigorous vortexing.

Erythrocyte Lysis by Plaque Material

Red cells were obtained from venous blood of a normal donor. The cells were incubated with extracts from vessels in a suspension containing 0.2 vol/vol% packed red cells and 1 mg/mL vessel lipid extracts in Hanks' balanced salt solution at 37°C. The amount of free hemoglobin was monitored spectrophotometrically for 72 hours[13] after centrifuging.

Glutathione/Glutathione Peroxidase Treatment

Equimolar amounts of glutathione were added to oxidized LDL or plaque lipids of known hydroperoxide content. Glutathione peroxidase was applied according to the manufacturer's instructions. Reduction of LOOHs took 1 hour for LDL and 16 hours for plaque lipids at 37°C.

Measurement of Dityrosine in Hemoglobin

Hemoglobin from minced complicated lesions was extracted by vortexing with saline. After centrifugation to remove insoluble material, the supernatant was precipitated and hydrolyzed. The residue was injected into a high-performance liquid chromatograph (Merck, Darmstadt, Germany) using a reverse-phase C18 column (4.6×150 mm, 5-mm particle size), and the eluate was monitored by fluorescence detection (excitation, 280 nm; emission, 410 nm). The mobile phase consisted of 20% methanol and 0.2% trifluoroacetic acid in water. Control dityrosine samples were prepared as described earlier.[21] Results are expressed as area under the curve.

Detection of Cross-Linked Hemoglobin by Western Blot

Hemoglobin (150 nmol) from complicated lesions was applied to 12.5% SDS-PAGE gel. Hemoglobin polymerization was detected using a chicken anti-human polyclonal hemoglobin antibody (ab17542, Abcam, Cambridge, United Kingdom).

Endothelial Cell Cytotoxicity Assay

Endothelial cell monolayers were exposed for 16 hours to lipid suspensions (2 mg/mL in Hanks' balanced salt solution) that were either untreated or treated with heme (5 μmol/L), heme and antioxidants (10 μmol/L butylated hydroxytoluene or 40 μmol/L α-tocopherol), or an iron chelator (100 μmol/L desferroxamine or the heme-binding protein hemopexin [5 μmol/L]). In additional experiments, the lipid suspension (2 mg/mL in Hanks' balanced salt solution) was treated with 10 μmol/L ferro- or ferrihemoglobin in the presence or absence of haptoglobin (20 μmol/L) or hemopexin (40 μmol/L) for 16 hours. Cell viability was determined by 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl-tetrazolium-bromide reduction.

Statistical Analysis

Statistical analysis was performed by the ANOVA test followed by the post hoc Tukey test for multiple comparisons. Significance is
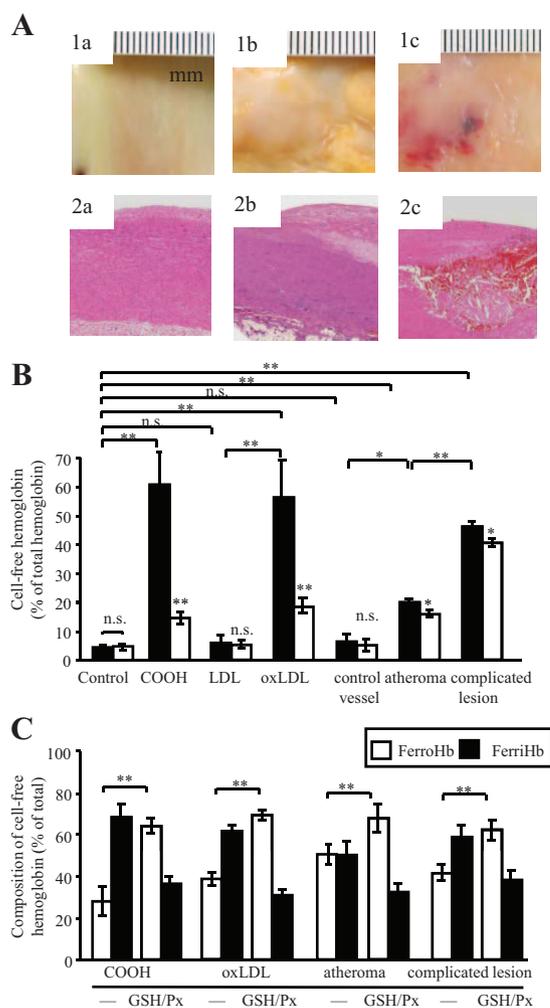

Figure 1. Hemolysis and hemoglobin oxidation are provoked by exposure to atheromatous lipid. A, Intact, normal arterial intima (control; 1a), atheromatous plaque showing irregular yellowish discoloration due to fibrofatty material (1b), and complicated plaque with a prominent hemorrhagic lesion (1c), representing a continuous spectrum of atheromatous changes of the aorta. Histopathology demonstrated no change in control (2a), extracellular intimal fat accumulation and cholesterol crystal formation in atheroma (2b), and red blood cell extravasation (hemorrhage) into the fatty plaque (2c). B, Cumene hydroperoxide (COOH; 50 μmol/L), LDL (250 μg/mL) oxidized by heme (1.25 μmol/L), and 1 mg/mL lipid from control (n=7), atheromatous (n=11), and complicated lesions (n=7) were added to red blood cell suspensions. Amounts of liberated hemoglobin were measured at 24 hours, with (empty bars) and without (filled bars) glutathione/glutathione peroxidase pretreatment (GSH/Px). n.s. indicates not significant. C, Composition of liberated hemoglobin (Hb) is expressed in percentage of total free hemoglobin.

indicated on the figures by 1 ($P<0.05$) or 2 ($P<0.01$) asterisks. Results are expressed as mean±standard deviation of at least 3 independent experiments.

