Hemin binding and oxidation of lipoproteins in serum: mechanisms and effect on the interaction of LDL with human macrophages[1]

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Abstract Most models of lipoprotein oxidation by free radicals have excluded macromolecular plasma components from the system. This limits their biological significance because oxidation of lipoproteins appears to occur in the intima in the presence of a plasma ultrafiltrate. Hemin, a product of in vivo hemoglobin degradation, binds and oxidizes purified lipoproteins. However, it is not known whether this occurs in the presence of plasma components that may sequester hemin. We found that hemin in serum diluted to protein levels of the extracellular fluid (10–30%) binds to low and high density lipoproteins (LDL, HDL) with association constants in the nmol/L range. In the presence of H_2O_2, hemin oxidizes both lipoproteins in diluted serum with formation of conjugated dienes, thiobarbituric acid reacting substances, and F_2-isoprostanes. This appeared to be caused by the high affinity of hemin with LDL and by the Fe^{3+} liberated that remains associated with the particles after hemin is degraded. Spectrophotometric and fluorescence experiments and electrophoresis of porphyrins complex with LDL indicated that the heme ring is buried in the lipoprotein surface-monolayer with the carboxylic groups in contact with positive regions of the protein and the solvent. Human macrophages associated and degraded 3- to 4-times more hemin-oxidized LDL in diluted serum than native LDL. It is possible then that at sites of LDL accumulation in the extracellular intima, hemin and H_2O_2 production could cause oxidation with potential atherogenic consequences for cellular lipoprotein processing. This may occur even when other macromolecules of the extracellular fluid are present.**—Camejo, G., C. Halberg, A. Menschik-Lundin, E. Hurt-Camejo, B. Rosengren, H. Olsson, G. I. Hansson, G-B. Forsberg, and B. Ylhen.** Hemin binding and oxidation of lipoproteins in serum: mechanisms and effect on the interaction of LDL with human macrophages. *J. Lipid Res.* 1998. **39:** 755–766.

Supplementary key words lipoprotein oxidation • hemin binding • serum components • conjugated dienes • macrophages • lipoprotein degradation

Free radical-mediated oxidation of unsaturated fatty acids, cholesterol, and proteins in lipoproteins may contribute to atherogenesis (1). Transition metals, free radical-generating synthetic molecules, lipoxygenases, and myeloperoxidase oxidize lipoproteins and these models have been extensively used to study such reactions and their effects on cells and tissues (2–4). However, no definitive agent has been identified as a biologically relevant pro-oxidant of lipoproteins (1–3). A current hypothesis is that oxidation of apoB-100-containing lipoproteins occurs through several pathways in the arterial intima after they are trapped by their specific association with extracellular proteoglycans (5–8). The amphipatic hemin is a protoporphyrin IX with a coordinated Fe^{3+}, formed in the in vivo breakdown of heme (protoporphyrin IX-Fe^{2+}), that is usually bound to hemoglobin and myoglobin. Hemoglobin and heme continuously leak from red blood cells in plasma and tissues in what is known as trivial hemolysis (9). Direct oxidation of LDL by hemoglobin from red blood cells in the presence of albumin has been documented (10). This oxidation results in apoB-100 cross-linking and TBARS formation (11, 12). The presence of H_2O_2 or other peroxides greatly acceler-

Abbreviations: apoB lipoproteins, lipoproteins containing apoB-100; LDL, low density lipoproteins; HDL, high density lipoproteins; VLDL, very low density lipoproteins; HSA, human serum albumin; PBS, phosphate-buffered saline with Ca^{2+} and Mg^{2+}; BHT, butylated hydroxytoluene; Na-EDTA, sodium salt of ethylenediamine-tetraacetic acid; PMSF, phenylmethylsulfonylfluoride; TBARS, thiobarbituric acid reacting substances; HMDM, human monocyte-derived macrophages.

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ates hemin oxidation of LDL and the resulting modified particles are cytotoxic (13, 14). Recently, with the use of surface- and core-located oxidizing fluorescent probes, it was shown that hemin changes the charge of purified LDL and oxidizes its core lipids more efficiently than free Fe^{3+} ions. This caused an increase in the LDL uptake by macrophages (15, 16).

The environment where oxidation of apoB-lipoproteins appears to occur, the extracellular intima, is bathed in what can be considered a dilute ultrafiltrate of blood plasma (17, 18). In developing lesions there is a progressive accumulation of apoB-lipoproteins in the extracellular compartment, probably retaining other macromolecular plasma components at a constant level (19, 20). In the intima, at albumin concentrations around 100 μmol/L, it is unlikely that enough free Cu^{2+} could be available to bind to trapped apoB-100 lipoproteins and to oxidize them (17, 21, 22). A similar situation exists for Fe^{3+} that will be bound mostly by transferrin instead of albumin and lipoproteins. Ceruloplasmin-bound Cu^{2+} can oxidize LDL in vitro; however, this is also efficiently inhibited by albumin (23). Most studies concerning in vitro oxidation of lipoproteins with transition metals, free radical generating systems, and cells in culture have been carried out in the absence of plasma macromolecular components or at very low concentrations. These conditions may limit the relevance of such models to the in vivo situation.

In normal situations the body has efficient systems to remove hemoglobin and heme produced intravascularly. Haptoglobin removes hemoglobin components to the reticuloendothelial system and hemopectin binds hemin that is then transported to the liver. However, under conditions that increase erythrocyte fragility, at sites of turbulent blood flow in arteries or at locations of intramural microhemorrages, this scavenging system may be saturated (9, 13, 14). Heme in neutral solutions and in the presence of oxygen is rapidly converted to hemin (24). Excess hemin in plasma is bound to hemopexin and albumin and this limits its oxidizing capacity (25). However, hemin and hemoporphyrins, because of their amphipathicity, have a high affinity with lipoproteins and cell membranes. Therefore, their partition between hemopexin, albumin, and lipoproteins should depend on the relative concentrations of the binding structures and its affinity with them (26). Hemoporphyrins, probably including hemin, are not totally removed from tissues to liver by hemopexin and albumin because they are found concentrated extracellularly in lipid deposits of atheromas and in macrophages. They are carried there possibly by lipoproteins (27, 28). Tissues can also eliminate hemin by opening of the porphyrin ring catalyzed by hemin oxidase, an enzyme that is up-regulated during oxidative stress (29). Recently a preliminary report showed that overexpression of hemin oxidase in LDL receptor-deficient mice reduced atherosclerotic lesions, thus implicating hemin in the process (30). We report here on binding mechanisms of hemin to lipoproteins and on its capacity to oxidize LDL and HDL lipoproteins in diluted serum. This is a model that may be close to the situation in extracellular intima. To evaluate whether the effect of hemin oxidation of LDL in diluted serum has biological consequences, we measured the lipoprotein uptake and degradation by human macrophages.

