Oxidation of Low-Density Lipoprotein by Hemoglobin Stems from a Heme-Initiated Globin Radical: Antioxidant Role of Haptoglobin

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ABSTRACT: Hemoglobin, known as a poor peroxidase, has been recently found to be a highly reactive catalyzer of low-density lipoprotein (LDL) oxidation resulting in oxidation of LDL lipids and covalent cross-linking of the LDL protein, apo B. We evaluated three possible mechanisms that may account for hemoglobin reactivity: oxidative activation by globin-dissociated hemin following its transfer to LDL; peroxidase-like reactivity of the ferryl iron active state in intact hemoglobin; and oxidation by a globin radical formed in oxidized hemoglobin. The first mechanism was ruled out because only a minor fraction of hemin was actually transferred to LDL in the process of oxidation. The second mechanism was excluded because hemoglobin ferryl, unlike ferryl of horseradish peroxidase, was not consumed in the process of LDL oxidation. Haptoglobin completely inhibited cross-linking of globin in hemoglobin/H_2O_2 mixtures but not in myglobin/H_2O_2, as well as cross-linking of apo B and oxidation of LDL lipids. Haptoglobin could not however abolish the hemoglobin ferryl state, a finding that further supported exclusion of the second mechanism. We conclude that the active species in hemoglobin-induced LDL oxidation is the globin radical, as suggested in the third mechanism. The present findings also show that haptoglobin functions as a major antioxidant thus protecting the vascular system.

An accumulating body of information testifies to the crucial role played by oxidized low-density lipoprotein (LDL)[1] in the pathogenesis of atherosclerosis (Steinberg et al., 1989; Esterbauer et al., 1992; Rice-Evans & Bruckdorfer, 1992; Hoff & Hope, 1995; Berliner & Heinecke, 1996; Hazell et al., 1996). While most *in vitro* studies have used copper to oxidize LDL, the *in vivo* trigger(s) of LDL oxidation are still a matter of controversy. Among the cells of the vascular system, neutrophils, endothelial cells and monocyte-derived macrophages have been shown to trigger LDL oxidation (Berliner & Heinecke, 1996). Among molecular components of the plasma, both globin-free hemin[2] and intact hemoglobin (Hb) were shown to function as active catalyzers of LDL oxidation (Balla et al., 1991; Paganga et al., 1992; Miller & Shaklai, 1994; Miller et al., 1996a). Oxidative reactivity of Hb toward vascular components is normally avoided by retention of Hb within the red blood cells. Nevertheless, as Hb concentration in the red cells is in the millimolar range, a minimal cell rupture—a frequent event even in healthy individuals—suffices to achieve local micromolar concentrations of extracellular Hb. In the event of trauma and/or medical treatment such as transfusion, hemolysis is more profuse and Hb concentrations may reach micromolar levels in whole plasma (Henry, 1991). Because Hb-mediated oxidation is accelerated by the presence of an oxidant like H_2O_2 (Miller et al., 1996a), Hb-derived LDL oxidation is expected to be more extensive under inflammatory conditions.

We recently demonstrated that the protein products of LDL oxidized by either hemin or intact Hb are covalent aggregates of apolipoprotein B (apo B) (Miller & Shaklai, 1994; Miller et al., 1996a). As each LDL particle contains one apo B molecule, intermolecular cross-linking of apo B should result in aggregation of LDL. Formation of oxidized LDL aggregates was shown to facilitate LDL uptake and accumulation in macrophages, a crucial event in the development of atherosclerosis (Berliner & Heinecke, 1996; Hoff & Hope, 1995; Jessup et al., 1992; O'Connell et al., 1994). In correlation with this sequence of events are recent findings indicating that the material of atherosclerotic lesions reacts with monoclonal antibodies raised against oxidatively cross-linked apo B (Hazell et al., 1996). On the basis of the above information, it was suggested that Hb should be considered as an *in vivo* trigger of vascular oxidations that lead to atherosclerosis.

The mechanism by which Hb exerts high oxidative reactivity toward LDL is yet unclear. The first option which comes to mind is that free iron, released from oxidized Hb, catalyzes LDL oxidation. However, non-heme iron has been found unable to induce any LDL oxidation (Balla et al., 1991). As Hb does not associate with LDL (Miller et al., 1996a), it cannot serve as a vehicle to insert into LDL free iron, trace amounts of which can be released upon interaction of Hb with a peroxide (Panter, 1994). Excluding Hb-dissociated iron as responsible for Hb reactivity leaves the option of heme-integrated iron as the key element in Hb oxidative reactivity. Although heme is tightly bound to globin in both Hb and myoglobin, these non-covalent bonds become weaker in oxidized Hb produced in the presence of a peroxide like H_2O_2 (Bunn & Jandl, 1968; Banerjee, 1962).

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[1] Abbreviations: LDL, low-density lipoprotein; Hb, hemoglobin; apo B, apolipoprotein B-100; HRP, horseradish peroxidase; PMSF, phenylmethylsulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TBARS, thiobarbituric acid reactive substances; CcP, cytochrome *c* peroxidase.

[2] The term "hemin" is used for the protein-free iron(III)-protoporphyrin IX, while the term "heme" is used for the protein-bound form independent of the iron oxidation state.

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This allows transfer of heme (Fe^{III}) to plasma proteins like albumin and hemopexin (Bunn & Jandl, 1968; Banerjee, 1962). Globin-free hemin readily associates with LDL and exerts marked oxidative activity (Balla et al., 1991; Miller & Shaklai, 1994). Thus, the high oxidative reactivity of Hb toward LDL may result from globin-free hemin transferred to LDL rather than from activity of the intact Hb molecule. On the other hand, Paganga et al. (1992), who first described Hb-induced LDL oxidation, observed a correlation between the levels of Hb ferryl state and oxidized LDL and suggested that ferryl-Hb is responsible for LDL oxidation. In agreement with this interpretation are conclusions of a recent study implying that in myoglobin, which reacts similarly to Hb, the peroxidase active site consists solely of the heme crevice in the process of linoleic acid peroxidation (Rao et al., 1994). Inconsistent with these conclusions were our observations that Hb (as well as myoglobin) is more active than horseradish peroxidase (HRP), a highly active conventional heme-peroxidase that provides a much higher ferryl reactivity than Hb or myoglobin (Miller et al., 1996a). A number of studies suggested that in H_2O_2-oxidized Hb and/or myoglobin, tyrosine, tryptophan, and possibly histidine-centered radicals are formed (Shiga & Imaizumi, 1975; Tew & Ortiz de Montellano, 1988; Giulivi & Davies, 1990; McArtur & Davies, 1993; Gunter et al., 1995; Gorbunov et al., 1995; Tschirret-Guth & Ortiz de Montellano, 1996). Accordingly, it was suggested that a surface globin radical rather than the ferryl center may be responsible for the high reactivity of Hb and myoglobin as catalyzers of LDL oxidation (Miller et al., 1996a).