Results

Hematomas occur in atheromatous lesions, and plaque material contains lipid oxidation products, including LOOH,[22] which not only can mediate the oxidation of hemoglobin but might also lyse intact red cells.[23] We therefore tested whether oxidized LDL and lipids from atheroma have hemolytic activity. Lipids of atheromatous (Figure 1A, 1b and 2b) and

ruptured complicated lesions (Figure 1A, 1c and 2c), as well as oxidized LDL, caused significant lysis of red cells within 24 hours (Figure 1B, filled bars). Moreover, the cell-free hemoglobin underwent oxidation (Figure 1C). Preincubation of lipid extract derived from atheroma, complicated lesion, or oxidized LDL with glutathione/glutathione peroxidase (which specifically reduced LOOH to alcohol by 35%, 38%, and 90%, respectively) significantly lowered the lytic effect (Figure 1B, empty bars). Oxidation of liberated hemoglobin was also reduced (Figure 1C, empty bars). These observations support the notion that red cells infiltrating an atherosclerotic lesion will undergo hemolysis and release free hemoglobin that is subsequently oxidized.

We reported earlier that both ferrihemoglobin and heme can mediate oxidative modification of LDL.[9] In contrast, ferrohemoglobin—in which the heme group is bound tightly—cannot initiate oxidation of LDL.[24] Therefore, we incubated lipids from atheromatous lesions with ferro- and ferrihemoglobin. Surprisingly, both ferro- and ferrihemoglobin induced the oxidative modification of atheromatous lesion lipids, as indicated by LOOH formation (Figure 2A). Because oxidatively modified LDL was shown to oxidize ferrohemoglobin[25] and the degree of oxidation strongly depended on the concentration of LDL-associated LOOHs (Figure 2B), we assessed whether LOOHs in the lipid extracts of atheromatous lesion might also oxidize ferrohemoglobin. Indeed, lipids derived from atheromatous lesions promoted the oxidation of ferrohemoglobin to ferrihemoglobin (Figure 2C). Treatment of oxidized LDL (Figure 2B) or atheroma lipids (Figure 2D) with glutathione/glutathione peroxidase lowered the LOOH content and inhibited the oxidation of hemoglobin.

As is true of intact hemoglobin, lipid extracts from atheromatous lesions exposed to heme also underwent lipid peroxidation as reflected by the accumulation of thiobarbituric acid-reactive substances (TBARs) and LOOHs (Supplemental Figure I, available online at http://atvb.ahajournals.org). The results suggest that hemoglobin-derived heme can promote oxidation within atheromatous lesions and that such oxidation requires oxidant species present in atheromatous plaque but not in normal vasculature.

Heme/iron-mediated oxidative modification of LDL can cause endothelial cytotoxicity[8,24] and—at sublethal doses—the expression of stress-response genes.[9,11–14] Therefore, we tested whether the same effect was observed when endothelial cells were exposed to lipids isolated from atheromatous lesions preexposed to heme or not (Figure 3A). As shown in Figure 3B, lipids from atherosclerotic lesions were cytotoxic to endothelium, an effect strikingly enhanced when lipids were preoxidized by exposure to heme. Equal amounts of lipid isolated from control blood vessels were not cytotoxic. At sublethal doses, atheroma lipid—whether pretreated with heme or not—induced the expression of the stress-responsive gene HO-1, at both mRNA (Figure 3C) and protein (Figure 3D) levels. In contrast, lipids from control blood vessels failed to affect the expression of HO-1 in endothelium. Pretreatment of heme-oxidized lipids with glutathione/glutathione peroxidase reduced the LOOH content (113 ± 30 versus 74 ± 22 nmol LOOH/mg extract, $P<0.01$) and inhibited the endothelial cytotoxicity by 25% ($P<0.05$). Furthermore,
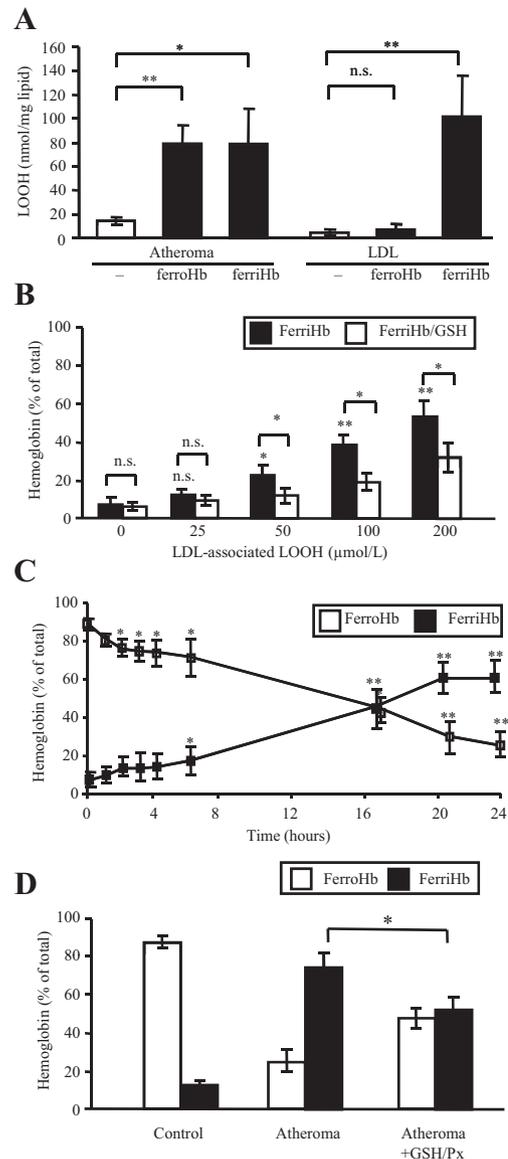