METHODS

Chemicals

Hemin and protoporphyrin IX were from Porphyrin Products (Logan, UT). Stock solutions of these substances were prepared the same day that they were used in 0.02 N NaOH. Solutions of H_2O_2, HSA (fatty acid free), BHT, probucol, EDTA, deuterium oxide, HEPES buffer, and cell culture grade PBS were purchased from Sigma Sweden AB. SeaKem agarose was from FMC Bioproducts (Rockland, ME). NaOH, KBr, and NaCl were suprapure grade from Merck (Darmstad, Germany). The kit for measurement of non-heme iron was purchased from Hoffmann-La Roche (Basel, Switzerland). A peptide containing the apoB-100 3145–3157 and 3359–3367 connected by three glycine (apoB-heterodimer) was synthesized and purified as described (31).

Serum and lipoprotein preparation

Blood obtained from apparently healthy subjects was used to prepare serum by low speed centrifugation under sterile conditions. Serum was used instead of plasma in all the experiments in order to have a controlled value of chelating agents. Once serum was obtained it was made 0.1 mg/ml in Na_2-EDTA. LDL was prepared by density gradient or differential centrifugation using D_2O buffers, containing 0.1 mg/ml Na_2-EDTA, to raise the serum density to 1.019 and 1.063 g/ml as described (32). This procedure allows fractionation of VLDL and LDL and its direct use in oxidation experiments without need for dialysis. When HDL was prepared, the density of serum was raised by addition of solid KBr. Density gradient centrifugations to separate VLDL, LDL, HDL, and lipoprotein-free serum were performed as described (32–34). For the cell culture experiments LDL was labeled with ^{125}I. The unbound radioactive iodine was eliminated by gel filtration (6). The methods for protein and cholesterol measurements were described previously (32).

Binding of porphyrins to lipoproteins, apoB segments, and serum proteins

The distribution of hemin and protoporphyrin within serum fractions was evaluated by adding the porphyrins to diluted serum at final concentrations of 50, 125, and 250 μmol/L. After 15 min equilibration the mixture was fractionated in the exponential density gradient described above. The solutions contained 10 μmol/L BHT and 0.1 mg/ml EDTA to block oxidation of hemin during fractionation. After ultracentrifugation for 24 h at 20°C, 0.4-ml fractions were collected and their cholesterol was measured. Hemin in the fractions was followed by its absorbance at 412 nm. Tubes containing equivalent fractions of serum with no porphyrin were used as references. Binding isotherms to purified LDL, HDL, and human serum albumin (HSA) were obtained by titrating a constant concentration of the porphyrins, 10 μmol/L, with increasing concentrations of the lipoproteins. Both components were equilibrated with PBS. After additions, the samples were incubated 15 min at 37°C to allow equilibration. After equilibration, the absorbance at 412 nm was read in thermostated cuvettes in a Lambda 12 spectrophotometer (Perkin-Elmer, Norwalk, CT). Binding of hemin to the apoB-100 heterodimer containing the positive segments 3145–3157 and 3359–3367 was followed by changes of the intrinsic fluorescence of the tryptophan in the peptide. The apoB-heterodimer and hemin were equilibrated in PBS and increasing concentrations of hemin were added to a fixed concentration of the peptide (20 μmol/L). The binding isotherms were analyzed with the general equations that assume ligand depletion proposed by Epps, Raub, and Kezdy (35) and with Klots plots using a non-linear least square curve-fitting program from GraphPad Software (San Diego, CA).

Quantum yield and fluorescence polarization measurements

The iron-free protoporphyrin IX fluorescent analogue of hemin was used as a probe of the region at which it binds to LDL, HDL, and HSA. The methods and analysis used followed those described by Ricchelli et al. (36–38) for the study of porphyrin interactions with membranes. Thermal titration of quantum yield and fluorescence polarization were carried out by preequilibrating 10 μmol/L of the porphyrin with 40 nmol/L of LDL and 100 nmol/L of HDL at 18°C for 15 min. The samples were then placed in a thermostated cuvette with a magnetic stirrer and a temperature measuring probe. The temperature was raised in 2.5°C steps and the fluorescence emission at 615 nm after excitation at 410 nm was followed. The same design was used for the thermal titration of fluorescence polarization of the phorphyrin. Both types of experiments were carried out in an LS-50B luminescence spectrometer from Perkin-Elmer (Norwalk, CT).

Measurements of lipoprotein oxidation and isoprostane production

TBARS production was measured with a microtiter plate version of the method (39). Conjugated diene formation in lipoproteins and oxidation of associated hemin were evaluated simultaneously by following the appearance of 235 nm absorbance and the decrease in absorbance at 415 nm, respectively. The experiments were performed in closed cuvettes in a Perkin-Elmer Lambda 12 spectrometer, equipped with an automatic cuvette changer thermostated at 37°C. The esterified isoprostane 8-epi-$PGF_{2\alpha}$ was measured in serum and serum lipoproteins, with a modification of the procedure described by Nourooz-Zadeh et al. (40). After alkaline hydrolysis of the lipids, the isoprostanes were isolated by three-stage solid phase extraction. Isoprostanes were separated as their pentafluorobenzyl ester, trimethylsilyl ether derivatives by selected ion monitoring gas chromatography, negative ion chemical ionization/mass spectrometry. [$^2H_4$]-8-epi-$PGF_{2\alpha}$ from Cayman Chemicals (Ann Arbor, MI) was used as internal standard.