In light of the currently available information, there are three candidate mechanisms that could possibly account for the catalytic activity of Hb in LDL oxidation. In the first, reactivity is derived from globin-free hemin released from Hb in the presence of H_2O_2. In the other two mechanisms heme initiates the oxidative reaction by intact Hb; oxidation then operates, according to one option, directly through the ferryl heme, and according to other, via the protein radical.

The common basis for the three mechanisms is that the unique relationship between the protein (globin) and heme in Hb/myoglobin underlies their high oxidative reactivity toward LDL. It was therefore thought that haptoglobin, which binds tightly to the globin part of the Hb (Nagel & Gibson, 1971; Hwang & Greer, 1980; McCormic & Atassi, 1990) yet permits the heme to function (Waks et al., 1963), might serve as a tool for analyzing the specific relationship of heme to globin in the presence of the peroxide H_2O_2. Using haptoglobin, we show here that the high Hb reactivity toward LDL is provided by the globin radical. Our findings also shed light on an antioxidant role of haptoglobin in the vascular system.

EXPERIMENTAL PROCEDURES

Chemicals. Human haptoglobin (from pooled plasma), bovine hemin, horseradish peroxidase type X (HRP), 5-dimethylaminonaphthalene-1-sulfonyl (dansyl) chloride, phenylmethylsulfonyl fluoride (PMSF), and Chelex-100 were purchased from Sigma Chemical Co., St. Louis, MO. Monobromobimane (mBBr) was purchased from Calbiochem, La Jolla, CA. Hydrogen peroxide was from Merck, Darmstadt, Germany. DEAE-Sepharose CL-6B was from Pharmacia, Uppsala, Sweden, and DE-52 cellulose was obtained from Whatman International, Maidstone, England. Chemicals for sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) were purchased from Bio-Rad Laboratories, Richmond, CA.

Isolation of LDL and Hb. LDL was isolated from human plasma by sequential ultracentrifugation as previously described (Schumaker & Puppione, 1986; Miller et al., 1996a). Hb was isolated from red blood cell lysates by ion-exchange chromatography using DEAE-Sepharose CL-6B (Hebbel et al., 1988) followed by gel chromatography on a Sephadex G-100 column to get rid of any residual catalase. Lack of catalase was judged by ferryl-Hb formation in presence of a small molar excess of H_2O_2. This Hb was verified spectrophotometrically as oxy-Hb (Antonini & Brunori, 1971). Met-Hb was prepared from the oxy-Hb (Antonini & Brunori, 1971) and before use, it was purified by admixing with DE-52 cellulose for 5 min followed by centrifugation to remove globin-free hemin contaminants (Ueno et al., 1982). Concentrations of Hb, myoglobin and HRP are expressed in heme equivalents throughout this study.

Association of Heme with LDL. Binding of heme to LDL was assessed fluorimetrically by exploiting fluorescence quenching due to energy transfer to bound heme (Förster, 1965; Miller et al., 1995). Intrinsic fluorescence of LDL protein tryptophans (using excitation/emission wavelengths of 285/340 nm, respectively) or extrinsic fluorescence of covalently bound fluorophores was followed. As extrinsic fluorophores, free amino or sulfhydryl groups of LDL were labeled by dansyl or mBBr, respectively (Solar et al., 1989; Kosower et al., 1979). 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 on ice with stirring, followed by dialysis (two changes) against PBS to remove unbound fluorescent dye. About 8−10% of the available amino groups were labeled by this procedure (Olofson et al., 1987). The fluorescence intensity of dansylated LDL was measured at 350/500 nm using an SLM-Aminco 8000 spectrofluorometer (SLM Instruments, Urbana, IL). Labeling with mBBr: mBBr in acetonitrile (at a final concentration of 8 μM, less than 1% acetonitrile in sample) was added to 2 μM (1 mg/mL) LDL solution in PBS. The mixture was incubated for 1 h at 37 °C, followed by dialysis (two changes) against PBS. All three available apo B sulfhydryl groups were labeled by this procedure (Sommer et al., 1991). The fluorescence intensity of mBBr-labeled LDL was measured at 380/460 nm.

To minimize quenching of the fluorophores due to light absorbance of hemin and/or Hb (trivial quenching), low concentrations of hemin and/or Hb were used and their contribution to quenching was subtracted. That indeed most of the fluorophore quenching resulted from radiationless energy transfer to heme was evident from the conserved emission pattern (Förster, 1965). Because energy transfer occurs only between chromophores in juxtaposition (Förster, 1965), quenching of fluorescence intensity served as an indication for heme binding with LDL. Because fluorescence quenching is not linear with the concentration of a quencher, calibration curves of fluorescence intensity versus distinct hemin concentrations were used for quantitation of bound heme.

Quantitation of Hb Oxidation States. In order to determine the distribution of the three Hb states, oxy (Fe^{II}), met (Fe^{III}), and ferryl (Fe^{IV}), within each reaction mixture, absorption was measured at wavelengths of 560, 576, and 630 nm using a HP8452A diode array spectrophotometer (Hewlett-Packard Co., Waldbronn, Germany). Absorbance at 700 nm served as the base line. The concentrations of the three Hb components, expressed in μM, were calculated using the reported extinction coefficients (Whitburn et al., 1982; Winterbourn, 1990) according to the following equations:

For reactions starting from met-Hb:

$$\text{met-Hb} = -116A_{576} + 409A_{630} \quad (1)$$

$$\text{ferryl-Hb} = 143A_{576} - 172A_{630} \quad (2)$$

For reactions starting from oxy-Hb:

$$\text{oxy-Hb} = 23A_{560} + 81A_{576} - 205A_{630} \quad (3)$$

$$\text{met-Hb} = -117A_{560} + 66A_{576} + 219A_{630} \quad (4)$$

$$\text{ferryl-Hb} = 149A_{560} - 86A_{576} + 72A_{630} \quad (5)$$

We avoided available equations for calculation of Hb oxidation that use the wavelength of 490 nm because β-carotene in LDL significantly contributes to absorption at this wavelength (Rice-Evans & Bruckdorfer, 1992), and thus its oxidative consumption masks the oxidative changes of Hb. Concentrations of HRP compound **II** were calculated using available extinction coefficients at 398 nm, an isobestic wavelength for compounds I and II, and at 430 nm, isobestic for compound I and resting HRP (Hayashi & Yamazaki, 1979).

$$\text{HRP compound II } (\mu\text{M}) = -4.7A_{398} + 19.7A_{430} \quad (6)$$

LDL Oxidation Experiments. LDL (600 μg/mL) was incubated for up to 3 h at 37 °C with several oxidation inducers (10 μM) in the presence of a 50% molar excess of H_2O_2. (Small excess of H_2O_2 was used in order to avoid destrucion of Hb.) Hb-induced oxidation products of apo B were followed by SDS−PAGE (Laemmli, 1970) with β-mercaptoethanol using 6% and 12% acrylamide bilayer slabs. Reactions containing Hb alone were run on homogenous 12% acrylamide slabs. Gels were stained with Coomassie Brilliant Blue R-250. Oxidation of LDL lipids was determined in terms of thiobarbituric acid reactive substances (TBARS) (Balla et al., 1991) and of conjugated dienes by absorbance at 234 nm (Esterbauer et al., 1992) using an Uvikon 930 spectrophotometer (Kontron Instruments, Zurich, Switzerland)

All experiments were performed at least in triplicate, and the data obtained are presented as mean values. In some cases a representative experiment is given.