Figure 2. LOOHs of atheroma lipids convert ferrohemoglobin to ferrihemoglobin. A, 200 μg/mL native LDL and 1 mg/mL lipid suspension from atheromatous lesions (n=16) were incubated with 10 μmol/L ferrohemoglobin (ferroHb) or ferrihemoglobin (ferriHb), and LOOH content was measured. B, LDL samples of different LOOH content were incubated with 10 μmol/L ferrohemoglobin for 90 minutes. Ferrihemoglobin ratios with and without (empty and filled bars) glutathione/glutathione peroxidase pretreatment (GSH/Px) are expressed as a percentage of total hemoglobin. C, Lipids (1 mg/mL) from atheromatous lesion were incubated with 10 μmol/L of ferrohemoglobin. Changes in ferrohemoglobin (□) and ferrihemoglobin (■) levels were monitored for 24 hours. D, Effect of glutathione/glutathione peroxidase pretreatment on the reaction of 1 mg/mL lipid from atheromatous lesion with 10 μmol/L of ferrohemoglobin at 24 hours.

the induction of HO-1 was decreased (153 ± 16 versus 105 ± 3 pmol of bilirubin formed per milligram of cell protein per 60 minutes, $P<0.01$) in endothelial cells.

In an attempt to explain the different effect of heme on lipid extracts of atheromatous lesions versus control blood vessels, we measured the amounts of lipid peroxidation products in control and atheromatous samples. Levels of conjugated dienes, LOOHs, and TBARs were significantly
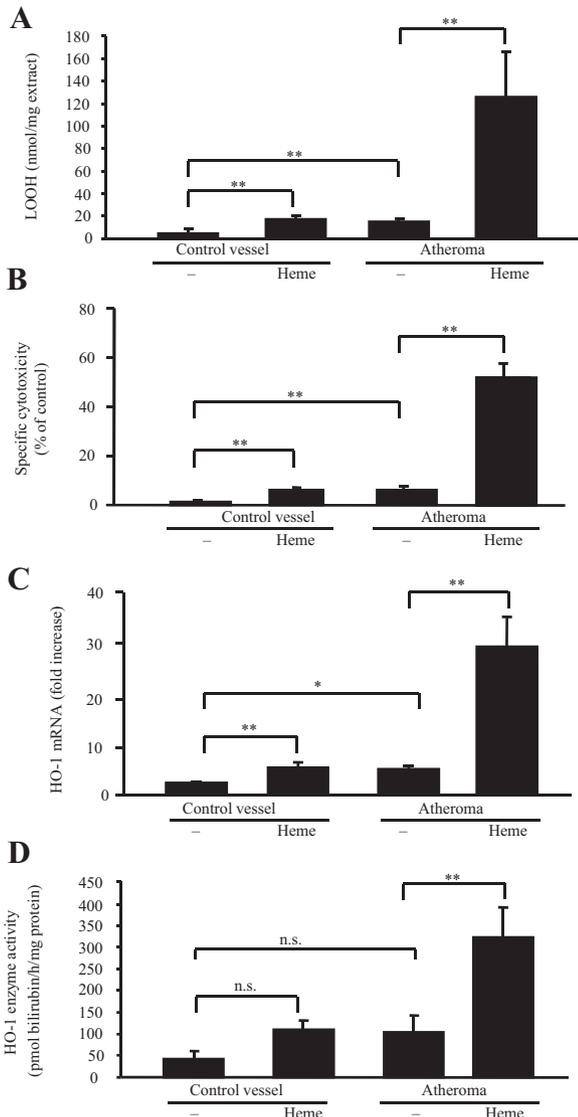
Figure 3. Heme with atheroma lipids augments lipid peroxidation and subsequent endothelial cell reactions. Lipids from atheromatous lesions (n=9) or controls (n=9) were treated with heme. LOOH content (A), specific cytotoxicity (B), HO-1 mRNA expression (C), and HO-1 enzyme activity (D) were measured. n.s. indicates not significant.
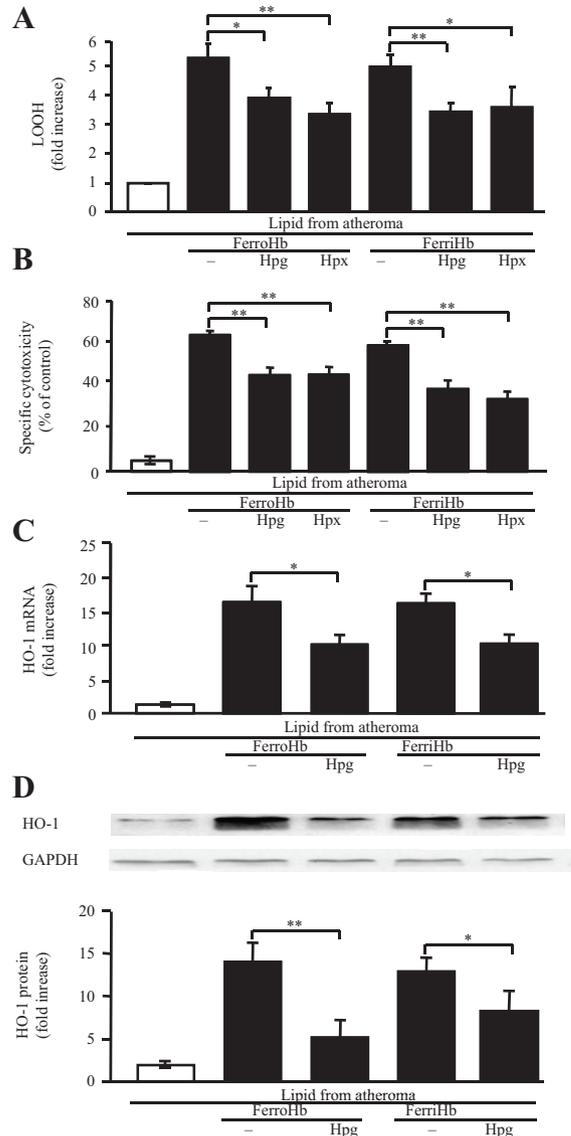

