# Mechanism of Low-Density Lipoprotein Oxidation by Hemoglobin-Derived Iron

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*Received November 7, 2002; Revised Manuscript Received February 10, 2003*

ABSTRACT: Excellular hemoglobin is an extremely active oxidant of low-density lipoproteins (LDL), a phenomenon explained so far by different mechanisms. In this study, we analyzed the mechanism of met-hemoglobin oxidability by comparing its mode of operation with other hemoproteins, met-myoglobin and horseradish peroxidase (HRP) or with free hemin. The kinetics of met-hemoglobin activity toward LDL lipids and protein differed from that of met-myoglobin and HRP, both quantitatively and qualitatively. Those differences were further clarified by analyzing heme transfer from the above-mentioned hemoproteins to LDL. It appeared that met-hemoglobin transferred most of its hemin to LDL, and the presence of $H_2O_2$ accelerated the process. In contrast, met-myoglobin partially released hemin, but only in the presence of $H_2O_2$, while HRP could not transfer heme at all. The minor amount of hemin transferred from met-myoglobin to LDL sufficed to trigger ApoB oxidation, forming covalent aggregates via inter-bityrosines. This indicated that heme bound to high affinity site(s) is responsible for oxidation. LDL components providing the sites were analyzed by binding heme−CO monomers to LDL. Soret spectra revealed that the high affinity site of monomeric hemin is located on the LDL protein, ApoB. The complex heme−CO−ApoB underwent instantaneous oxidation to hemin−ApoB, and the bound hemin then slowly disintegrated in conjunction with LDL oxidation. Hemopexin prevented LDL oxidation by trapping hemoprotein transferable heme. We concluded that met-hemoglobin exerts its oxidative activity on LDL via transfer of heme, which serves as a vehicle for iron insertion into the LDL protein, leading to formation of atherogenic LDL aggregates.

The high rate of atherosclerosis in modern society calls for understanding the mechanisms involved on a molecular basis. Oxidation of low density lipoproteins (LDL)[1] has been shown to play a central role in this process. Therefore, in the last two decades an exploding research field relating LDL oxidation to atherosclerosis has immerged. On the basis of the analysis of atherosclerotic lesions, the transition metals iron and copper were suggested to play a central role as triggers of LDL oxidation in vivo (*1*). Copper is a scarce metal in the blood system appearing in the form of ceruloplasmin, which is not considered a pro-oxidant (*2*). Iron, in contrast, exists in the blood at extremely high (millimolar) concentrations, in the form of hemoglobin. The latter is constantly released from the shielding erythrocytes under a variety of stress conditions in a process called trivial hemolysis, yielding met ($Fe^{3+}$) hemoglobin. As if ignoring the higher likelihood of iron reactivity in the vascular system, copper has been used as an oxidation trigger in most in vitro studies, related to the link between LDL oxidative modification and atherosclerosis. This should not come as a surprise because in vitro experiments with iron salts fail to induce LDL oxidation (*3*, *4*), due to iron's poor solubility in phosphate-containing physiological buffers (*5*). In light of copper's unavailability and iron's inactivity, the established hypothesis linking these redox active metals to early stages of atherosclerosis was recently even questioned (*6*).

Unlike free iron, solid indications point to excellular hemoglobin involvement in LDL oxidation and atherosclerosis (*7*, *8*). A positive correlation was demonstrated between elevated levels of oxidized LDL and intravascular rupture of erythrocytes in uremic patients undergoing hemodialysis who are prone to atherosclerosis (*8*−*10*). The description of a heme oxygenase-1-deficient patient whose blood contained both excellular hemoglobin (met form) and oxidatively modified LDL (*11*, *12*) pointed to a central role of met-hemoglobin in the oxidative modification of LDL. Ample research suggested ways by which hemoglobin and its twin hemoprotein, myoglobin, exert LDL oxidation (*13*−*16*). In the presence of a peroxide like $H_2O_2$, the two oxygen binding hemoproteins yield an active ferryl ($Fe^{IV}$) iron state typical of peroxidases, although their peroxidase activity on small substrates is low (*17*, *18*). Both hemoglobin and myoglobin were found to be active oxidizers of LDL and thus were assumed to act via their ferryl active heme state (*19*, *20*).

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[1] Abbreviations: ApoB − apolipoprotein B; APS − ammonium persulfate; BSA − bovine serum albumin; CD − conjugated dienes; D-LDL − dansylated low-density lipoprotein; Hb − hemoglobin; Hb-CO − carboxyhemoglobin; HO-1 − heme oxygenase-1; Hx − hemopexin; HRP− horseradish peroxidase; LDL − low-density lipoprotein; Mb − myoglobin; MDA − malondialdehyde; metHb − methemoglobin; metMb − metmyoglobin; MPO − myeloperoxidase; OD − optical density; oxyHb − oxyhemoglobin; PBS − phosphate buffer saline; PMSF − phenylmethylsulfonyl fluoride; SDS−PAGE − sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TBARS − thiobarbituric acid reactive substances; UV absorbance − UVA; Vit E − vitamin E (α-tocopherol).

10.1021/bi020647r CCC: $25.00 
Published on Web 05/20/2003

However, the increased activity of myoglobin and more so of hemoglobin toward LDL, as compared to the classic horseradish peroxidase (HRP), called for further explanations. Involvement of LDL lipid peroxides was proposed by some researchers (*14*, *15*) and globin radicals by others (*21*−*23*). On the basis of studies with myoglobin, it was suggested that heme bound covalently to globin is the active component, responsible for the high oxidability of the oxygen binding hemoproteins toward LDL (*24*, *25*). However, unlike myoglobin the interaction of $H_2O_2$ with hemoglobin does not yield any heme−globin covalent product (*26*). Recent studies suggested that globin-free heme is responsible for hemoglobin peroxidation activity, but claimed that Mb is not active at all (*12*). Thus, despite accumulated information, it is still unclear whether the oxidative activity of Hb and Mb toward LDL differ, and if so, in which manner.

Protein-free hemin was shown to be very active as an LDL pro-oxidant, and at as low as a few micromolar concentration, if given enough time, addition of extrinsic peroxide is not required (*27*, *28*). Unlike hemoglobin and myoglobin, the hydrophobic hemin associates with LDL, and thereby can act directly as an oxidizer of lipids and protein in LDL (*16*). Hemin, like copper, was reported to trigger increased light absorbance in UV range referred to formation of CD (lipid conjugated dienes) in three-phase kinetics: lag time, fast propagation, and termination. Regarding protein oxidation, covalent cross-linking of ApoB is the dominating product in hemin, as well as in hemoglobin and myoglobin triggered oxidation. Such a product opposes degradation of the protein in LDL oxidized by copper (*27*−*29*).