RESULTS

Can Heme Be Transferred from Hb to LDL During Oxidation?

In a previous study we showed that free hemin, but not intact Hb, can bind to LDL (Miller et al., 1996a). Thus, any association of heme with LDL in a Hb/LDL mixture should reflect transfer of globin-free hemin to LDL. Therefore, to search for heme transfer from Hb to LDL under oxidative conditions, we compared the time course of heme association with LDL in the presence of H_2O_2 when added as free hemin and when added as Hb (oxy- or met-). The amount of LDL-associated heme was assessed by fluorescence quenching of dansylated LDL (for details see experimental section). At the same time, we monitored the time-dependent oxidation of LDL lipids, as expressed by conjugated dienes (Figure 1). Binding of free hemin to LDL was instantaneous (shorter than the fluorometer response time) and complete, but lipid oxidation developed only after a lag phase of about 1 h (Figure 1A). Admixing of oxy-Hb and LDL with H_2O_2 did not result in either heme tranfer or LDL lipid oxidation within a time period of about 20 min (Figure 1B). After this lag time, a low heme transfer (~10%) was observed concomitantly with a burst of lipid oxidation (Figure 1B). Met-Hb behaved similarly to the oxy form, except that the lag phase was shorter and the fraction of LDL-bound heme was somewhat larger (Figure 1C). There was no fluorescence quenching nor lipid oxidation in the hemin/Hb-lacking samples. In the same experiments, replacement of dansyl by the intrinsic LDL fluorophore, tryptophan, or by mBBr yielded similar results (not shown). The small fraction of LDL-bound heme in the oxy- and met-Hb reaction

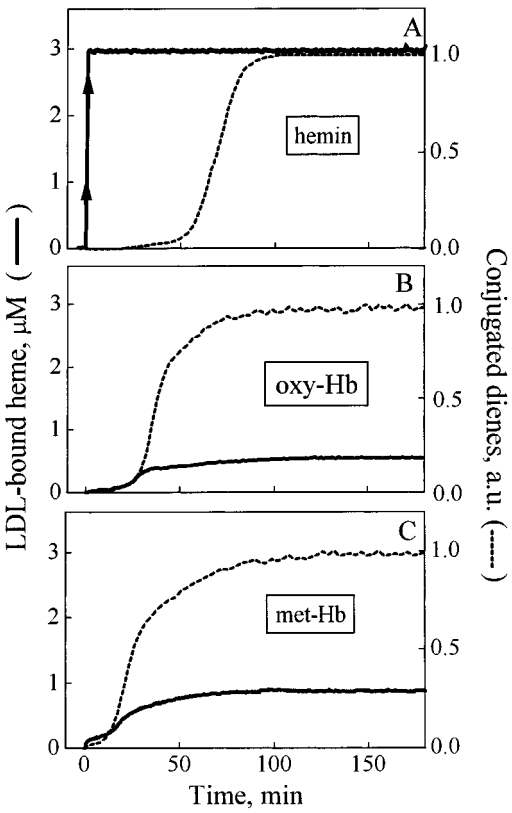

FIGURE 1: Time courses of heme association with LDL and of oxidation of LDL lipids. Dansyl-labeled LDL (200 μg/mL) was mixed with 3 μM hemin (A), oxy-Hb (B), or met-Hb (C), and 4.5 μM H_2O_2 at 37 °C. Time course of dansyl fluorescence intensity (excitation/emission at 350/500 nm, respectively) in these reaction mixtures was measured concomitantly with that of absorbance at 234 nm and 700 nm. Concentrations of LDL-bound heme (solid traces) was calculated from the fluorescence quenching, as described in Experimental Procedures. The optical density at 234 nm, representing the estimated amount of conjugated dienes (dashed traces), was normalized to a maximal value in each case. Absorbance at 700 nm was used as a base line.

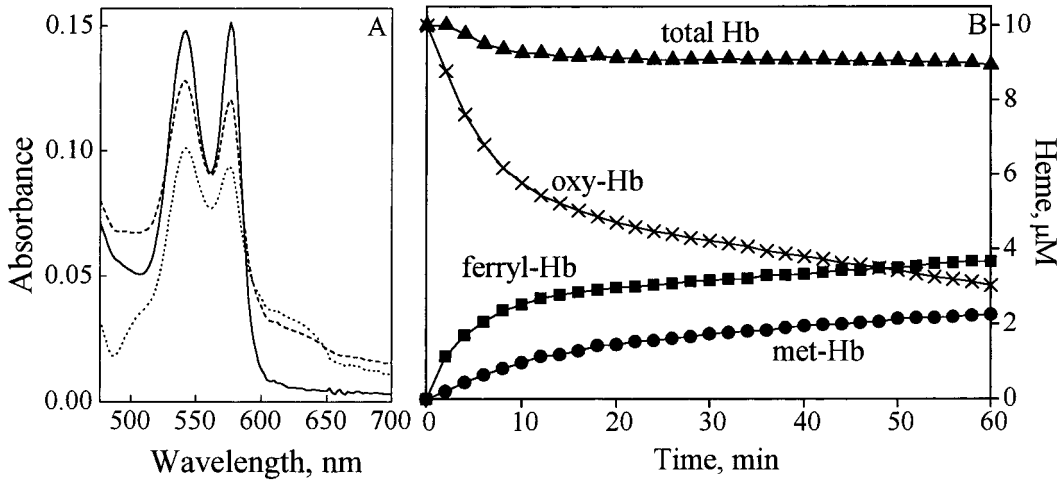

FIGURE 2: Oxidation states of heme iron during the reaction of oxy-Hb with LDL/H_2O_2. (A) Visible spectra of the reaction mixture containing oxy-Hb (10 μM), LDL (600 μg/mL) and H_2O_2 (15 μM) at 37 °C were measured at time zero (solid), after 10 min (dashed) and after 60 min (dotted). A sample containing LDL only was used as a base line. Note that the decrease in absorption at 490 nm in the 60-min spectrum reflects oxidative destruction of β-carotene in LDL. (B) Time-dependent changes in Hb oxidative states in the same mixture as in (A) were calculated according to equations shown in the Experimental section. Total amount of Hb was calculated as the sum of oxy-, met-, and ferryl- forms.

mixtures (Figure 1B and C, respectively), estimated from fluorescence quenching, is probably an overestimation because it may include quenching of the probe fluorescence due to its own oxidation. Indeed, fluorescence quenching occurred simultaneously with lipid oxidation (Figure 1B and C). Based on these results, we conclude that negligible heme transfer from Hb to LDL, or none at all, occurred under the oxidative conditions employed.