Agarose electrophoresis

Electrophoresis of LDL and serum lipoproteins was performed in a Bio-Rad 192 cell (Hercules, CA) in the "submarine mode" as described (32). Increasing concentrations of hemin and protoporphyrin IX (0, 10, 20, 40, 80, and 100 μmol/L) final concentrations were added to 2-ml serum samples diluted to 20% with PBS that contained 10 μmol/L BHT and 0.2 mg/ml EDTA. After incubation for 30 min at room temperature, 10-μl aliquots were used for electrophoresis. The remaining diluted serum was immediately fractionated using differential centrifugation in D_2O solutions to isolate LDL in a TLA 120.2 rotor of a Beckman tabletop ultracentrifuge (Fullerton, CA) (32). Aliquots (10 μl) of the floated LDL containing 0.5 mg protein/ml were used for electrophoresis. The serum samples were stained with Sudan black B and the purified lipoproteins with Coomassie brilliant blue.

Cell culture

VLDL/LDL-depleted human serum was prepared by ultracentrifugation, diluted to 10% in PBS, and sterilized by filtration. ^{125}I-labeled LDL was added to the VLDL/LDL-depleted serum (10 μg protein/ml, 300 cpm/μg). Three aliquots of this serum were incubated with *a*) 50 μmol/L hemin, *b*) 50 μmol/L hemin plus 100 μmol/L H_2O_2, and *c*) no additions, used as control. To half of each aliquot 10 μmol/L probucol was

added. All the aliquots were incubated in sterile conditions at 37°C for 16 h. Human monocyte-derived macrophages (HMDM) were obtained and purified as described (6). After 8 days in culture in RPMI-1640 medium, supplemented with 10% calf serum, in 16-mm dishes, the adherent cells were washed 3 times with PBS. The experimental human serum diluted to 10% and containing the lipoprotein dilutions was added to the cells. Each experimental condition was run in four dishes. After 4 h, cell uptake of the labeled lipoprotein was measured and cell protein was evaluated after washing and hydrolysis (6). LDL degradation was evaluated in the same experiments as suggested by Sakai et al. (41). Non-specific binding was subtracted from dishes that contained a 20-fold excess of unlabeled LDL. The methods used for cell protein determination and counting have been described previously (6).

RESULTS

Hemin distribution in serum

In separate experiments, hemin and protoporphyrin were added to diluted serum and allowed to equilibrate. To control the oxidation of hemin during ultracentrifugation, the serum and gradient solutions were made 10 μmol/L in BHT and 0.1 mg/ml EDTA. **Figure 1** shows how the absorbance of hemin at 412 nm follows the distribution of VLDL, LDL, and HDL cholesterol. However, approximately 40% of hemin remains associated with serum proteins with density above 1.210 g/ml. Figure 1 shows the results for 125 μmol/L hemin, but a similar distribution was observed at 50 and 250 μmol/L. Addition of the non-iron-containing protoporphyrin IX to the same serum resulted in a distribution almost identical to that of hemin (data not shown). The results indicate that in serum diluted to 20% the porphyrins are distributed about evenly between lipoproteins and non-lipoprotein proteins. Binding of hemin to LDL in diluted serum can also be monitored by the change in electrophoretic mobility of the lipoprotein. **Figure 2** presents agarose gels from 20% diluted serum incubated with the indicated increasing amounts of hemin (A) or protoporphyrin IX (B) and of the LDL isolated from the same serum samples (C, hemin; D, protoporphyrin IX). The concentration-dependent increase in LDL electrophoretic mobility caused by binding hemin or protoporphyrin was visible in serum and in the isolated lipoprotein. No increase in

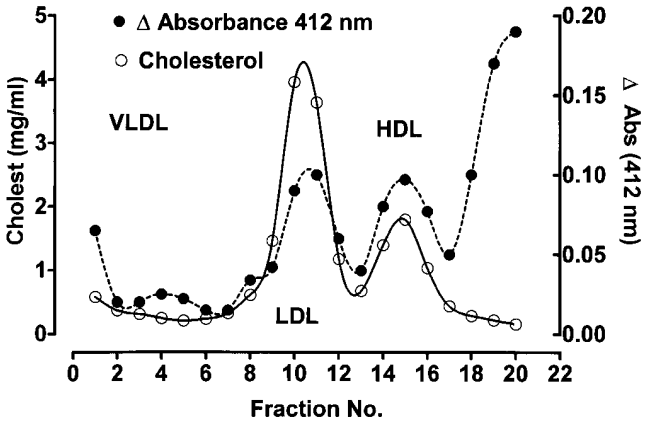

Fig. 1. Distribution of 125 μmol/L hemin equilibrated with serum diluted to 20% of its original concentration. To preserve hemin during the fractionation, the solutions contained 10 μmol/L BHT and EDTA. The diluted serum solution, 4 ml, was fractionated in an exponential KBr density gradient and 0.4-ml fractions were collected for measurement of cholesterol and hemin content (absorbance at 412 nm).

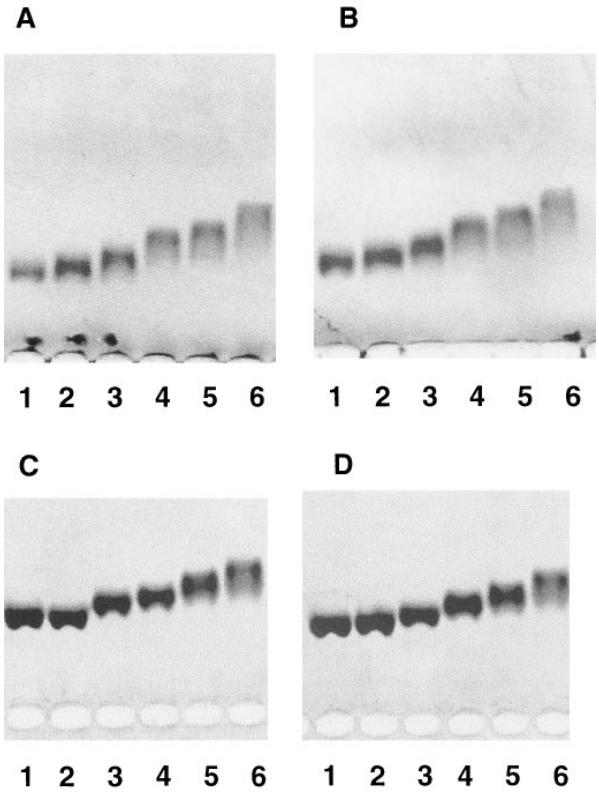

Fig. 2. Agarose electrophoresis of 20% diluted serum incubated with increasing amounts of hemin or protoporphyrin IX and of the isolated LDL from this serum. Diluted serum, containing 10 μmol/L BHT and 0.2 mg/ml EDTA, was treated with 0, 10, 20, 40, 80, and 100 μmol/L, 1 to 6, respectively, with hemin (A) or protoporphyrin IX (B). Aliquots were used for electrophoresis. LDL was isolated from the same serum and aliquots from hemin-treated serum (C) and protoporphyrin IX-treated serum were used for electrophoresis. Serum was stained with Sudan black B and isolated LDL with Coomassie brilliant blue.