Figure 4. Oxidation of atheroma lipids by hemoglobin is inhibited by haptoglobin or hemopexin. Atheroma lipids (n=16) were incubated with ferrohemoglobin (ferroHb) or ferrihemoglobin (ferriHb) in the presence or absence of haptoglobin (Hpg) (n=5) or hemopexin (Hpx) (n=3) for 16 hours. LOOH content (A), specific cytotoxicity (B), HO-1 mRNA (C), and protein expression (D) were measured.

higher in atheromatous lesions compared with controls (Supplemental Table I). Elevated cholesterol, oxycholesterol, lysophospholipid, and decreased phosphatidylserine were found in atheromatous lipids compared with controls (Supplemental Table II). Moreover, lipids extracted from atheromatous lesions contained 1.9 times as much as fatty acids than control extracts. Significantly lower amounts of polyunsaturated fatty acid were found, likely reflecting enhanced oxidation of polyunsaturated fatty acid within the lesions.

The chemical changes exerted by heme on lipids isolated from atheromatous lesions were attenuated by antioxidants such as butylated hydroxytoluene, α-tocopherol, the iron chelator deferoxamine, and the heme binding protein hemopexin[26] (Supplemental Figure IIA). Because haptoglobin stabilizes the binding of heme to globin and inhibits heme release from hemoglobin,[27] we exposed lipid derived from

atheromatous lesions to hemoglobin in the presence of haptoglobin. Hemoglobin-mediated oxidative modification of lipid extracted from atheromatous lesions was inhibited by haptoglobin (Figure 4A). Moreover, the heme-binding protein hemopexin also suppressed the oxidation of lipid by ferro- and ferrihemoglobin, indicating the necessity for heme release from ferrihemoglobin for this oxidative process. Inhibition of lipid oxidation by either haptoglobin or hemopexin reduced the cytotoxicity (Figure 4B) and HO-1 induction caused by sublethal amounts of pretreated atheromatous lesion lipids (Figure 4C and 4D).

Because ruptured complicated lesions contain hemoglobin and desferroxamine prevented lipid oxidation, we next measured levels of iron and lipid peroxidation products in extracts of these lesions. Concentrations of iron, conjugated dienes, and LOOHs
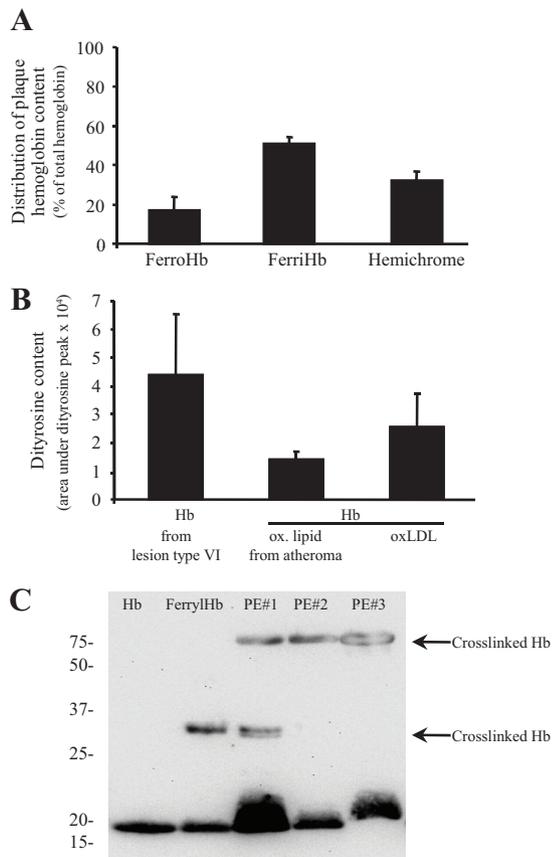
Figure 5. Hemoglobin (Hb) derived from complicated lesions is oxidized. A, Hemoglobin composition was determined. B, Dityrosine content of oxidized hemoglobin was measured by high-performance liquid chromatography. Oxidation of hemoglobin was induced by heme pretreated lipid from atheromatous lesion (n=3) or oxidized LDL. C, Hemoglobin polymers were detected in H_2O_2-treated hemoglobin and in hemoglobin obtained from complicated lesions (PE#1, PE#2, PE#3).