Effect of LDL on H_2O_2-Triggered Hb Oxidation States

In any mechanism by which Hb exerts oxidative activity toward LDL, heme should be involved. Thus, we first examined the heme moiety of Hb for oxidative changes that could be best followed by visible absorption. Representative time-dependent spectral changes of oxy-Hb incubated with LDL and H_2O_2 are shown in Figure 2A. The time-dependent changes in the levels of oxy-, met-, and ferryl-Hb, calculated as described in the experimental section, are demonstrated in Figure 2B. Oxy-Hb was diminished as a result of formation of some met-Hb and more so of ferryl-Hb. Other than a negligible initial reduction, the total amount of heme in solution, as calculated by the sum of the three Hb forms, was preserved. This observation is consistent with our previous conclusion that LDL oxidation occurs without transfer of heme to LDL.

The formation of oxidatively active ferryl-Hb during the process of LDL oxidation by Hb is in agreement with previous findings (Paganga et al., 1992). Nevertheless, the extent of involvement of LDL was unclear. We therefore compared the time course of ferryl-Hb appearance in the absence and in the presence of LDL under identical conditions. The results showed that the presence of LDL had only a minor effect on the pattern of ferryl-Hb appearance when either the oxy- or the met-Hb form was applied (Figure 3A and B).

To facilitate the analysis, LDL oxidation was performed under the same conditions except that Hb was replaced by HRP, the heme peroxidase shown to trigger LDL oxidation as well (Wieland et al., 1993). Under our reaction conditions of minimal excess of H_2O_2, the HRP compound **II** (Fe^{IV}),

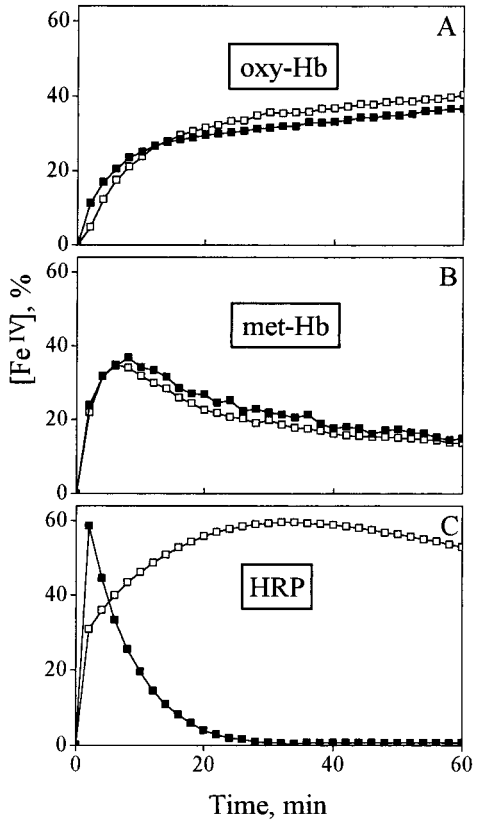

FIGURE 3: Effect of LDL on the time course of ferryl-Hb and HRP compound **II** formation. Hb (oxy- or met-; 10 μM) or HRP (3 μM) was incubated with 50% excess of H_2O_2 in the absence or presence of LDL (600 or 180 μg/mL in the case of Hb or HRP,respectively) at 37 °C. Time course of the heme Fe^{IV}, ferryl-Hb, or HRP compound **II**, calculated as described in the Experimental section, is presented as a percentage of total hemoprotein concentration. Open and filled squares indicate reaction mixtures in the absence and in the presence of LDL, respectively.

an analog of ferryl-Hb (Fe^{IV}), is formed (Hayashi & Yamazaki, 1979). We therefore followed the evolution of compound **II** in HRP/H_2O_2 mixtures in the absence and in the presence of LDL. In the absence of LDL compound **II** increased, reaching a maximal steady state value, whereas in the presence of LDL the compound **II** level rapidly

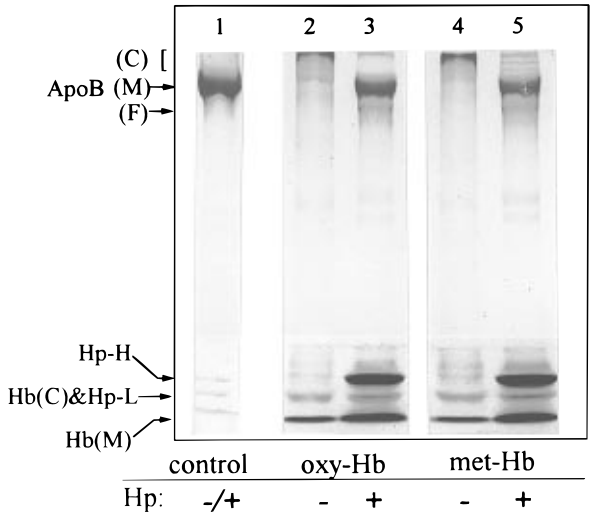

FIGURE 4: Effect of haptoglobin on protein pattern changes in LDL oxidized by Hb. LDL (600 μg/mL) was incubated for 3 h at 37 °C in the presence of H_2O_2 (15 μM) without or with either oxy-Hb (10 μM) or met-Hb (10 μM), in the presence or absence of haptoglobin (0.35 mg/mL), as indicated. Protein products were visualized by SDS−PAGE. Apo B (C), (M), and (F) indicate, respectively, cross-linked, monomer and fragment of apo B; Hb-(M) and Hb(C) indicate, respectively, monomer and cross-linked Hb; Hp-L and Hp-H indicate light and heavy chains of haptoglobin, respectively.

declined (Figure 3C). The consumption of HRP compound **II** by LDL indicated that LDL was oxidized by HRP catalysis via a regular heme peroxidase mechanism, namely, by direct electron transfer from the substrate to the excited heme iron. In contrast, the failure of LDL to decrease the ferryl-Hb level indicated a likely difference in the mechanisms. Although it is possible that heme iron was re-activated by lipid peroxides during LDL oxidation thereby reaching a balance between ferryl consumption and formation, this should be a case for both Hb and HRP. Therefore, the ferryl heme is probably not directly involved in the Hb catalytic mechanism of LDL oxidation. We designed further experiments in order to investigate the role of globin in the Hb-induced LDL oxidation.