conjugated dienes or TBARS was detectable over the values measured for the LDL samples containing no porphyrins, conjugated dienes <0.2 μmol/mg apoB protein, and TBARS <2 nmol/mg apoB protein. Therefore, the increase in negative charge was caused by the porphyrin binding and not by oxidation. With some sera, protoporphyrin IX was slightly more effective than hemin in altering LDL mobility. This is expected because of the larger net negative charge of the porphyrin without Fe^{3+}.

Binding isotherms of porphyrins with LDL, HDL, and HSA

Binding of porphyrins to LDL, HDL, and HSA induces a hyperchromic change in their Soret absorption band between 380 and 420. This change can be conveniently used to evaluate binding isotherms, especially because in this region the proteins do not absorb. We used titration of a fixed concentration of hemin with increasing concentrations of LDL, HDL, and HSA to avoid the disruption of the lipoproteins at high porphyrin concentrations. **Table 1** presents the dissociation constant (K_d), maximal binding (B_t), and Hill number for the association of hemin and protoporphyrin to LDL. Their similarity suggests that it is the porphyrin ring that controls the association. Representative isotherms for the binding of hemin or protoporphyrin IX to LDL are given in **Fig. 3**. The parameters for the association of hemin with HDL and HSA are also given in Table 1. For calculations we used 2.5×10^6, 3.0×10^5, and 6.7×10^4 daltons for the apparent molecular masses of LDL, HDL, and HSA, respectively. On this base, the affinity of hemin or protoporphyrin IX for LDL is about 6 times higher than for HDL and 20 times higher than that for HSA. However, if expressed per mg of lipid, the affinity of the porphyrins with LDL and HDL is similar. Hemin binds also to the synthetic heterodimer containing the positive regions 3145–3157 and 3359–3367 of apoB-100. This suggests that association of porphyrins with LDL may involve positively charged segments of the protein and surface lipids of the particle at the same location. The Hill number, calculated from the slope of Klotz plots, suggests a homogeneous binding region in LDL, HDL, and the apoB heterodimer. HSA appears to have more than one class of binding site for hemin. The calculated B_t indicates that at saturation the sites in LDL can bind approximately 50 porphyrins per particle and 5 in HDL. HSA appears to be saturated with two hemin molecules and the apoB heterodimer with two (Table 1).

Oxidation of LDL and HDL

Hemin and H_2O_2 added to serum diluted to 10 or 20% with PBS caused formation of conjugated dienes production of TBARS that began after 10–30 min of the incubation. The amount of TBARS produced was moderate, reaching 8–12 nmol/ml after 16 h. Elimination of VLDL from serum did not alter conjugated diene formation, but elimination of LDL reduced this to approximately 40–60%, depending on the LDL/HDL ratio. Elimination of LDL plus HDL almost abolished formation of conjugated dienes and TBARS. This indicates that the main source of them are the lipids from LDL and HDL. **Figure 4** shows the formation of conjugated dienes in 20% diluted serum containing LDL and HDL when incubated with 10 and 100 μmol/L hemin and 250 μmol/L H_2O_2. It can be observed that conjugated diene formation, Δ absorbance at 236 nm,

TABLE 1. Binding parameters for the association of hemin and protoporphyrin IX to lipoproteins, human serum albumin, and apoB-100 segments

System	K_d[b]	B_t	Hill Coefficient
		mol porphy./mol	
LDL/hemin	49 ± 5 nmol/L	45–65	0.91–1.31
LDL/protoporphyrin IX	22 ± 5 nmol/L	40–60	0.89–1.32
HDL/hemin	291 ± 26 nmol/L	4–6	0.70–1.10
HSA/hemin	1000 ± 220 nmol/L	1.8–2.3	1.68–2.52
Hemin/apoB-heterodm.[a]	3.6 ± 1 μmol/L	1.5–2.0	0.70–1.10

The values represent averages ± standard deviations of four separate titrations with lipoproteins obtained from two subjects, except in the case of HSA where a single lot of the protein was used. In the case of the porphyrins, 10 μmol/L was titrated with increasing concentrations of LDL, HDL, or HSA.

[a] A fixed concentration of the apoB heterodimer, 20 μmol/L, was titrated with increasing concentrations of hemin in PBS at 37°C.

[b] The equation used was: $f = \alpha Wo + (\beta - \alpha)/2[Wo + Bo + K_d - (Wo + Bo + K_d)^2 - 4B_t]^{1/2}$; where f, the change in spectral properties, is related to the complex concentration by the equation: $f = \alpha W + \beta C$; where W is the ligand concentration whose optical properties are followed; C is the complex concentration; and α and β are proportionality constants. Wo and Bo are the analytical concentrations of the ligands and K_d the dissociation constant. B_t is the maximal binding and is equal to WoBo; see Epps, Raub, and Kezdy (35).