The above-summarized information, drawn from intensive research on the subject of heme/hemoprotein induced oxidation, leaves us in a state where it is not even clear if hemoglobin and myoglobin act by similar mechanisms (*12*, *18*, *20*−*24*). This mist originates from vague information on whether hemoglobin and/or myoglobin high reactivity as triggers of LDL oxidation stems from the intact molecules or rather from oxidative disintegration products such as heme and iron. In the current study, the issue of oxidative activity of hemoglobin toward LDL was revisited by systematic comparison of identical LDL samples peroxidized by hemoglobin, myoglobin and HRP, as well as free hemin. We provide evidence that the high oxidative activity of excellular hemoglobin toward LDL resides in the transfer of heme monomers to a high affinity site on the LDL protein, ApoB. The protein bound heme is unstable in the oxidative lipid particle milieu and the released iron within the particle catalizes the LDL oxidation. This way hemoglobin acts as a "Trojan horse" in oxidative modification of the particle, specifically its protein. The analysis of hemoglobin stemmed LDL oxidation provides a molecular basis for the "iron hypothesis" (*30*).

## MATERIALS AND METHODS

*Materials.* Bovine hemin, equine myoglobin, bovine catalase, horseradish peroxidase type X (HRP), 5-dimethylaminonaphthalene-1-sulfonyl (dansyl) chloride, α-tocopherol and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co., St. Louis, MO. Hemopexin was isolated from human plasma as previously described (*31*). Hydrogen peroxide was from Merck, Darmstadt, Germany. DE-52 cellulose and CM-52 cellulose were obtained from Whatman International, Maidstone, England. PD-10 desalting columns were purchased from Amersham Pharmacia Biotech, Buckinghamshire, England. Chemicals for sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) were purchased from Bio-Rad Laboratories, Richmond, CA.

*Isolation of LDL.* LDL was isolated from human plasma by sequential ultracentrifugation as previously described (*32*) and eluted through two PD-10 columns to remove EDTA and KBr. The concentration of LDL was determined as protein concentration by the Lowry method (*33*). Enrichment of LDL with vitamin E (1 mg/mL) was achieved by addition of α-tocopherol in ethanol to isolated LDL. The mixtures were then incubated at 37 °C for 3 h. Control experiments were carried out with LDL samples to which the same volume of ethanol (without α-tocopherol) was added.

*Isolation of Hemoglobin.* Hemoglobin was isolated from red blood cell lysates by ion-exchange chromatography using CM-52 cellulose (*34*) followed by desalting on PD-10 columns. Lack of catalase was determined from ferrylHb formation in the presence of an equimolar amount of $H_2O_2$. This Hb was verified spectrophotometrically as oxyHb (*35*). Since iron appears in HRP as $Fe^{3+}$ and Mb iron under air conditions is also in its $Fe^{3+}$ form (met), Hb was used throughout this study in its met form as well. MetHb was prepared from the oxyHb (*35*) and before use it was purified by admixing with DE-52 cellulose for 5 min followed by centrifugation to remove globin-free hemin contaminants (*36*). Concentrations of metHb, metMb, catalase, and HRP were expressed in heme equivalents throughout this study.

*Heme Solutions*. Commercial hemin was freshly prepared as a stock solution by dissolving in 5 mM NaOH. Undissolved remains were removed by centrifugation at 12000*g* for 10 min. The concentration of hemin in this stock solution was determined using the extinction coefficient $\epsilon_{385} = 58.4$ $mM^{-1}$ $cm^{-1}$ in 5 mM NaOH (*37*). For the production of heme−CO, the buffer was pre-equilibrated with 100% CO gas for 15 min. Several granules of dry sodium dithionite followed by hemin (in 5 mM NaOH) were sequentially added. The heme−CO formation was verified spectrophotometrically and the concentration was calculated using $\epsilon_{407} = 147$ $mM^{-1}$ $cm^{-1}$ (*38*). The freshly made solution was immediately used since it was found to undergo rapid changes with time.

*Heme Transfer from Hemoproteins to LDL.* Binding of heme to LDL was assessed fluorimetrically by exploiting fluorescence quenching due to energy transfer to bound heme (*21*, *39*). Intrinsic fluorescence of LDL protein tryptophan (using excitation/emission wavelengths of 280/332 nm, respectively) or extrinsic fluorescence of covalently bound fluorophores was followed. As extrinsic fluorophores, free amino and alcohol groups of LDL were labeled by dansyl (*40*). It should be emphasized that LDL was labeled on the day of the experiment and used immediately afterward, since dansylation is known to increase LDL's susceptibility to oxidation with time. Freshly dansylated LDL was found to retain a similar oxidation kinetic pattern as determined from UVA formation within 4 h of preparation. Dansylation procedure: dansyl chloride in acetone (at a final concentration of 16 μM, less than 1% acetone in sample) was added to 0.8 μM (0.4 mg/mL) LDL in 5 mM $NaHCO_3$-containing

saline. The mixture was incubated for 45 min in ice with stirring, followed by desalting against PBS on PD-10 columns to remove unbound fluorescent dye. About 8−10% of the available amino groups were labeled by this procedure (*41*). The fluorescence intensity of dansylated LDL was measured at $\lambda$ex = 338/em = 504 nm using an SLM-Aminco 8000 spectrofluorimeter (SLM Instruments, Urbana, IL). To minimize quenching of the fluorophores by light absorbance of the hemoproteins (trivial quenching), low (micromolar) concentrations were used and any trivial contribution to quenching (quenching within the mixing time of few seconds) was subtracted. The conserved emission pattern established that indeed most of the fluorophore quenching resulted from radiationless energy transfer to heme (*39*). Since energy transfer occurs only between chromophores in juxtaposition (*39*), quenching of fluorescence intensity served as an indication for heme binding with LDL.

*Quenching of LDL Fluorescence Intensity*. It was used as a tool for assessment of heme translocation to LDL. The term "fluorescence intensity" in the *y*-axis of Figure 5 stands for $100 \times (F_0 - F_t)/(F_0 - F_\infty)$, where $F_0$ is the fluorescence intensity of LDL prior to addition of hemin and/or hemoprotein; $F_\infty$ - fluorescence intensity of LDL 4 h after addition of free hemin; $F_t$ is fluorescence intensity of LDL at any time after addition of a hemoprotein.