Effect of Haptoglobin on LDL Oxidation by Hb: Comparison with Various Oxidation Promoters

Haptoglobin forms a complex of extremely high affinity with Hb via a well-characterized globin site (Nagel & Gibson, 1971; Hwang & Greer, 1980; McCormic & Atassi, 1990). We therefore used haptoglobin as a tool to elucidate the possible role of globin in Hb-induced LDL oxidation. We assumed that if the globin is indeed involved in Hb-derived oxidation, binding of haptoglobin should affect Hb reactivity. LDL was incubated with oxy- or met-Hb and H_2O_2, in the absence or in the presence of haptoglobin, under conditions shown previously to yield oxidation of LDL lipids and cross-linking of the LDL protein, apo B (Miller et al., 1996a). We found that in haptoglobin-containing reaction mixtures most of the apo B failed to cross-link and remained in its monomeric form (Figure 4, compare lane 3 with 2 and lane 5 with 4). This was true for both oxy- and met-Hb reactions, although to a lesser degree for the latter, in which a trace of cross-linked protein could still be observed. Haptoglobin also strongly inhibited LDL lipid oxidation

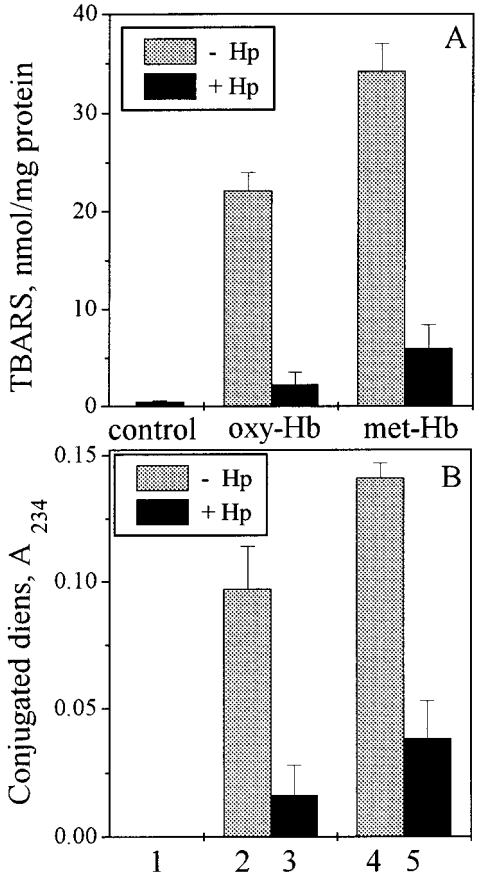

FIGURE 5: Effect of haptoglobin on oxidation of LDL lipids by Hb. TBARS (A) and conjugated dienes (B) were measured in the same reaction mixtures as in Figure 4. Conjugated dienes were assessed by absorbance of 40-fold diluted samples at 234 nm. Identical samples without LDL were used as references.

induced by Hb, as indicated by the amounts of TBARS and conjugated dienes (Figure 5A and B, compare lane 3 with 2 and lane 5 with 4). We concluded that haptoglobin reacts as an efficient inhibitor of oxidative cross-linking of LDL protein. It should be noted that in haptoglobin-containing reaction mixtures, an additional protein band was present below the apo B monomer representing a fragment of apo B (Figure 4, lanes 3&5). The position of this fragment suggests that it is half the size of apo B monomer according to molecular weight standards. It is noteworthy that the same molecular weight fragment appeared as a product of HRP-derived oxidation of LDL (Miller et al, 1996a; Figure 6, lane 8).

To determine whether haptoglobin inhibition is specific for Hb catalysis, we carried out further experiments in which Hb was replaced by other agents known to trigger LDL oxidation, namely myoglobin, free hemin, copper and HRP, in the absence and in the presence of haptoglobin. In these reactions, the pattern of oxidized LDL protein was practically unchanged by haptoglobin (Figure 6, lanes 3, 5, 7, and 9 as compared to lanes 2, 4, 6, and 8, respectively). Lipid oxidation parameters also indicated no inhibition by haptoglobin (Table 1), except for HRP reactivity, which was partially reduced in the presence of haptoglobin but still much less relative to the Hb reactivity. Because these results clearly indicated that haptoglobin is a specific antioxidant of Hb, the next experiments were aimed at determining the effect of haptoglobin on the Hb heme site.

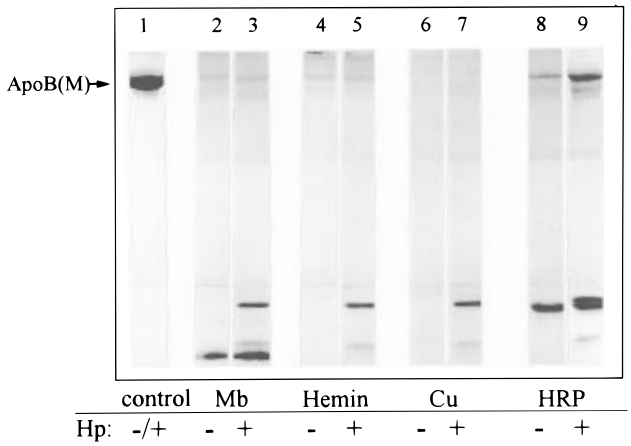

FIGURE 6: Effect of haptoglobin on protein pattern changes in LDL oxidized by met-myoglobin, hemin, copper and HRP. LDL (600 μg/mL) was incubated for 3 h at 37 °C in the presence of H_2O_2 (15 μM) alone or with met-myoglobin (Mb; 10 μM), hemin (10 μM), Cu_2SO_4 (20 μM) or HRP (10 μM) in the presence or absence of haptoglobin (0.35 mg/mL), as indicated. Protein products were visualized by SDS−PAGE. Apo B (M) indicates apo B monomer. The top of lanes 2−5 shows residual cross-linked apo B.

Table 1: Effect of Haptoglobin on Oxidation of LDL Lipids by Myoglobin, Hemin, Copper, and HRP

	TBARS, nmol/mg of protein		conjugated dienes, A_{234}	
sample	− Hp	+ Hp	− Hp	+ Hp
LDL alone	0.4 ± 0.2	0.4 ± 0.2	0	0
myoglobin/H_2O_2	23.3 ± 1.8	21.3 ± 2.1	0.17 ± 0.02	0.14 ± 0.02
hemin/H_2O_2	36.7 ± 3.8	37.0 ± 3.3	0.10 ± 0.03	0.10 ± 0.03
$CuSO_4$	73.3 ± 9.3	63.2 ± 9.1	0.31 ± 0.04	0.31 ± 0.03
HRP/H_2O_2	16.1 ± 3.6	8.1 ± 1.7	0.22 ± 0.05	0.14 ± 0.06

[a] TBARS and conjugated dienes were measured in the same reaction mixtures as presented in Figure 6. Conjugated dienes were assessed by absorbance of 40-fold diluted samples at 234 nm. Identical samples without LDL were used as references.