were elevated by ≈2-fold in ruptured complicated lesions, as compared with atheromatous lesions (0.433±0.075 versus 0.185±0.096 nmol Fe/mg tissue; 0.047±0.019 versus 0.021± 0.003 A_{234} conjugated dienes/mg tissue and 0.465±0.110 versus 0.248±0.106 nmol LOOH/mg tissue, respectively), and complicated lesions contained 5.6 times more TBARs than atheromatous lesions (0.028±0.012 versus 0.005±0.001 nmol/mg tissue). Importantly, hemoglobin derived from ruptured complicated lesions was mainly oxidized (Figure 5A). Moreover, the amounts of a protein oxidation marker, dityrosine,[21] were elevated in complicated lesions (Figure 5B), whereas atheromatous lesions did not contain detectable dityrosine. Supporting the assumption that hemoglobin was oxidized by LOOHs, we found that dityrosine also forms in hemoglobin exposed to oxidized LDL and oxidized atheroma lipids, whereas hemoglobin oxidized by $K_3Fe(CN)_6$ did not contain this marker (not shown). Accumulation of dityrosine was accompanied by the formation of cross-linked hemoglobin (Figure 5C), a hallmark of precedent formation of ferrylhemoglobin.

Discussion

Atheromatous lesions are prone to disruption, leading to hematoma or hemorrhage. Erythrocytes can also enter devel-

oping atherosclerotic lesions through the porous neovasculature in the vasa vasorum underlying atherosclerotic plaque.[1–3] The results reported here indicate that once exposed to oxidized plaque material, erythrocytes are lysed, the liberated hemoglobin is oxidized, and heme dissociates from the resultant ferrihemoglobin. The free heme can be oxidatively cleaved by oxidized plaque components through reaction with LOOHs. This engenders the release of iron, which can further promote the oxidation of plaque lipids through redox cycling reactions. The result of these chemical reactions is the formation of deleterious oxidized "gruel" that, among other things, leads to endothelial oxidative stress and ultimately to cytotoxicity.

These oxidation reactions involve initial interactions between the excess lipid in the vessel wall (mainly derived from LDL) and heme and heme-derived iron. Previously we have shown that heme can enter the lipid moiety of LDL and induce iron-dependent lipid peroxidation.[8] Here we demonstrate that lipids isolated from human atheromatous lesions—which are already in an oxidized state—can be further oxidized in the presence of heme, whereas this effect is not observed using lipids isolated from normal vasculature. Heme treatment of the atheroma and its extracted lipid increased the amount of conjugated dienes, LOOHs, and TBARs. Kinetics of the reaction between heme and atheroma lipid in the presence or absence of antioxidants and an iron chelator were similar to the reactions between LDL and heme, suggesting that the mechanisms of the 2 reactions are similar. The heme-binding protein hemopexin, which likely prevents heme:lipid interactions and blocks the oxidative scission of heme,[26] significantly inhibited the oxidative reactions. This serum protein, present at remarkably high concentrations in plasma (≈1 g/L), binds heme with extraordinary avidity (dissociation constant [K_d] less than 1 pmol/L) and promotes its clearance.

Importantly, atheroma lipid, whether exposed to heme or not, is cytotoxic, whereas lipids isolated from control vessels do not exert toxicity toward endothelial cells. We have found that atheroma lipids, when oxidized by heme, are highly cytotoxic to human endothelial cells, and hemopexin reduced this cytotoxicity. The inhibition provided by desferroxamine supports the idea that as heme-mediated oxidation of plaque material proceeds, there is a concomitant increase in free iron from the heme molecule.