*LDL Oxidation Parameters.* For thiobarbituric acid reactive substances (TBARS) (*28*) and SDS−PAGE (*42*), LDL (600 $\mu$g/mL) was incubated for up to 4 h at 37 °C with each hemoprotein (10 $\mu$M) in the presence or absence of $H_2O_2$ (10 $\mu$M). Oxidation products of ApoB were followed by SDS−PAGE with $\beta$-mercaptoethanol using 4.5−12% acrylamide bilayer slabs. Gels were stained with Coomassie Brilliant Blue R-250. For UV absorbance and fluorescent products formation, LDL (100 $\mu$g/mL) was incubated for up to 4 h at 37 °C with different hemoproteins (3 $\mu$M) in the presence or absence of $H_2O_2$ (3 $\mu$M). The kinetics optical density or absorbance in the UV region was followed by measuring absorption spectra every 270 s in the 200−700 nm range.

Lipids oxidation kinetics in LDL was measured by monitoring time-dependent increase in 234 and 268 nm, the maximal or a shoulder of absorbance typical for CD, a product of oxidized lipids (*43*). Production of LDL aggregates along with oxidation will contribute to absorbance. Because light scatter is proportional to 1/(wavelength),[4] the contribution of light scatter at such short wavelength is large. In addition while absorption is linear with concentration, light scatter increases with the amount and size of aggregates not linearly. Both free hemin and hemoglobin induce LDL aggregates upon oxidation (*27*, *50*). Thus, the OD (optical density) at 234 and 268 nm reflect only partially the amount CD formed. Increased UV absorbance (UVA) can still serve as an indication for LDL oxidation by these two inducers but not quantitatively. Throughout the study UVA at 234 nm was used as an indication for LDL oxidation reaction but in experiments employing high LDL concentrations 268 nm was used to reduce contribution from light scatter.

Quantitative comparison of lipid oxidation was assessed as TBARS since this method uses absorption at 532 nm, where light scatter contribution to absorption is negligible. Spectrophotometers used were Uvikon 930 Kontron Instruments, Zurich, Switzerland, and UV/VIS 920 spectrophotometer (GBC, Dandenong, Australia). Bityrosines (*27*) and protein−lipid conjugates (*44*) formation were monitored fluorimetrically (using excitation/emission wavelengths of 327/400−440 nm) using an SLM-Aminco 8000 spectrofluorimeter (SLM Instruments, Urbana, IL).

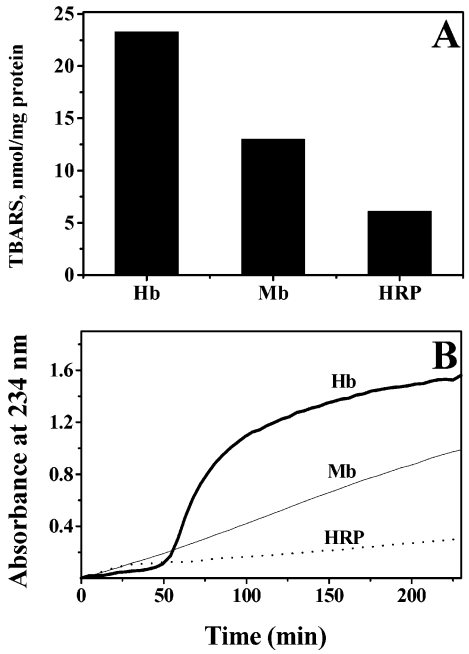


FIGURE 1: Oxidation of LDL lipids by Hb, Mb, and HRP. Reactions were carried out at 37 °C in PBS buffer (pH = 7.4). (A) Amount of TBARS formed within 3.5 h. Reaction mixtures contained 600 $\mu$g/mL LDL, 10 $\mu$M $H_2O_2$, and 10 $\mu$M hemoprotein. (B) Time-dependent elevation of absorbance at 234 nm. Reaction mixtures contained 100 $\mu$g/mL LDL, 3 $\mu$M $H_2O_2$, and 3 $\mu$M hemoprotein: Hb-solid bold line, Mb-solid line, and HRP-dotted line.

## RESULTS

*Oxidation of LDL by Hb as Compared to Other Hemoproteins*. Freshly prepared LDL was incubated simultaneously with equimolar concentrations ($\mu$M range) of each of the three hemoproteins, Hb, Mb (met-forms), and HRP, as well as $H_2O_2$. Figure 1A shows a representative experiment comparing the maximal amount of TBARS formed by the three hemoproteins under identical conditions. As can be seen, Mb induced more TBARS than HRP, but only half of the amount triggered by Hb. Lipid oxidation was also followed using continuous UVA monitoring of increased absorbance at 234 nm, reflecting both CD formation and light scatter of LDL aggregates formed in Hb induced reactions. Figure 1B demonstrates a representative experiment comparing UVA mediated by each of the three hemoproteins. The results indicate that after 3.5 h the relative reaction yield as reflected by increased OD at 234 nm was similar to that exhibited by TBARS, namely, Hb > Mb > HRP. However, the kinetic pattern of the three hemoproteins differed significantly. Oxidation reactions induced by Mb and HRP appeared as a continuous UVA increase, practically monophasic. In contrast, Hb induced a slow OD increase during the lag time and termination phases and much faster increase, in a middle propagation phase.

Previous studies have shown that Hb as well as Mb induces covalent aggregates of the protein ApoB, while the hemoperoxidase HRP, like copper, leads to fragmentation of the

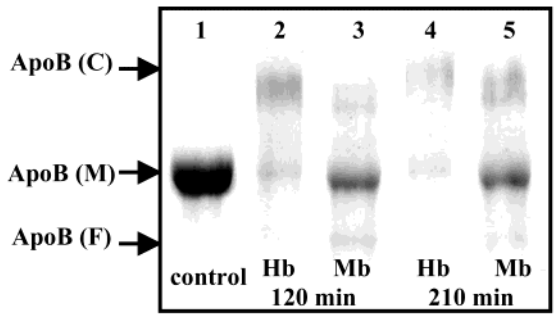


FIGURE 2: Oxidative modification of LDL protein (ApoB) by Hb as compared to Mb. Protein pattern was visualized by SDS−PAGE. LDL (600 $\mu$g/mL) was incubated for 3.5 h at 37 °C in PBS buffer (pH = 7.4). Reaction mixture contained 10 $\mu$M $H_2O_2$ and 10 $\mu$M of one of the hemoproteins. ApoB (C), (M), and (F) indicate cross-linked, monomer, and fragment of ApoB, respectively.