Effect of Haptoglobin on the Level of Fe^{IV} Active Intermediates of Hb and HRP During LDL Oxidation

Binding of haptoglobin to Hb leaves the heme iron free to interact with other molecules, such as O_2 (Waks et al., 1963). It was unclear, however, whether haptoglobin binding affects ferryl state formation. We therefore followed the effect of haptoglobin on the ferryl-Hb state during the process of LDL oxidation. Formation of ferryl in both oxy- and met-Hb was not inhibited but rather increased by haptoglobin (Figure 7A and B). The increase in ferryl level in oxidation triggered by oxy-Hb was minor, and was more pronounced in the case of met-Hb. This phenomenon may be attributed to the strengthening association between heme and globin in haptoglobin-bound met-Hb (Bunn & Jandl, 1968). The level of HRP compound **II** was also slightly increased in the presence of haptoglobin (Figure 7C). The fact that haptoglobin had no effect on the ferryl state in both Hb and HRP suggests that haptoglobin binding to Hb has no specific impact on the its ferryl state.

Effect of Haptoglobin on Oxidation of Guaiacol by Hemoglobin and HRP

The presence of ferryl species is not always correlated with catalytic activity of heme in peroxidases (Ortiz de Montellano, 1992). We therefore next followed the effect of haptoglobin on oxidation of guaiacol, a substrate known to

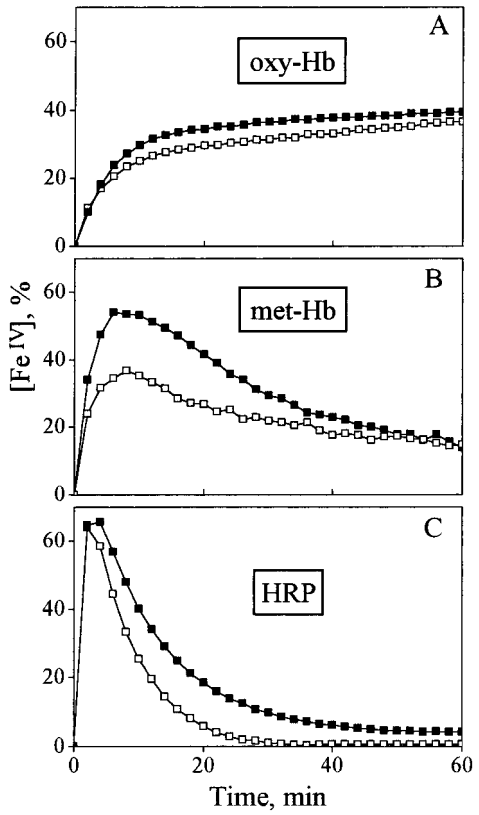

FIGURE 7: Effect of haptoglobin on the time course of ferryl-Hb and HRP compound **II** formation. Hb (oxy- or met-; 10 μM) or HRP (3 μM) was incubated with 50% excess of H_2O_2 and with LDL (600 or 180 μg/mL in the case of Hb or Hp, respectively) in the absence or presence of haptoglobin (0.35 or 0.116 mg/mL for Hb or HRP, respectively) at 37 °C. Time course of the heme Fe^{IV}, ferryl-Hb or HRP compound **II**, calculated as described in the Experimental section, is presented as a percentage of total hemoprotein concentration. Open and filled squares indicate reaction mixtures in the absence and in the presence of haptoglobin, respectively.

be oxidized in the heme active site (Ortiz de Montellano, 1992). In parallel with appearance of a guaiacol oxidation product, a consumption of the Hb and HRP Fe^{IV} state was observed (not shown). The presence of haptoglobin had practically no effect on guaiacol oxidation by HRP (Figure 8C). When guaiacol oxidation was triggered by either oxy- or met-Hb, not only did the presence of haptoglobin not diminish the oxidative reactivity of the hemoglobins, but it increased their oxidative efficiency (Figure 8A and B). This effect is consistent with the stability conferred by haptoglobin on the Hb ferryl state, as demonstrated in Figure 7. The discrepancy between the effects of haptoglobin on Hb-induced oxidation of LDL and of guaiacol further confirm that the heme site of Hb does not directly contribute to LDL oxidation.

Effect of Haptoglobin on Globin Cross-Linking by H_2O_2

It is known that in the absence of additional substrates, Hb oxidation by H_2O_2 results in covalent self-cross-linking of globin (Alloiso et al., 1982). Our finding that haptoglobin binding enhances the peroxidase reactivity of the Hb heme site raised the question of whether it would have a similar effect on the reactivity of globin? The reaction of globin cross-linking requires a higher level of H_2O_2 than that employed in our experiments. To avoid the bolus addition of large amount of H_2O_2, we used a glucose oxidase/glucose

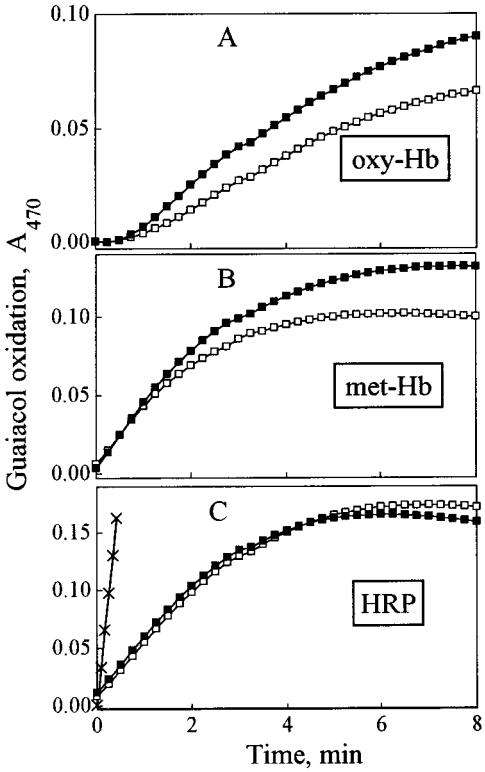

FIGURE 8: Effect of haptoglobin on oxidation of guaiacol by Hb and HRP. Guaiacol (1 mM) was incubated at 37 °C in the presence of H_2O_2 (50 μM) with either Hb (oxy- or met-; 10 μM) or HRP (1 nM) in the presence or absence of haptoglobin (0.35 mg/mL). Appearance of an oxidation product was followed by absorbance at 470 nm. Open and filled squares indicate reactions in the absence and presence of haptoglobin, respectively. As reactions were carried out with a small excess of H_2O_2 over heme, the guaiacol oxidation mode was nonlinear when both Hb and HRP were employed. Increasing H_2O_2 concentrations to those usually used in HRP catalytic reactions (1 mM) resulted in a much higher oxidation rate with a linear kinetic mode (crosses).