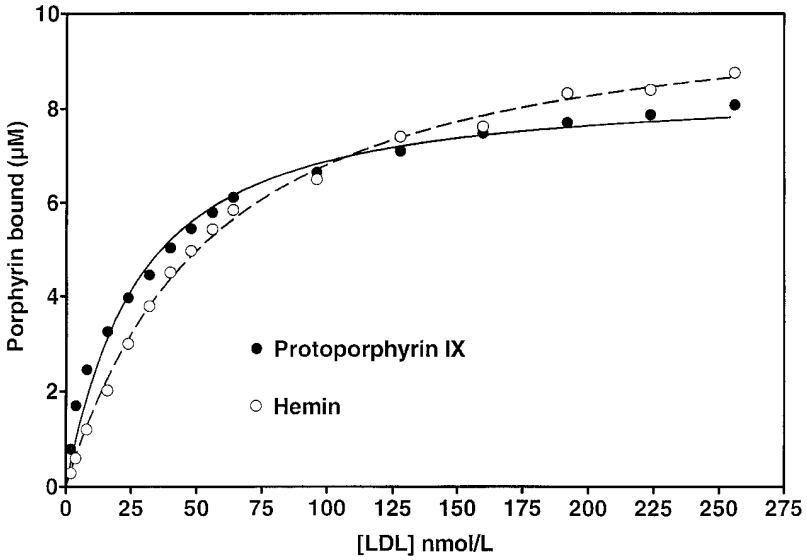

Fig. 3. Binding isotherms of hemin and protoporphyrin IX to human LDL. A constant amount of the porphyrins was titrated with increasing concentrations of the lipoprotein. The points represent the experimental data and the curve the non-linear regression fitted to the points.

follows the negative Δ absorbance at 407 nm that monitors free radical-mediated disappearance of the Soret band of the porphyrin ring. We tested the hemin-mediated formation of conjugated dienes in diluted serum depleted of VLDL, LDL, and HDL to which increasing concentrations of LDL or HDL were added. **Figure 5** shows the results obtained after 60 min incubation at 37°C of serum diluted to 20% with PBS that contained 50 μmol/L hemin and 250 μmol/L H_2O_2. At equivalent protein concentrations, HDL appears to cause higher conjugated diene formation. However, it should be noted that at equal lipoprotein protein values, lipoprotein-depleted serum with added HDL has 3–5 more lipoprotein particles than lipoprotein-depleted serum containing LDL. This suggests that, per particle, LDL and HDL support similar conjugated dienes production in diluted serum. The oxidation of lipoproteins by 50 μmol/L hemin and 250 μmol/L H_2O_2, as measured by conjugated dienes or TBARS, diminished with increased concentrations of serum and

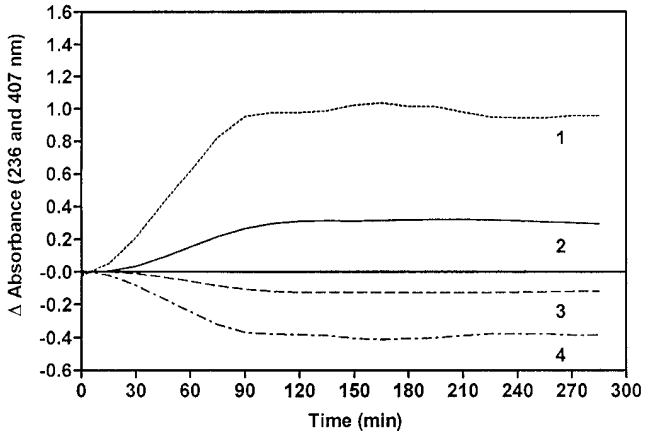

Fig. 4. Conjugated diene formation in serum. Increase in conjugated dienes, absorbance at 236 nm (curves 1 and 2), and disappearance of hemin, decrease in absorbance at 407 nm (curves 3 and 4) were monitored for 300 min at 37°C, in VLDL-depleted serum diluted to 20% with PBS and incubated with 10 μmol/L (curves 2 and 3) and 100 μmol/L hemin (curves 1 and 4) and 250 μmol/L H_2O_2. The reference cell contained the same VLDL-depleted serum with hemin but no H_2O_2. All samples contained 0.1 mg/ml EDTA.

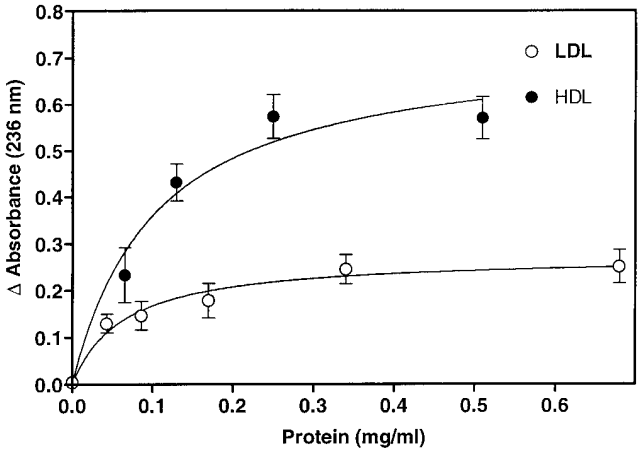

Fig. 5. Oxidation of increasing concentrations of LDL or HDL added to VLDL, LDL, and HDL-depleted serum diluted to 10%. Diene formation was followed until it became stable, 60 min. The increase in absorbance at 236 nm is plotted as function of added LDL- or HDL-protein concentration. The bars represent the range of duplicate determinations. Oxidation was induced by addition of 50 μmol/L hemin and 250 μmol H_2O_2. The samples contained 0.1 mg/ml EDTA.

was not detectable beyond 35–40% diluted serum, even after 120 min incubation. The F_2-isoprostane, 8-epi-$PFG_{2\alpha}$, appears to be a marker of free radical-mediated in vivo oxidation of arachidonic acid (42). It was interesting, therefore, to study whether this marker was a product of lipoprotein oxidation induced by hemin. Serum depleted of VLDL was diluted to 20% with PBS and oxidized for 16 h with 150 μmol/L hemin and 750 μmol/L H_2O_2. LDL and HDL were isolated by differential ultracentrifugation, and the lipids of total serum, LDL, and HDL were analyzed for the presence of esterified 8-epi-$PFG_{2\alpha}$. For the hemin-treated, VLDL-depleted serum, the increase in lipid-bound isoprostanes was 3- to 4-times the levels of the control serum (2–4 pmol/ml). When hemin and H_2O_2 were added, the value was 4- to 6-times that of control. The F_2-isoprostane produced was mainly associated with LDL and HDL isolated from diluted serum. The values measured in the isolated LDL and HDL are given in **Fig. 6**. It can be observed that hemin alone was sufficient to produce a substantial amount of F_2-isoprostane and that H_2O_2 potentiated the effect.