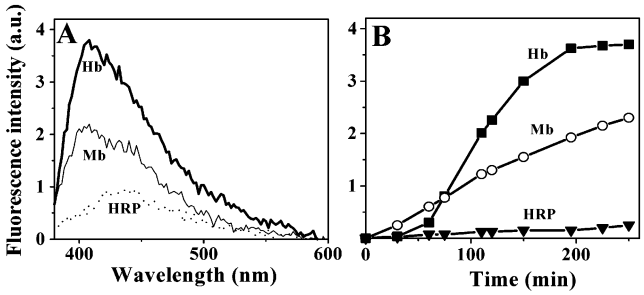


FIGURE 3: Fluorescence characteristics of LDL oxidized by various hemoproteins**.** Reactions were carried out at 37 °C, at pH 7.4 (in PBS buffer) and contained 100 $\mu$g/mL LDL, 3 $\mu$M $H_2O_2$, and 3 $\mu$M hemoprotein. (A) Emission spectra of the reaction mixtures following 4 h of incubation (upon excitation at 327 nm): Hb, solid bold line; Mb, solid line; and HRP, dotted line. (B) Kinetic pattern of the reaction at 400 nm: Hb (■), Mb (●), and HRP (▲).

protein (*16*). To evaluate differences in ApoB oxidative modification by Hb and Mb, we also followed time-dependent changes in the protein pattern of Figure 1A reaction mixtures in SDS−PAGE. We observed that the formation of ApoB covalent aggregates by Mb is less efficient than that induced by Hb. The representative results in Figure 2 show that for this specific LDL, ApoB monomer was almost completely consumed in the Hb containing reaction, but only partial consumption occurred in the Mb containing one. Although both Hb and Mb triggered mainly formation of ApoB covalent aggregates, some fragments appeared as well. However, in the Mb triggered reactions the ratio of fragments to aggregates was larger than that in the Hb induced ones (lanes 3 and 5, Figure 2).

The findings presented in Figures 1 and 2 indicate that although the kinetic pattern of Mb reactivity towards LDL lipids resembles that of the peroxidase HRP, oxidative modification of ApoB is reminiscent of Hb reactivity. To assist analysis of these data, additional parameters indicative of oxidative changes in the LDL protein were followed. As described in previous literature, oxidative modification of ApoB involves typical fluorescence chromophores: lipid−protein conjugates at 430−440 nm region (*44*) and bityrosines at ∼400 nm (*27*). Figure 3A demonstrates the emission spectra of chromophores formed after 4 h in LDL modified by equimolar $H_2O_2$ and one of the three hemoproteins under reaction conditions stated in Figure 1B. As seen, the fluorescence products of the three reaction mixtures differed: Hb and HRP induced a major emission peak, around 400 and 440 nm, respectively, while the Mb-induced reaction exhibited a heterogeneous spectrum composed of these two chromophores. The kinetics of fluorescence intensity at 400 nm in these reactions is shown in Figure 3B. The differences in the kinetic pattern between Hb and Mb triggered reactions (HRP does not contribute) resemble those observed in UVA formation, namely, a three-phase pattern and a higher intensity in the case of Hb and a monophase rate in the Mb-mediated reaction (compare Figures 3B and 1B). Taken together, these data indicate that HRP induces formation of protein−lipid conjugates only, Hb triggers mainly formation of bityrosines, and Mb forms a much smaller amount of bityrosines with a relatively high contribution of lipid−protein conjugates.

*Hb-Mediated LDL Oxidation Is Anchored in Heme Transfer.* Our previous studies, which showed similar fluorescence characteristics of LDL oxidation products in reactions mediated by Hb and free hemin (formation of bityrosines), might indicate the possible involvement of globin-free hemin in Hb-mediated LDL peroxidation (*27*). This option gained support by a recent study demonstrating that hemoglobin oxidized in plasma can exert LDL oxidation via released heme (*12*). However, this possibility was previously ruled out based on the finding that free hemin-induced oxidation was slower as compared to Hb-triggered one (*21*). The plasma proteins, albumin and hemopexin, provide sites for hemin monomers binding. It was shown that those completely structurally different proteins bind hemin with the same kinetics, indicating that the rate of hemin binding to protein depends on dissociation of heme dimers (or higher aggregates) (*45*). Thus, if LDL oxidation relies on availability of hemin monomers, the rate-limiting step of the process should be hemin dimers dissociation. This option was evaluated by continuous monitoring UVA reflecting LDL oxidation (see details in Materials and Methods) triggered by globin-free hemin. Hemin concentration was 3 $\mu$M as in Hb and lower expected to yield increased fraction of monomers. At all hemin concentrations, a three-phase kinetic pattern of increased UVA appeared: lag-time, fast propagation, and slower termination. While the maximal absorbance decreased with total hemin concentration employed, the lag phase became markedly shorter at hemin concentrations of 1 $\mu$M and below, from ∼110 to ∼55 min (Figure 4). At low concentration hemin exists as monomers and the lag time reflects consumption of the LDL antioxidants only. The extension of the lag-time at 3 $\mu$M therefore reflects also the slow dissociation of hemin dimers to monomers (*44*). Thus, hemin monomers associated with LDL are responsible for its oxidative reactivity. The monomerization stage will be spared if the hemin source is a hemoprotein.

To assess transfer of hemin from Hb and other hemoproteins directly to LDL, the following experiments were designed. Freshly prepared LDL was labeled with dansyl (see Materials and Methods), a fluorescence label used previously to follow hemin binding to LDL (*21*). Time-dependent fluorescence intensity of dansylated LDL was monitored after admixing hemin, either in a globin-free form or as one of the following hemoproteins: Hb, Mb, HRP, and catalase. The final amount quenched by free hemin was taken as 100% and the relative quenching by the hemoproteins ($Fe^{3+}$) was assessed as described in Materials and Methods. The results

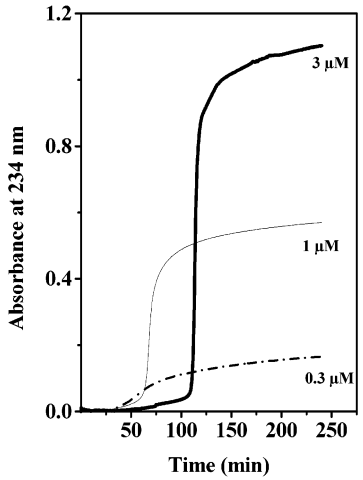


FIGURE 4: Dependence of LDL oxidation rate on globin-free hemin concentration. UVA kinetics measured at 234 nm is shown. Reactions were carried out at 37 °C at pH = 7.4 (PBS buffer) and contained 100 $\mu$g/mL LDL and free hemin: 3 $\mu$M, solid bold line; 1 $\mu$M, solid line; 0.3 $\mu$M, dashed line.