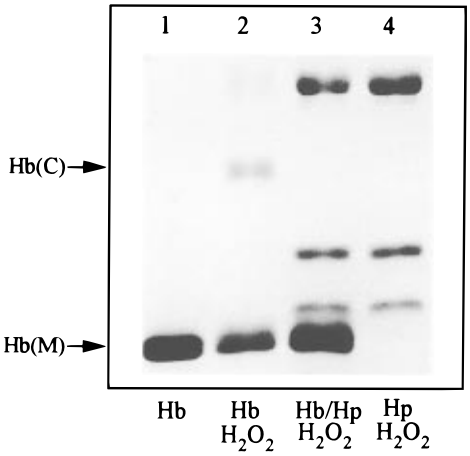

FIGURE 9: Effect of haptoglobin on H_2O_2-triggered cross-linking of globin. Oxy-Hb (25 μM) was incubated for 3 h at 37 °C with H_2O_2 in the presence or absence of haptoglobin (0.88 mg/mL), as indicated. Constant production of H_2O_2 at the rate of 5 μM/min was generated by 0.85 μg/mL glucose oxidase plus 5 mM glucose. Sample 4 was haptoglobin alone incubated with or without H_2O_2. Samples were run on SDS−PAGE. Hb (M) and (C) indicate monomer and cross-linked Hb, respectively.

system which yields constant production of a small amount of H_2O_2 (Hanan & Shaklai, 1995). Appearance of a cross-linked globin was observed on SDS−PAGE (Figure 9, lane 2 as compared to 1). Haptoglobin completely inhibited globin cross-linking in the Hb/H_2O_2 reaction mixture (lane 3). In contrast to Hb, oxidative self-cross-linking of myoglobin was not affected by haptoglobin (data not shown). This is in correlation with the failure of haptoglobin to inhibit LDL oxidation by myoglobin (Figure 6, Table 1). These data can be explained by the inability of myoglobin to bind with haptoglobin (Hwang & Greer, 1980; McCormic & Atassi, 1990). A comparison of effects of haptoglobin on the oxidative reactions of globin in Hb and myoglobin, both in the absence and in the presence of LDL, suggests a major role for globin in Hb-derived LDL oxidation.

DISCUSSION

Both of the oxygen binding proteins, Hb and myoglobin, have long been known to catalyze oxidative reactions but with low reactivity (Everse et al., 1994). Previous data appeared to support the concept that the unexpectedly high oxidative reactivity of Hb toward LDL as a substrate may stem from free hemin detached from Hb under oxidation conditions. This notion was based on the following information from previous studies: (a) in Hb/H_2O_2 mixtures heme tends to become detached from the globin (Bunn & Jandl, 1968; Banerjee, 1962); (b) globin-free hemin, unlike intact Hb, readily associates with hydrophobic and amphipatic components including LDL and the LDL-associated hemin is extremely active as an LDL oxidizer (Balla et al., 1991; Miller & Shaklai, 1994; Miller et al., 1995); (c) Hb-derived LDL oxidation is inhibited by hemopexin, a high affinity heme-binding protein (Miller et al., 1996b). However, the present study provides unequivocal evidence that free hemin is not the source of the oxidative reactivity of Hb. Rather than preceding LDL oxidation, heme transfer was always concurrent with its commencement (Figure 1B and C). While LDL oxidation by bound hemin was preceded by a lag phase of 1 h (Figure 1A), the Hb-derived LDL oxidation always appeared after a shorter lag phase (e.g., Figure 1; Miller et al., 1996a). Finally, this study showed that under conditions that lead to LDL oxidation by Hb, only insignificant heme transfer occurs from either oxy- or met-Hb to LDL (Figure 1B and C). The amount of heme transferred to LDL cannot account for its oxidation.

Having excluded the concept of globin-detached heme as the source of Hb oxidative reactivity, we considered further mechanisms in which the core of the reactivity might derive from the intact Hb molecule. It is generally agreed that the oxygen-binding proteins Hb and myoglobin exert low peroxidase reactivity and that the mechanism is the same as that of a classical peroxidase like HRP (Ortiz de Montellano, 1992; Everse et al., 1994). The prototype peroxidase mechanism as applied to Hb is described below by eqs 7−12. In the first stage, a two-electron oxidation of a hemoprotein by one H_2O_2 molecule occurs (eqs 7−9). When a Fe^{II}-hemoprotein is the resting state, an intermediate ferryl species (Fe^{IV}) is produced (eq 7). In all reactions of the current study, oxy-Hb [Hb(Fe^{II})O_2] reacted in essentially the same manner as met-Hb [Hb (Fe^{III})], only more slowly. The difference in rate was attributed to comproportionation between oxy-and ferryl-Hb producing met-Hb (eq 8). In the case of a Fe^{III}-hemoprotein, one oxidation equivalent produces a ferryl iron (Fe^{IV}) and the second equivalent produces a radical (designated as Hb^+) located on either porphyrin or

protein (Ortiz de Montellano, 1992) (eq 9). These equations are given below:

Stage 1

$$Hb(Fe^{II})O_2 + H_2O_2 + 2H^+ \rightarrow Hb(Fe^{IV}) + O_2 + 2H_2O \quad (7)$$

$$Hb(Fe^{IV}) + Hb(Fe^{II})O_2 \rightarrow 2Hb(Fe^{III}) + O_2 \quad (8)$$

$$Hb(Fe^{III}) + H_2O_2 + 2H^+ \rightarrow Hb^+(Fe^{IV}) + 2H_2O \quad (9)$$

In the second stage of the oxidation process, $Hb^+(Fe^{IV})$ reverts to the resting state in two steps, where one electron is drawn from one substrate molecule in each step (eqs 10 and 11) to yield the oxidized substrate (eq 12). (AH denotes a substrate, while A_2 is an oxidized substrate product.) The equations are given below:

Stage II

$$Hb^+(Fe^{IV}) + AH + OH^- \rightarrow Hb(Fe^{IV}) + {}^{\bullet}A + H_2O \quad (10)$$

$$Hb(Fe^{IV}) + AH + OH^- \rightarrow Hb(Fe^{III}) + {}^{\bullet}A + H_2O \quad (11)$$

$$2\,{}^{\bullet}A \rightarrow A_2 \quad (12)$$

The results of the present study indicate that in the case of LDL oxidation, a final oxidative product is achieved in the form of cross-linked apo B and oxidized lipids. However, the reaction does not proceed via eq 11, which demands consumption of ferryl-Hb at the expense of the oxidized substrate. The fact that Hb-derived LDL oxidation was not followed by any reduction in the ferryl state (Figure 3) indicates that no electron transfer from LDL to the heme iron occurred in the oxidation process. The experiments involving haptoglobin-bound Hb provide additional evidence for lack of direct correlation between heme-iron and LDL in Hb-induced oxidation. In these experiments, inhibition of both lipid and protein oxidation in LDL by haptoglobin was followed by an increase rather than the expected reduction in the ferryl-Hb level (Figure 7).