Even after LDL is floated through a density gradient for 24 h, hemin remains associated with the lipoprotein and can be detected by the band at 405 nm, if protected with BHT and EDTA. **Figure 7-A** presents the distribution of LDL in a density gradient after incubation of 2 ml of 2 μmol/L of the lipoprotein with 50 μmol/L hemin and 10 μmol/L BHT and no H_2O_2. A small amount of TBARS and non-heme iron, measured as Fe^{2+}, could be detected associated with the lipoprotein. Fractionation of a similar hemin-containing aliquot to which 100 μmol/L of H_2O_2 was added but no BHT is shown in Fig. 7-B. Hemin was no longer detected (absorbance at 405 nm) and a substantial increase in the TBARS and free Fe^{2+} was measured. Part of the TBARS and iron leaked from the LDL but appreciable amounts remained associated with the lipoprotein. This suggests that oxidative degradation of the porphyrin ring released Fe^{3+} inside the lipoprotein particle.

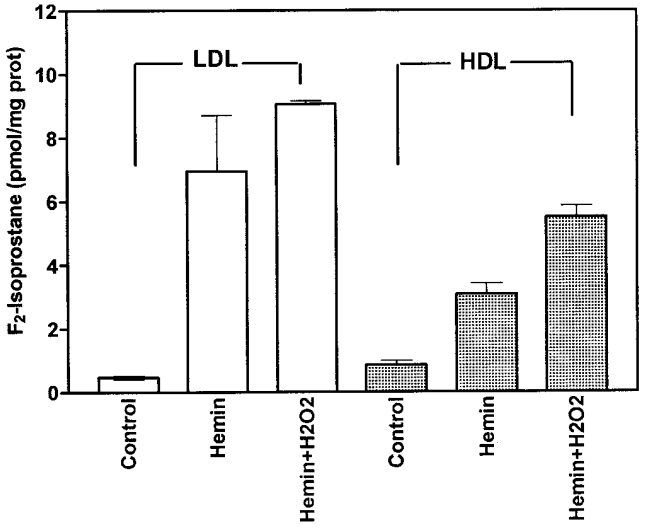

Fig. 6. Content of 8-epi-$PFG_{2\alpha}$ in lipids of LDL and HDL isolated from diluted serum oxidized with hemin. The lipoproteins were isolated from 20% diluted VLDL-depleted serum after incubation with 150 μmol/L hemin and 150 μmol/L hemin plus 750 μmol/L H_2O_2 for 16 h at 37°C. The samples contained 0.1 mg/ml EDTA. The F_2-isoprostane was isolated from total lipids and characterized by gas chromatography/mass spectrometry. The values are averages of duplicates and the bars represent range of duplicate measurements.

Porphyrins as probes of the association with lipoproteins

Hemin has no fluorescence because of the electron withdrawal effect of the coordinated iron. However, protoporphyrin IX has an emission band at 630 nm when excited at 412 nm. The binding isotherms of

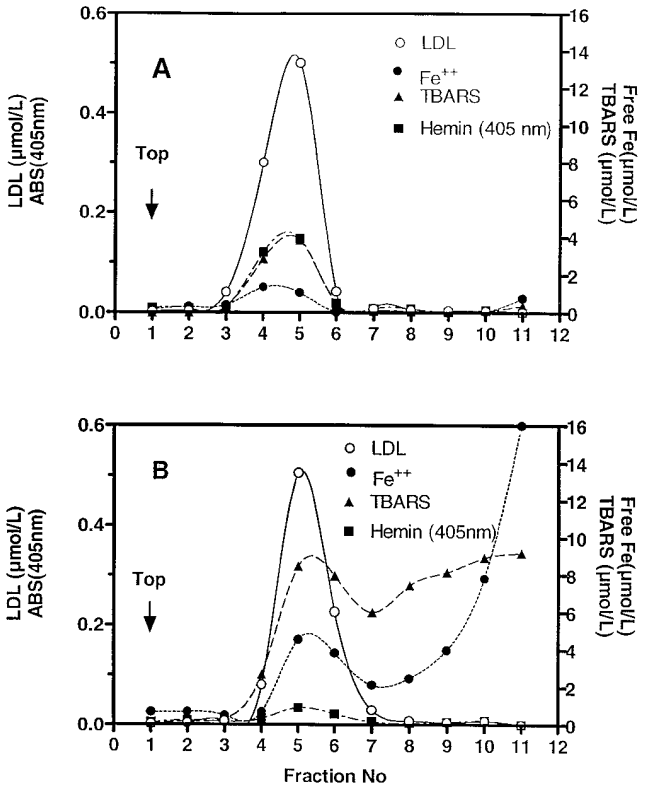

Fig. 7. Effect of oxidation on the association of hemin and LDL. Density gradient centrifugation of LDL incubated with hemin in conditions that prevent oxidation (A) or allow oxidation (B). In A, the incubated LDL contained 10 μmol/L BHT and in B, no BHT but 100 μmol/L H_2O_2. The lipoproteins were fractionated in PBS-deuterium oxide density gradients, and protein, TBARS, free iron, and absorbance at 405 (hemin) nm were measured in 1-ml fractions collected after 24 h centrifugation.

hemin and protoporphyrin IX with isolated LDL and HDL are similar. This and the structural similarities of these porphyrins suggested that protoporphyrin IX could be used as a probe of their association with lipoproteins. The emission spectra of protoporphyrin IX is markedly enhanced and shifted to higher wavelengths after association with LDL and HDL. This change provides a sensitive tool to explore the interactions. The quantum yield of porphyrins bound to the lipoproteins should be altered by changes in the structure of its binding pocket. **Figure 8** shows the effect of increasing the temperature on the quantum yield at 640 nm expressed as a ratio to the corrected emission at 20°C. LDL-bound porphyrin reports a transition in the lipoprotein taken place between 35 and 45°C. No change in the porphyrin fluorescence was induced by increase in temperature when bound to HDL. The porphyrin alone shows only a gradual increase in emission with temperature, probably due to changes in aggregation. Binding to the lipoproteins causes an increase in the fluorescence polarization of protoporphyrin IX because the association with the macromolecules reduces the tumbling rate of the heme ring. Thermal titration of fluorescence polarization, plotted as a ratio of polarization observed at different temperatures to that at 20°C, also shows for the LDL-bound porphyrin a transition between 35 and 45 degrees (not shown). As with the quantum yield measurements, no transition was evident in the fluorescence polarization of protoporphyrin IX associated with HDL.