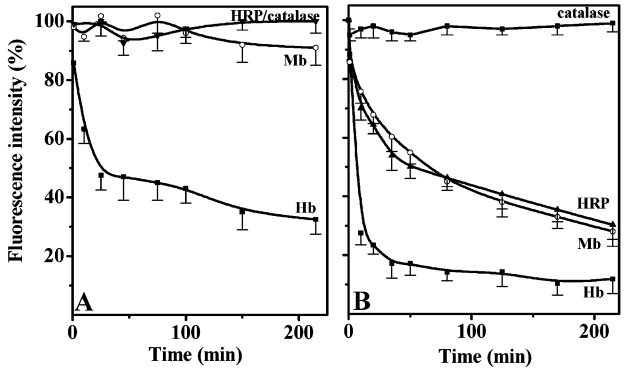


FIGURE 5: Time-dependent quenching of dansyl fluorescence intensity in LDL. (A) Reactions were carried out at 37 °C, at pH 7.4 (in PBS buffer) and contained 100 $\mu$g/mL of dansylated LDL (details concerning dansylation are found in Materials and Methods) and 3 $\mu$M hemoprotein: Hb (■), Mb (○), HRP (▼), catalase (●). (B) Reaction mixtures are as in (A) with additional 3 $\mu$M $H_2O_2$. For details of analysis see Materials and Methods.

presented in Figure 5A show that of all four hemoproteins only Hb caused a time-dependent reduction of fluorescence intensity (~70% that of free hemin) while Mb, HRP, and catalase did not affect the LDL fluorescence intensity. In the presence of $H_2O_2$ (equimolar to heme), the results were different (Figure 5B): the rate of fluorescence intensity quenching by Hb/$H_2O_2$ was faster in comparison to absence of the peroxide, reaching a maximal value (~85% that of hemin) in less than an hour. Catalase remained inactive, but both Mb and HRP triggered fluorescence quenching, although lower and slower than Hb. Under the above experimental conditions $H_2O_2$ alone did not affect the fluorescence intensity of dansylated LDL. Thus, quenching by Mb/$H_2O_2$ and HRP/$H_2O_2$ could be attributed either to heme transfer or peroxidase activity of the hemoproteins. To differentiate between these two options, we used hemopexin (Hx), which forms a stable, high affinity complex with hemin and allows its transfer from methemoglobin (*46*). Advantage was taken of the fact that the Soret band of heme-Hx is at 413 nm while those of HRP, Hb, and Mb (met forms) are relatively blue shifted (403, 405, and 408 nm, respectively). Indeed, the difference spectra in Figure 6 demonstrate hemin transfer

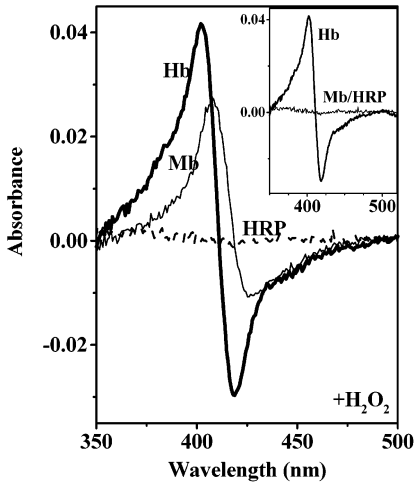


FIGURE 6: Heme detachment from hemoproteins expressed as transfer to Hx. Shown are difference spectra achieved by extraction between initial and final reaction spectra. Reactions were carried out at 37 °C, at pH 7.4 (in PBS buffer) and contained 0.75 $\mu$M hemoprotein, 2.25 $\mu$M Hx in the presence of 0.75 $\mu$M of $H_2O_2$: Hb, solid bold line; Mb, solid line; and HRP, dotted line. Inset: as in main, but in absence of $H_2O_2$.

from Hb and Mb to Hx in preseence of $H_2O_2$, but not from HRP. In the absence of peroxide, heme transfer to Hx occurred from Hb only (inset). In light of the information provided in Figure 6, the fluorescence quenching in Figure 5B should be interpreted as follows: the presence of $H_2O_2$ triggers heme transfer from Mb to LDL, but not from HRP. Thus, the fluorescence quenching inflicted by HRP should be attributed only to its peroxidase activity, bleaching the probe either directly or via oxidized lipids in LDL.

*Spectral Characteristics of the LDL High Affinity Site for Heme.* Heme can associate with a variety of amphipathic sites provided by lipid clusters (*47*) and as a monomer with specific sites in proteins (*48*). As a lipoprotein, LDL potentially offers lipids, protein, or shared heme-binding sites (*49*). The results of the current study have indicated that monomerization of hemin is a prerequisite to accomplish its oxidative activity toward LDL. Assuming that oxidation follows association with LDL, supplementary experiments were required to characterize the high affinity sites of hemin in LDL. To assess binding to the highest affinity sites only, hemin concentration lower than the LDL protein stoichiometry was used (1 $\mu$M hemin and 1.6 $\mu$M (800 $\mu$g/mL) of LDL, ApoB). Hemin was mixed with LDL by adding an aliquot from freshly prepared water phase stock solution. A new Soret absorption spectrum developed within a few minutes, reaching a maximum at 403 nm within approximately half an hour (inset of Figure 7), as shown in a previous study (*27*). The spectra of hemin in buffer alone and in LDL containing buffer are shown in the inset to Figure 7. Hemin ($Fe^{3+}$) was monomerized in one part of the stock solution by turning it into heme−CO (see methods). The Soret of heme−CO in buffer appeared at 407 nm (Figure 7) as published in previous literature (*51*). Admixing heme−CO aliquot with LDL containing buffer resulted in an instantaneous red-shifted Soret band with a maximum at 422 nm (Figure 7). Since heme−CO exists only as a monomer in the water phase, the latter shift (from 407 to 422 nm) is indicative of binding of the heme−CO to a specific site in LDL. The heme−CO−LDL complex was extremely short-