Because hemoglobin, when oxidized, releases its heme,[28] we asked whether ferrihemoglobin might also modify the lipids of artery walls. After a coincubation of ferrihemoglobin and atheroma lipid, peroxidation products were formed to an extent similar to that of heme treatment. Both the hemoglobin-binding protein haptoglobin[27] and the heme-binding protein hemopexin inhibited such oxidative modification of lipids, indicating the importance of heme loss and scission in hemoglobin-provoked oxidation of lipids derived from atheromatous lesions. Ferrohemoglobin also exerted the same effect on plaque extracts. Thus, it appears that these extracts oxidize ferrohemoglobin to ferrihemoglobin, thereby leading to heme instability and heme-mediated initiation of lipid peroxidation.
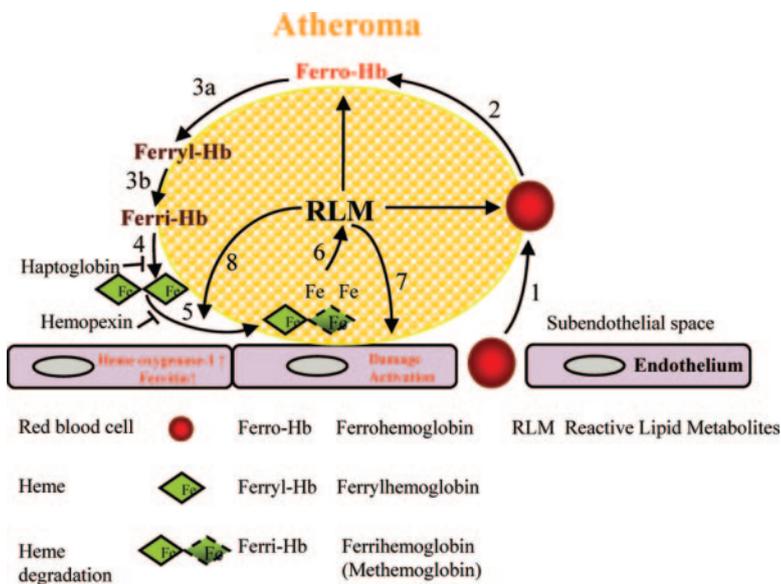
Figure 6. A model for red cell-mediated progression of atherogenesis. 1, Infiltration by red blood cells of the atheromatous lesion. 2, Erythrocyte lysis and liberation of ferrohemoglobin (Ferro-Hb) by lipids of atheroma. 3, Oxidation of ferrohemoglobin to ferrylhemoglobin (Ferryl-Hb) (a) and to ferrihemoglobin (Ferri-Hb) (b). 4, Release of heme from ferrihemoglobin. 5, Heme uptake by atheroma lipid. 6, Amplification of lipid oxidation in atheroma. 7, Damage and activation of endothelium induced by reactive lipid metabolites of atheroma. 8, Induction of HO-1 and ferritin by atheroma lipid and heme.

HO-1, a key antioxidant enzyme that exerts cytoprotective effects in endothelial cells,[8,11–14] also plays an important role in preventing the development of atherosclerosis. Elevated amounts of HO-1 were found in macrophages and medial smooth muscle cells of human atherosclerotic lesions.[15] The central importance of HO-1 in atherosclerosis is highlighted by the case of a HO-1-deficient boy who experienced severe atherosclerosis.[19]

Earlier, we found that HO-1 induction occurs when endothelial cells are treated with sublethal amounts of LDL oxidized by ferrihemoglobin-derived heme.[24] Now, we demonstrate that heme and hemoglobin-treated atheroma lipids also induce HO-1 in endothelial cells exposed in sublethal doses. The degree of HO-1 induction is partially a function of LOOH levels of the lipid as inhibition of lipid oxidation moderates its HO-1 inducibility. Supporting this, 13-HPODE, a hydroperoxide derivative of linoleic acid, acts as a transcriptional factor for HO-1 via a regulatory element in the promoter region of the human HO-1 gene.[29]

Supporting the relevance of our model of plaque development, we examined complicated lesions containing hematomas. Lipids of these plaques were highly oxidized, as reflected by increased amounts of conjugated dienes, LOOHs, and TBARs. Earlier chemical investigations of gruel from advanced lesions revealed that it contains ceroid-like insoluble material composed mainly of hydroxyapatite, iron, and calcium. These materials are cytotoxic to macrophages, and this was ameliorated by chelating iron and calcium.[6] Gruel from advanced plaque also contains large amounts of organic soluble carbonyls and aldehydes that are also cytotoxic.

Hemoglobin derived from hematomas in complicated lesions was mainly present in the oxidized form (ferrihemoglobin). Under inflammatory conditions, ferrihemoglobin is formed in erythrocytes exposed to activated polymorphonuclear cells.[30] Cell-free hemoglobin is readily oxidized to ferrihemoglobin by oxidants such as H_2O_2, produced by activated polymorphonuclear cells or monocyte/macrophages. Hemoglobin can also be oxidized by reacting with LOOHs.[25] In addition to ferrihemoglobin, these reactions

generate ferrylhemoglobin,[31] an unstable oxidized form of hemoglobin detected in humans under physiological[32] and pathophysiologic conditions.[33] This highly unstable oxidized form of hemoglobin (ferryl state, FeIII/IV=O) rapidly returns to the ferric (FeIII) state through protein electron transfer in which the α-chain tyrosine 42 acts as a redox center, cycling between the tyrosine and the tyrosyl radical while delivering electrons to ferryl heme.[10,34,35] Tyrosyl radicals can react with each other to generate dityrosine, leading to inter- and intramolecular cross-linking and formation of hemoglobin multimers. We recently found that, unlike other forms of oxidized hemoglobin, ferrylhemoglobin acts as a potent proinflammatory agonist in endothelial cells, leading to the upregulation of adhesion molecules that support the recruitment of macrophages into the vessel wall.[36] Detection of dityrosine and cross-linked hemoglobin in complicated plaques suggests that LOOH-mediated oxidation of hemoglobin occurs within the lesion. Further evidence for this mechanism is the finding that oxidation of hemoglobin with either LDL or atheroma lipid with elevated LOOH levels also led to the formation of dityrosine residues in the globin moiety of hemoglobin.