Macrophages uptake of hemin-oxidized LDL in diluted serum

LDL oxidized by hemin and hemin–H_2O_2 was taken up and degraded more efficiently than control, untreated LDL (**Fig. 9**). Probucol reduced uptake and degradation of LDL treated with hemin–H_2O_2 to the levels of LDL treated only with hemin. This suggests that probucol eliminates the modifications caused by hemin–H_2O_2 but not those induced only by hemin. As shown in Fig. 2, hemin binding without oxidation causes an increase in the negative charge of LDL. This alteration may be responsible for the enhancement of macrophage uptake and degradation caused by hemin even without detectable oxidation of the LDL.

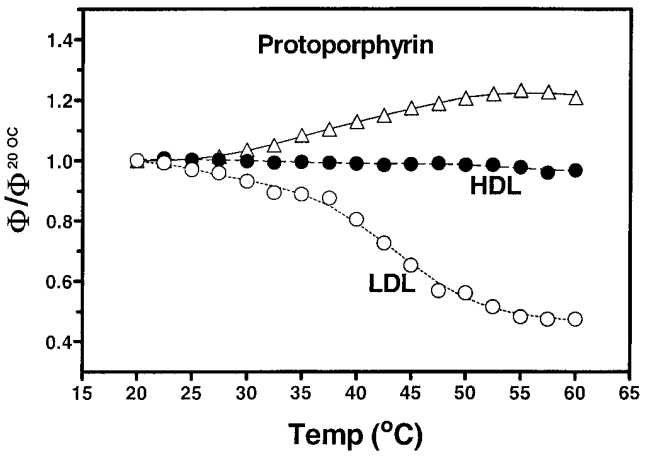

Fig. 8. Thermal titration of the quantum yield of protoporphyrin IX associated to LDL and HDL. The values are expressed as the ratio of the yield at each temperature to that at 20°C.

DISCUSSION

Free radical-mediated oxidation of lipids and proteins occurs in the atherosclerotic arterial intima (3). How many of these processes are the consequence or cause of atherogenesis is unknown. Furthermore, several free radical generating systems that may oxidize lipoproteins in the intima microenvironment are under study. Hemoglobin from ruptured erythrocytes oxidizes LDL in vitro (12, 43), probably through a pathway that involves a ferric/ferryl cycle of the heme iron and hydroperoxides (12, 44, 45). Blood lipoproteins may be the vehicles that take them to atherosclerotic lesions and tumors (46). This suggestion is based on data showing that non-iron-containing porphyrins associate in plasma with LDL and HDL lipoproteins and not only with albumin and hemopexin (26). There is an extensive literature for the use of lipoprotein complexes as targeting mechanisms for the delivery of synthetic non-iron-containing porphyrinoids to tumors as photosensi-

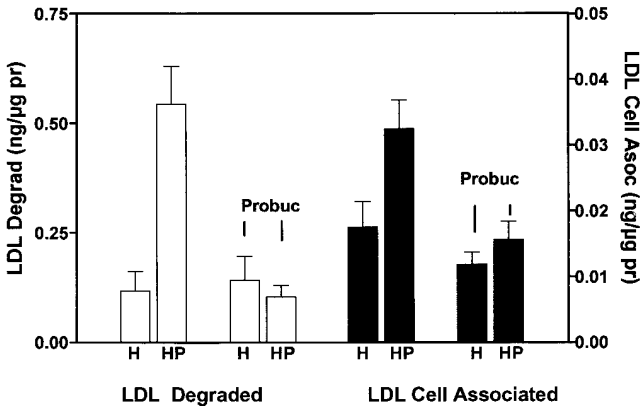

Fig. 9. Uptake and degradation of LDL by human monocyte-derived macrophages (HMDM) from diluted serum preincubated with hemin. Aliquots of the 10% diluted serum were preincubated with hemin (H) or with hemin and H_2O_2 (HP) in the absence or in the presence of 10 µmol/L probucol. LDL uptake and degradation is expressed as values (mean ± SD, n = 4) above those of control dishes, serum with no hemin.

tizers for photodynamic therapy (46). Atherosclerotic lesions also concentrate porphyrins and, although not specifically documented, probably hemin. Measurements of iron-containing porphyrins in tissues are difficult because they do not fluoresce and probably because they are rapidly destroyed once involved in free radical-mediated oxidations (13).

We showed that the Fe^{3+}–hemin in diluted human serum binds to LDL and HDL with high affinity and remains associated with the lipoproteins even after ultracentrifugal fractionation under a density gradient (Fig. 1). Similar results were reported for porphyrins containing no iron (26). However, in order to detect hemin after density gradient centrifugation, it was necessary to block its oxidative destruction with lipophilic antioxidants. The binding affinity measured for the hemin/lipoproteins associations (nmol/L) is lower than the one reported for hemin/hemopexin (pmol/L) (47). But the fact that hemin is found associated with lipoproteins when added to serum or plasma indicates that in this environment lipoproteins can compete effectively with hemopexin and albumin for the binding of the porphyrins (26). In diluted serum, hemin was found associated with LDL and HDL and it catalyzed formation of conjugated dienes, a moderate amount of TBARS, and isoprostaglandins, specifically 8-epi-$PGF2_\alpha$, that largely remain associated with the lipoproteins. The oxidation of purified LDL by hemin has been studied previously (10, 11, 13, 15, 16). However, we believe that this is the first time that LDL and HDL oxidation by hemin has been demonstrated in the presence of other serum components that should exist in the extracellular compartment of the arterial intima (17, 18).