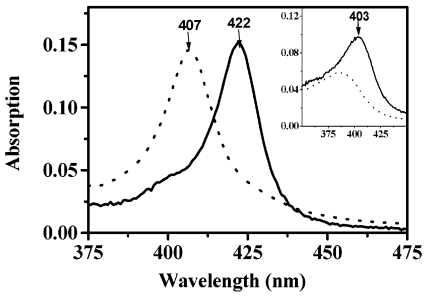


FIGURE 7: Binding of heme−CO to LDL. Absorbance spectra of 1 $\mu$M heme−CO; alone (dashed line) or in the presence of 0.8 mg/mL (1.6 $\mu$M) LDL (solid line). Inset: Absorbance spectra of 1 $\mu$M hemin; alone (dashed line) or in the presence of 0.8 mg/mL (1.6 $\mu$M) LDL (solid line). Details concerning heme−CO preparation can be found in Materials and Methods. Buffer PBS, pH = 7.4.

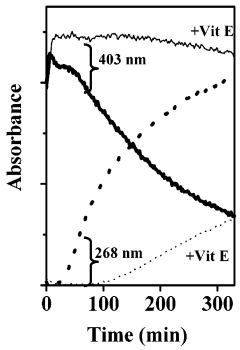


FIGURE 8: Correlation between heme disintegration and susceptibility of LDL to oxidation. Heme−CO (1 $\mu$M) was associated with 0.8 mg/mL LDL protein (1.6 $\mu$M ApoB) at 37 °C in CO-saturated PBS buffer. Part of the LDL was enriched with 1 mg/mL α-tocopherol (VitE). Time dependence of absorbance at 268 nm (dotted lines) and 403 nm (full lines) are shown. Absorbance scales: each unit stands for 0.1 OD at 268 nm and 0.0185 OD at 403 nm.

lived and within a few minutes was completely transformed into hemin−LDL, as judged from conversion of the Soret 422 nm band of heme−CO−LDL to hemin−LDL characterized by 403 nm. The 422 nm spectrum demonstrated in Figure 7 was obtained within 10−15 s, the shortest possible time for injecting/mixing plus spectral screening. Although more stable, the typical Soret of hemin−LDL decayed and finally was completely abolished. This scenario of events is demonstrated by 403 nm kinetics in Figure 8 (bold solid line) including a fast initial increase followed by a slower decrease. It should be emphasized that in the above process the heme iron ($Fe^{2+}$) lost the CO ligand along with undergoing oxidation to $Fe^{3+}$, thus forming hemin−LDL. Enrichment of LDL with a large excess of vitamin E was required to gain steady 403 nm Soret absorbance (solid regular line). Even under these oxidation-protective conditions, oxidation of $Fe^{2+}$ to $Fe^{3+}$ (thereby conversion of heme−CO−LDL to hemin−LDL) could not be inhibited. These changes are observed as fast initial increase in OD 403 nm (Figure 8 regular full line). In conjunction with measuring kinetics at 403 nm, time-dependent changes in UVA region followed in the same reaction mixtures at 268 nm (dashed lines, Figure 8). The latter wavelength was used instead of 234 nm used in Figures 1 and 4 to reduce the large light scatter contribution at the high LDL concentration used in these experiments (800 $\mu$g/mL as compared to 100 $\mu$g/mL in experiments 1 and 4). As seen, the vitamin E-enriched LDL exhibited an

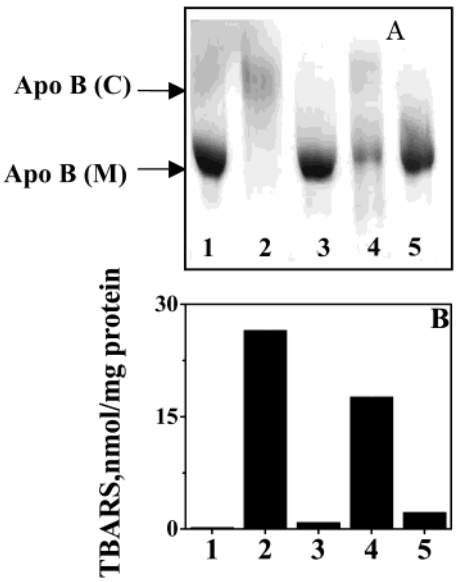


FIGURE 9: Effect of Hx on LDL oxidation induced by Hb as compared to Mb. LDL (600 $\mu$g/mL) was incubated at 37 °C for 4 h with the following compounds: alone or in the presence of 10 $\mu$M $H_2O_2$ (lane 1); 10 $\mu$M Hb + 10 $\mu$M $H_2O_2$ (lane 2); 10 $\mu$M Hb + 10 $\mu$M $H_2O_2$ + 30 $\mu$M Hx (lane 3); 10 $\mu$M Mb + 10 $\mu$M $H_2O_2$ (lane 4); 10 $\mu$M Mb + 10 $\mu$M $H_2O_2$ + 30 $\mu$M Hx (lane 5). (A) Oxidative protein modifications were visualized by SDS−PAGE. The top part of the gel is shown. ApoB (C) and (M) indicate cross-linked and monomer of ApoB, respectively. (B) Oxidative lipid modification as followed by TBARS formation.

extended lag time (100 min as compared to 25 min), followed by a slow increase in UVA.