Overall, our results support the concept that erythrocytes invading atheromatous lesions are lysed by lipid oxidation products within the lesions. Ferrohemoglobin released by this event is converted to ferryl- and ferrihemoglobin by the same oxidized materials. This, in turn, destabilizes the heme group, promoting its release from the globin, and the free hydrophobic heme group readily enters atheroma lipid. Oxidative scission of the heme group leads to iron release and a feed-forward process of further plaque lipid oxidation. The inhibition of heme release from hemoglobin by haptoglobin and sequestration of heme by hemopexin suppress hemoglobin-mediated oxidation of lipids of atheromatous lesions and attenuate subsequent endothelial cell damage (Figure 6). These events likely amplify the oxidation of plaque components that are cytotoxic for endothelial cells and, quite likely, infiltrating phagocytic cells, which would otherwise help resolve these lesions.

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Disclosures

None.

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SUPPLEMENTAL METHODS

Chemicals

All reagents were purchased from Sigma (St Louis, MO) unless otherwise specified.

Cell culture

Primary human umbilical vein endothelial cells (HUVECs) were cultured as previously described.[1,2]

Hemoglobin and hemopexin preparation

Purified ferrohemoglobin was prepared from lysed fresh human red cells using ion-exchange chromatography.[3] Human hemopexin was isolated following procedures described by Vretblad and Hjorth.[4]

Preparation and oxidation of low density lipoprotein

LDL was isolated from plasma derived from EDTA-anticoagulated venous blood of healthy volunteers by gradient ultracentrifugation (Beckman Instruments, Palo Alto CA).[5] Oxidation of LDL was carried out by the addition of heme (5 µmol/L) and H_2O_2 (75 µmol/L).[1]

Determination of neutral lipids and fatty acid.

Lipid extracts were redissolved in chloroform and spotted onto a Silica gel G (Merck, Darmstadt, Germany) plate. Neutral lipids were determined by thin layer chromatography as described.[6] Quantification was performed using QuantiScan software (Biosoft, Cambridge, UK). Neutral lipids and phospholipids were identified and quantitated using analytical grade standards (Sigma). For fatty acid analyses, the lipid extracts were hydrolyzed and methylated.[7] Fatty acid methylesters were injected into a Hewlett Packard 5890 gas

chromatograph coupled to a Hewlett Packard 5970 mass spectrometer (Palo Alto, CA, USA). Results are expressed as mean ± SD of mol% from three independent experiments.

Measurement of lipid peroxidation parameters, heme and iron in blood vessel samples

Conjugated diene content, total lipid hydroperoxides, thiobarbituric acid-reactive substances (TBARs), iron and heme were determined spectrophotometrically as described earlier.[1]

Lymphoblastoid cell culture and cytotoxicity assay

Human HO-1 deficient and control immortalized lymphoblastoid cell lines (LCLs) were generous gifts from Akihiro Yachie (Kanazawa University, Japan). LCLs were cultured in RPMI 1640 medium containing 15% FBS.

Lymphoblastoid cell suspensions were pretreated with heme (5 µmol/L, 60 min), then exposed to lipid suspensions (2 mg/mL in HBSS) for 16 hours. Following this, the cell suspension was centrifuged (400 x g, 10 min), the supernatant was removed and 0.5 mg/mL MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl-tetrazolium-bromide) in HBSS was added in order to assess cell viability. After an additional 6 hours, the MTT solution was removed, dimethyl-sulfoxide (DMSO) was added and absorbance at 570 nm was measured.

Real time RT-PCR to determine HO-1 mRNA

Confluent endothelial cells grown in 6-well plates were exposed to lipid suspensions (1 mg/mL) for 1 hour. Total RNA was isolated from cells using the RNAzol STAT-60 (TEL-TEST Inc., Friendswood, TX). HO-1 and cyclophilin (housekeeping gene) levels were measured by real time PCR using fluorescent TaqMan probes following reverse transcription.[8]

Western blot for HO-1 protein

Confluent endothelial cells grown in 6-well plates were exposed to pretreated lipid suspensions described above for 1 h. HO-1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein amounts were determined.[9]

HO enzyme activity assay

HO activity in endothelial cell microsomes was measured by bilirubin formation after treating the cells grown in Petri dishes as above.[2]

SUPPLEMENTAL RESULTS

Lipid extracts from atheromatous lesions exposed to heme also underwent lipid peroxidation as reflected by the accumulation of TBARs and lipid hydroperoxides. Concentrations of TBARs and lipid hydroperoxides reached a maximum at 12 hours and remained high for 24 hours (supplemental Fig I A and B). In the course of lipid peroxidation, the heme absorbance decreased (supplemental Fig I C) accompanied by an increase of 'free' iron of 0.62 ± 0.27 nmol/mg indicating (probably oxidative) scission of the porphyrin ring. In contrast, no significant increases in either TBARs or lipid hydroperoxides occured in lipid extracts from control blood vessels and heme was not destroyed.

The chemical changes exerted by heme on lipids isolated from atheromatous lesions were attenuated by antioxidants such as BHT, α-tocopherol, the iron chelator deferoxamine, and the heme binding protein, hemopexin. Inhibition of heme-induced lipid oxidation (supplemental Fig II A) reduced both cytotoxicity (supplemental Fig II B) and induction of HO-1 expression at the mRNA and protein levels (supplemental Fig II C and D).