In terms of conjugated dienes and F_2-isoprostane production, LDL and HDL in serum were similarly susceptible to hemin oxidation (Figs. 4, 5). Both parameters appeared related to lipid content of the particles. The isoprostane 8-epi-$PGF2_\alpha$ may be a stable marker of the in vivo free radical oxidation of esterified arachidonic acid, probably involving iron-porphyrins (48). F_2-isoprostanes have potent vasoconstrictor actions acting through thromboxane-like receptors (49). Recently, F_2-isoprostanes were found associated with susceptibility to iron-mediated endogenous oxidation of serum from premature infants (42). Perhaps more relevant to the potential role of hemin as a pro-oxidant in the arterial intima are the results of Praticò et al. (50) showing that human atherosclerotic plaques accumulate F_2-isoprostanes. It remains to be studied whether these substances associated with lipoproteins have effects in macrophages or arterial cells. Oxidation of diluted serum with hemin and H_2O_2 caused a significant increase above the basic binding and degradation of labeled LDL by human macrophages (Fig. 9). In hemin-treated serum, both LDL and HDL carry oxidized lipids and F_2-isoprostanes. We do not know which of these carriers may be a donor of such compounds for cells. However, it is possible that by exchange of the oxidized phospholipids among the lipoproteins, LDL, via specific receptors, remains the main vehicle. The biological effects of free radical-mediated oxidation of HDL are much less characterized than those of LDL. It has been suggested that HDL takes up peroxides from oxidized LDL and therefore diminishes its further oxidation (1). In serum, LDL and HDL appeared similarly susceptible to hemin-caused oxidation. However, our experiments with macrophages were not designed to study the effect of the oxidized HDL. It should be interesting to analyze the potential actions of hemin-oxidized HDL on cells in the presence of different concentrations of LDL.

The site and extent of insertion of dicarboxylic porphyrins in phospholipid membranes and liposomes depend on their protonation state (51). At neutral pH, and at the surface monolayer of LDL and HDL, the planar porphyrin ring should be in hydrophobic pockets. The ionized propyl carboxyls, on the other hand, should be in close contact with positive surface charges of the apoB-100. The absorption spectra of protoporphyrin IX and hemin and the emission band of protoporphyrin IX upon association with LDL and HDL show similar alterations. These suggest that the general polarities of the porphyrin-binding regions are alike in the two particles. When associated with LDL, thermal titration of the porphyrin quantum yield and its fluorescence polarization reported the LDL transition associated with the cholesteryl ester core (52). On the other hand, changes in electrophoretic mobility of LDL induced by association with the porphyrins, without oxidation, suggest that the porphyrin carboxyls are titrating surface charges (Fig. 2). This is also supported by binding of protoporphyrin to positively charged segments of apoB-100 that should reside in the LDL surface (Table 1) (31, 53, 54). The high affinity association of dicarboxylic porphyrins to other proteins with segments rich in arginine and lysine has been described (55). The results from the changes in fluorescence of the porphyrin and the non-oxidative mediated increase in electrophoretic mobility of LDL can be explained with a model similar to that proposed by Richelli (51) for phospholipid vesicles with positive charges. In our model the arginine and lysines of apoB-100 could provide the positive charges. In the LDL surface monolayer, at regions where lysine and arginine-rich segments of apoB-100 exist, the porphyrin ring may be intercalated within fatty acid chains of phospholipids and free cholesterol. The porphyrin tetrapyrrole ring may be deep enough to sense transitions of the cholesteryl esters core. On the other hand, the carboxyl side

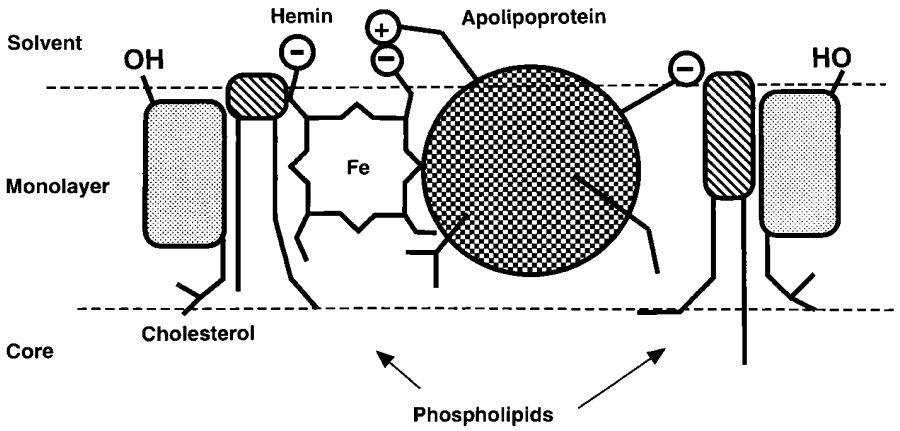

Fig. 10. Scheme of the surface monolayer of LDL at sites where dicarboxylic porphyrins are inserted. The carboxylic groups of the porphyrins may interact with lysine or arginine side chain amino groups of the hydrophylic side of the apoproteins at the surface of the lipoproteins. By doing this or by exposing new carboxylic groups to the solvent, the inserted porphyrins may effectively increase the negative surface charge of the particles, increased electrophoretic mobility at pH 8.4. The hydrophobic ring should be inserted deep enough to reach non-polar regions of the surface monolayer to account for the observed shift in spectral properties.

chains should be close to the surface interacting with positive surface side chains of the apoB-100. This model is also supported by results of fluorescence quenching experiments, suggesting that in the association with LDL hematoporphyrin interacts with both the protein and lipids (56). An alternative explanation is that some heme molecules bind to the positive apoB-100 segments and others are inserted deep into the surface lipid monolayer close to the core. With the heme carboxylic residues ionized, at neutral pH, this appears not energetically favorable. Furthermore, the binding isotherms for both LDL and HDL suggest a single class of binding sites with a Hill number close to 1. A diagram of the proposed model of the insertion site is presented in **Fig. 10**. Tribble et al. (16) proposed that the rapid destruction of the porphyrin ring, that follows very closely diene formation and oxidation of core-residing probes, liberates Fe^{3+} that then becomes the core oxidant. Our data, indicating that a substantial amount of heme-liberated iron in LDL does not readily exchange with the solvent, are in line with this idea. In summary, the results presented show that hemin, even in the absence of added peroxides, can produce oxidation products of arachidonic acid, F_2-isoprostanes, in LDL and HDL in diluted serum. With added peroxides, heme in diluted serum oxidizes the lipoproteins to induce formation of conjugated dienes and low levels of TBARS. The alterations on the LDL particle caused an increase in its uptake and degradation by macrophages. Together with lipid oxidation products, LDL may therefore be a vehicle for internalization of Fe^{3+} into the cells, a situation that could contribute to the cytotoxicity of hemin-oxidized lipoproteins, as suggested by Yuan, Brunk, and Olsson (57). The experiments support the hypothesis that at sites of LDL retention in the extracellular intima, heme may be a pro-oxidant of pathological significance, even in the presence of other plasma components.

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