*Protection of LDL from Hb Oxidation by Hemopexin.* Having indicated that even VitE enrichment cannot completely rescue LDL from an oxidative attack, we searched for vasculature components that can inhibit hemin transfer from metHb to LDL. Previous studies indicated that hemin can transfer from hemoglobin in met form to Hx (*46*) and can also inhibit Hb-induced oxidation (*50*). On the basis of the current observations, it appears that prevention of Hb-derived LDL oxidation by Hx might relate to its ability to compete with LDL on heme transfer from Hb. Dissociation of the hemin monomer from metHb is a slow process relative to its association with LDL, and thus expected to be the rate-limiting step in its transfer to LDL and Hx. Previous estimation from fast kinetics binding of heme−CO to LDL (ref *51*, Table 2) did not consider the instantaneous oxidative transformation of heme−CO−LDL to hemin−LDL revealed in the current study. Thus, the distribution of heme between LDL and Hx was estimated using transfer studies. We observed that in mixtures of metHb, LDL, and Hx, the Soret spectrum was shifted immediately to that of hemin−Hx (data not shown). We therefore followed oxidation of the LDL lipids (as TBARS) and protein (by SDS−PAGE), in the presence of hemopexin, by the hemoproteins which potentially can deliver hemin to LDL, Hb, and Mb. Oxidative cross-linking induced by both hemoproteins was completely abolished in the presence of Hx (Figure 9A: compare lanes 3 and 2 and 5 and 4). In Hb containing reaction mixtures, lipids oxidation was practically inhibited by Hx (Figure 9B: 1 ± 0.2%). However, in the Mb-mediated LDL oxidation, lipids were partially oxidized, even in the presence of Hx (Figure 9B: 11 ± 0.5%). These results clearly indicate that

LDL-trapped hemin is completely responsible for Hb-triggered LDL oxidation and partially for Mb-induced oxidation.

## DISCUSSION

In light of a long-standing suggestion that LDL oxidation correlates with atherosclerosis, it is only natural to suspect that the iron carrying hemoproteins, which topographically might contact LDL in the vasculature, trigger the process. The major in vivo candidates reported by many studies as active oxidation mediators are hemoperoxidases: myeloperoxidase (*52*) or HRP (*53*), as its model, and the oxygen binding proteins, myoglobin (*15*) and hemoglobin (*20*). Another common assumption is that globin-free hemin is responsible for the iron-derived LDL vascular damage (*12*, *28*). While it was accepted that hemoperoxidases operate intactly via their heme-iron site, the surprisingly high peroxidative activity of the oxygen binding proteins had a variety of explanations. This situation relates to the enormous information accumulated throughout the years on peroxidatively active candidates that might be derived from the oxygen-binding hemoproteins. Globin radicals (*21*, *54*, *55*), covalently cross-linked heme−globin (*24*, *25*), globin-detached hemin (*12*, *56*) as well as free iron liberated from oxidatively disintegrated hemin (*57*) were suggested as such candidates. Attempts were made to use all these options to provide grounds for a mechanism by which hemoglobin and myoglobin iron oxidative activity impairs the vascular system (*12*, *15*, *21*, *22*). We feel that the results of the present study have clarified the picture.

The results of Figures 1−3 show that Hb activity toward LDL lipids and protein differs from that of Mb and HRP, both quantitatively and qualitatively. The qualitative differences are demonstrated by the completely dissimilar kinetic pattern of UVA triggered by Hb as compared to HRP. Hb-induced kinetics is composed of a lag time, a burst phase, and slower termination, while that of HRP is monophasic. In fact, we previously demonstrated that the LDL protein oxidative products triggered by Hb and HRP vary completely: cross-linking of ApoB by Hb but its fragmentation by HRP (*16*). The results of the present study indicate that cross-linking of ApoB by Hb is linked to bityrosines formation, while degradation of the protein by HRP correlates with formation of lipid−protein conjugates (Figures 2 and 3). Mb exhibits mixed features of Hb and HRP activity (Figures 1−3).

It thus appeared that the mechanisms by which the three hemoproteins operate differ. The differences were clarified by analyzing heme transfer from the above-mentioned hemoproteins to LDL. It appeared that Hb ($Fe^{3+}$) is capable of transferring large amounts of hemin into LDL, both in absence and presence of external peroxides (Figure 5). In the absence of $H_2O_2$, Mb and HRP were unable to transfer heme to the lipoprotein. Upon peroxide addition, no hemin was transferred from HRP, but Mb did exhibit a partial heme release. We therefore concluded that the differences demonstrated by the three hemoproteins regarding the LDL oxidative pattern are derived from their ability to transfer hemin. Thus, Mb inactivity reported recently by Jeney and co-workers can be explained by the lack of heme transfer in the absence of sufficient peroxide under the conditions used by them (*12*).

The hemoproteins used in this study to induce LDL oxidation can be divided into two groups based on their UVA kinetic patterns: the first including HRP and Mb and the second Hb. In the first group activity resides in the intact hemoproteins and shows continuous elevation of the absorbance resulting mainly from formation of conjugated dienes. As a classic catalyst HRP remains intact using external peroxide as well as internal LDL peroxides (*69*). Mb, a much poorer peroxidase than HRP when acting on small substrates, turns more efficient toward LDL lipids. This might be explained by support gained from the globin radical formed in the process of Mb peroxidation. In the second group, presented by hemoglobin, we observed a completely different kinetic pattern of UVA propagation reminiscent of globin-free hemin triggered changes. In both cases, there are two slow UVA phases and a middle, much faster UVA elevation. Kinetic dissimilarities in the action of free hemin and hemoglobin hemin can be explained on the basis of the rates by which the hemin monomer reaches its high affinity site where it acts fast. Globin-free hemin associates fast with the LDL surface but requires monomerization to attach to its activity site. Thus, the three kinetic phases include: no activity, sharp UVA increase, and termination. In the hemoglobin-induced reaction, hemin is ready to be delivered as a monomer, reaching fast the high activity site but the rate of accomplishment of the reaction is dependent on the slower rate of heme transfer. As a result typical UVA kinetics (Figure 1B) consists of three phases but appears less segregated. In this case, the overlapping heme transfer rate results in the first phase of immediate minimal UVA elevation, a slower propagation phase and a longer termination phase as compared to free hemin triggered reaction. It is interesting to note in this context that under extreme conditions such as harsh acidification which allow heme transfer from myoglobin, the three-phase kinetic of UVA elevation appears in LDL peroxidation by this hemoprotein as well (*13*).

An important point to note is that the size of the signal of the middle, fast phase, in the case of hemoglobin and free hemin has a significant contribution from the light scatter of LDL aggregates (formed as oxidative product), while the contribution of conjugated dienes is only minimal in this case. As stated in the methods, the light scatter from the aggregates highly depends on their size which in turn depends on the concentration of LDL particles in the reaction mixture. At high concentration of LDL the light scatter from aggregates might already contribute to the UVA of the lag phase, and the constantly growing aggregates could possibly add long-lasting increased UVA. This is best seen by variations in the UVA kinetic pattern in Figures 1 and 8. The lag phase in Figure 8 is short, and there is no clear separation between propagation and termination phases.