We have previously shown that HO-1 has a crucial role in preventing human lymphoblastoid cells from oxidized LDL mediated cytotoxicity.[8] Therefore, we tested whether HO-1 deficient cells might be more sensitive to heme-oxidized atheromatous lesion lipid as well. We have compared the cytotoxic effect of oxidized lipid on a lymphoblastoid cell line derived from a HO-1 deficient child and from control individuals. Indeed, heme-treated lipid from atheroma was more cytotoxic to the HO-1 deficient cells than to the control lymphoblast cells at all examined concentrations (supplemental Fig III).

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SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure I.

Heme induces peroxidation of lipid extract derived from atheromatous lesions; in parallel heme degradation occures. Lipid extracts (1 mg/ml) derived from atheromatous lesion and control were reacted with heme (5 µmol/L) at 37 ^{o}C. TBARs **(A)** and LOOH **(B)** contents of lipids and heme **(C)** for atheromatous (●) and control (○) lesions were measured. Figure is a representative of five separate experiments.

Supplemental Figure II.

Oxidation of lipids of atheromatous lesions and subsequent endothelial reactions are inhibited by antioxidants and by hemopexin. Lipids from atheromatous lesions (n=11), were treated with heme alone or in the presence of BHT, α-tocopherol, DFO or hemopexin (n=5) for 16 hours. Lipid hydroperoxide **(A)**, cytotoxicity **(B)**, HO-1 mRNA **(C)** and protein **(D)** in endothelial cells were measured.

Supplemental Figure III.

HO-1 deficient cells are more sensitive to oxidative damage caused than control cells. Lymphoblastoid cells of a HO-1 deficient patient and control were treated with oxidized atheroma lipids and cytotoxicity was measured.
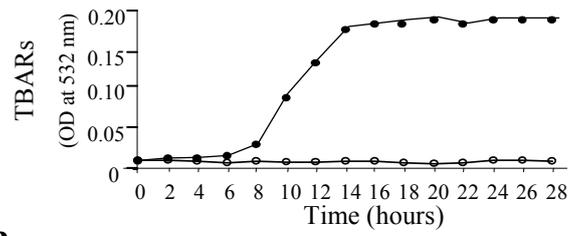
SUPPLEMENTAL TABLES

Table I. Chemical analysis of parameters indicative of oxidative stress in vessel wall samples. Products of lipid peroxidation, iron and hemoglobin contents of controls and atheromatous lesions were measured.

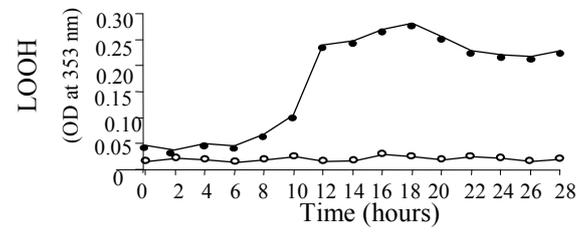|	Control (n=20)	Atheroma (n=33)	Significance (p<0.05)
Conjugated dienes (A_{234}/mg tissue)	0.006 (±0.002)	0.021 (±0.003)	0.002
LOOH (nmol/mg tissue)	0.003 (±0.003)	0.248 (±0.106)	0.016
TBARs (nmol/mg tissue)	0.002 (±0.001)	0.005 (±0.001)	0.021
Iron (nmol/mg tissue)	0.059 (±0.027)	0.185 (±0.096)	0.048
Hemoglobin(nmol/mg tissue)	not detectable	not detectable	n.s.

Table II. Lipid and fatty acid composition of lipid extracts derived from control and atheromatous lesions. Amounts of neutral lipids, phospholipids, and fatty acids (saturated fatty acids – SFA, monounsaturated fatty acids – MUFA, polyunsaturated fatty acids - PUFA) were measured.

		Control (n=20)	Atheroma (n=33)	Significance (p<0.05)
Lipid composition (mol%)	Cholesterol	16.9±4.3	42.6±11.2	0.01
	Oxy-sterol	2.05±0.5	5.14±1.2	0.04
	Diacylglycerol	0	1.17±0.2	0.04
	Triacylglycerol	54.4±12.5	35.8±18.2	n.s.
	Free fatty acids	17.3±5.4	8.06±4.5	n.s.
	Lyso-phospholipid	2.61±0.6	4.42±0.8	0.03
	Phosphatidylserine	6.61±1.3	2.85±2.2	0.04
Fatty acid composition (mol%)	C14:0	0	0.63±0.6	0.03
	C16:0	28.7±5.6	27.1±8.2	n.s.
	C18:0	10.98±4.3	10.1±7.6	n.s.
	C16:1	0	2.56±0.92	0.04
	C18:1	18.31±5.2	28.84±8.3	0.02
	C20:1	0.1±0.2	3.91±1.1	0.04
	C18:2	39.58±8.9	21.9±7.6	0.02
	C20:3	0.78±0.5	1.54±1.2	n.s.
	C20:4	1.54±0.9	1.21±1.1	n.s.
	Total SFA	39.68	37.83	n.s.
	Total MUFA	18.41	35.31	0.02
	Total PUFA	41.9	24.65	0.02

A

B

C

A

B

C

D