Once realizing that heme transfer from Hb to LDL is responsible for its oxidation, we analyzed its potential high affinity binding sites in LDL. As already presented in a variety of studies, hemin can associate with proteins and lipids, both present in LDL. We previously described hemin binding to LDL, but the sites were not identified (*27*). Taking into consideration that a variety of sites are involved, we concentrated in this study on the highest affinity sites in the light of their responsibility for trapping residual excellular Hb hemin. The spectral light absorption characteristics of

LDL-associated hemin described in this study coincide with our conclusion that hemin binds only as a monomer to LDL (inset to Figure 7), but could not differentiate between lipids and protein potential sites. To ensure binding of heme only as a monomer to LDL, heme−CO was used. Sub-micromolar concentrations of heme−CO, as compared to molarity of LDL single protein were employed to emphasize only spectral characteristics of the high affinity heme-site in LDL. Our study revealed that the Soret spectrum of LDL-bound heme−CO at 422 nm does not correlate with lipid-bound heme−CO, since the latter is characterized by the Soret absorption at 415 nm (*58*). In contrast, the Soret peak at 422 nm fits association of heme with the LDL protein since it is included in the region of other proteins which are not in the category of hemoproteins but provide high affinity sites for heme−CO, such as hemopexin, myosin, calmodulin, and guanylate cyclase (*59*−*61*).

The finding that hemin binds to the LDL protein sheds light on the presumably contradicting results regarding Hb oxidative potential toward LDL and vesicles composed of lipids only. While Hb triggers oxidation of LDL lipids at physiological pH, it fails to produce the same effect on lipids in protein-devoid systems under the same conditions (*63*). This is explained in light of the requirement of heme transfer from Hb. Because lipids have much lower affinity for hemin, they fail to compete with globin, unless the heme−globin bond is weakened by reduction of the pH. In fact, hemin which associates with a variety of proteins in lipid-devoid systems can induce peroxide-dependent oxidation of these proteins, which results in bityrosine and protein aggregate formation, only that in these cases an increased peroxide amount is required as compared to LDL. Examples are red cell membrane proteins, such as spectrin, and muscle proteins, such as myosin (*60*, *64*). The LDL particle is unique in providing high affinity protein sites and the lipid peroxides that are mingled in the particle surface. It should be emphasized that favored oxidation of ApoB in LDL by an active oxidant is not unique to hemin. It was recently demonstrated that peroxynitrite, an in vivo relevant oxidant, attacks preferentially the protein (*65*).

Unlike other proteins known to associate with hemin, such as myosin, the complex hemin−ApoB is destroyed in the oxidation process, and even a high affinity ligand like CO cannot protect its destruction (Figure 8). Large amounts of the strong endogenous antioxidant VitE also fail to prevent transformation of heme−CO−ApoB to hemin−ApoB. It appears that hemin disintegration is a prerequisite for its activity. We reason that Hb exerts its oxidative activity on LDL via iron, released from disintegrated hemin, attached to the protein. Thus, it appears that Hb-derived iron reacts with LDL similarly to copper via binding to ApoB, followed by both lipid and protein oxidation, only that copper can reach its binding site directly, while iron requires the Hb heme as a vehicle. The above conclusion calls for a comparison between the oxidative activity of these two metals. The main difference applicable for in vitro experiments complies to the chemical nature of the metals, copper being water soluble as opposed to iron (e.g., insoluble as phosphate available usually in buffers). Thus, copper hits its protein-binding site directly and lipid oxidation lag time is derived from antioxidant consumption only. Iron, on the other hand, requires mediation by hemoproteins available in vivo, and the lag time of its activity involves transfer from hemoprotein, mostly Hb. Although both metals operate via binding to the ApoB site (*5*), the sites are different as determined from different oxidative products. Copper oxidation results in lipid−protein conjugate formation, which is parallel to the degradation of the protein, similarly to the HRP oxidative activity.

The results of the current study correlate with our previous indication (*50*) that the Hb oxidative activity can be inhibited by hemopexin. The present study provides the mechanism for Hx inhibition by showing that Hx competes well with LDL for heme transfer. This is true for both Hb and Mb globin-released heme (Figure 9). Thus, the present study establishes that unlike albumin (*66*), hemopexin is an efficient plasma component that will prevent Hb mediated oxidative pathology. Indeed, the existence of hemopexin not only in circulation, but also in various cellular compartments, like the brain, points to its ubiquitous activity as a heme trap (*67*).

An important issue to be pointed out relates to the concept of oxidatively damaged LDL as being unequivocally atherogenic. Recent studies have shown that oxidation of LDL components by itself does not suffice to turn LDL into an atherogenic form and their aggregation is required (*68*). Unlike the vastly analyzed copper oxidized LDL, the heme transfer mechanism, by which Hb (and to a lesser extent Mb) operates to oxidize LDL, results in an irreversible LDL aggregation. The experiments of the present study involving Mb, which transfers minor amounts of hemin (Figures 2 and 3), indicate that the high activity of iron released from disintegrated hemin has a major contribution to LDL aggregation, namely, atherogenic oxidation.

Finally, the current study has shown that the high activity of hemoglobin stems from heme transfer to LDL protein and further oxidative disruption of the hemin to release iron, thereby propagating the peroxidation process. Whether or not such a process will take place under a given situation in vivo depends on the blood system. Two plasma proteins protect the vascular system from Hb-induced LDL oxidative modification, haptoglobin and hemopexin (*21*, *50*). Therefore, the Hb derived pathology will take place only in their absence. Two situations should be considered. The first relates to periodic appearance of high level of excellular Hb overcoming the inhibiting proteins capacity. The second comprises pathological conditions existing in chronic hemolytic anemias where constant low level hemoglobin is released to the vascular system. The first situation might exist in individuals treated by hemodialysis which are known to develop atherogenesis (*9*). In the second category, no direct correlation to atherosclerosis exists although oxidative modification of LDL has been reported in hemolytic anemia like $\beta$-thalassemia (*70*). In the hemolytic anemias hemopexin and haptoglobin are low but not completely absent. This is because most of the hemoglobin is removed by spleen and liver macrophages which recognize the deteriorated red cells prior to their vascular rupture. In a situation like hemodialysis there is massive mechanic rupture of healthy red cells with no apoptotic signals to be removed by spleen macrophages. Thus, under such conditions the vascular system is expected to lack defense and heme transferred from the methemoglobin finds its way to the vasculature LDL.

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BI020647R