To ensure that the heme active site does not directly participate in LDL oxidation, we also tested the effect of haptoglobin on oxidation of the small classical substrate for heme-peroxidases, guaiacol. As stated, unlike LDL, oxidation of guaiacol by Hb consumes the Hb ferryl. Moreover, in correlation with the observed lack of any effect on the ferryl level by haptoglobin, Hb-induced guaiacol oxidation was also unaffected by haptoglobin (Figure 8). Thus, Hb-induced oxidation of guaiacol and of LDL as substrates occur by different mechanisms. Our results clearly show that, unlike in oxidation induced by Hb, in HRP-induced oxidation of both guaiacol and LDL the ferryl-iron is the electron acceptor (Figures 3 and 8). It should be remembered that the reactivity of Hb as a peroxidase via the ferryl toward guaiacol is much lower (several orders of magnitude) than that of HRP (Waks et al., 1963). Our results indicate that this relatively low Hb reactivity probably occurs in the case of oxidation of LDL by Hb via ferryl as well. The protein pattern of oxidized LDL shown in Figure 4 demonstrates that when Hb-derived apo B cross-linking was abolished by haptoglobin, a small amount of an apo B fragment appeared. The size of this fragment concurs with the product in HRP-oxidized LDL that was previously suggested to be an apo B molecule degraded into two equal fragments (Miller et al., 1996a; Figure 6, lanes 8 and 9). This product may be related to LDL oxidation by both Hb and HRP via ferryl. That this fragment was seen only in the presence of haptoglobin is probably related to the increased reactivity of Hb ferryl-iron by haptoglobin (Figure 7). Some of this product might also be produced in the absence of haptoglobin and not detected because of sensitivity limits of the method or as a result of participation of the fragments in the cross-linked main product. In any case, this minor reactivity of Hb via the ferryl heme-iron merely serves to indicate that the main product of Hb-oxidized apo B was produced by a different mechanism.

The specific oxidative reactivity of Hb (and of myoglobin) lies in the ability of these two hemoproteins to undergo autoxidation of the globins to form globin-globin dimers in the presence of H_2O_2 (Tew & Montellano, 1988; Giulivi & Davies, 1990; Tschirret & Ortiz de Montellano, 1996). Thus, Hb and myoglobin should actually be defined as pseudoenzymes when categorized as heme-peroxidases. In both H_2O_2-oxidized Hb and H_2O_2-oxidized myoglobin, the second oxidizing equivalent has been defined as a tyrosine- and/or tryptophan-centered globin radical (Tew & Montellano, 1988; Giulivi & Davies, 1990; McArtur & Davies, 1993; Gorbunov et al., 1995). Therefore, $Hb^+(Fe^{IV})$ should be designated as ${}^{\bullet}$globin-heme(Fe^{IV}) and eq 9 should be rewritten as follows:

$$\text{globin-heme}(Fe^{III}) + H_2O_2 + 2H^+ \rightarrow {}^{\bullet}\text{globin-heme}(Fe^{IV}) + 2H_2O \quad (13)$$

The globin radical was also suggested to be directly formed from oxy-Hb (Giulivi & Davies, 1990). Following reaction 7, the ferryl-Hb transforms to a form of globin radical/heme-(Fe^{III}):

$$\text{globin-heme}(Fe^{IV}) \rightarrow {}^{\bullet}\text{globin-heme}\ (Fe^{III}) \quad (14)$$

Irrespective of the heme iron state, the intermediate globin radical terminates in autoxidation as a globin cross-linking product; thus, two globin radicals yield a globin-globin dimer. Globin dimer yield from the short-lived globin radical is poor (Figure 9). However, this radical can initiate radical propagation in LDL via the apo B:

$${}^{\bullet}\text{globin-heme}\ (Fe^{IV}) + \text{apo B} \rightarrow \text{globin-heme}\ (Fe^{IV}) + {}^{\bullet}\text{apo B} \quad (15)$$

Availability of apo B radicals located on tyrosine or/and tryptophan residues has been reported (Kalayanaraman et al., 1995; Giessaul et al., 1996). Termination of the apo B radicals accounts for appearance of the cross-linked apo B protein in high yield (Miller et al., 1996a):

$$2n\,{}^{\bullet}\text{apo B} \rightarrow (\text{apo B} - \text{apo B})_n \quad (16)$$

It should be noted that at this stage it is not clear whether apo B radical is formed directly from the globin radical or indirectly through other LDL radicals such as α-tocopheroxyl (Kalayanaraman et al., 1995). The latter does not seem to occur in Hb-induced LDL oxidation because, as shown in this study HRP, found to initiate a LDL α-tocopheroxyl

radical (Kalayanaraman et al., 1995), does not mediate apo B cross-linking (Figure 6).

The possibility that the globin radical participates in Hb-(or myoglobin-)derived oxidation of other substrates, e.g., isolated lipids, has already been discussed (Newman et al., 1991; Yoshida et al., 1994). Newman et al. (1991) reported an electron spin resonance signal ascribed to a globin radical during myoglobin-induced oxidation of lipids. In a previous study from our laboratory it was suggested that myoglobin uses the globin radical in its efficient catalysis of myosin oxidation. Nevertheless, to the best of our knowledge the hypothesis of the globin radical-derived oxidation has not yet been proved. In the present study, using the tool of haptoglobin in Hb-induced LDL oxidation, we provide direct evidence for oxidative reactivity of the globin radical.

Our results show that upon Hb-haptoglobin binding, the globin radical, loses its ability to be terminated by forming globin dimers (Figure 9). In addition, haptoglobin-bound Hb loses the ability to initiate the apo B protein radicals that are terminated by apo B cross-linking (Figure 4). On the other hand, our results show that similar functions in myoglobin, namely oxidative self-cross-linking and induction of apo B cross-linking, are not inhibited by haptoglobin. As haptoglobin is incapable of binding to the myoglobin backbone, we conclude that attachment of haptoglobin to the globin is a prerequest for inhibition of the globin radical. Hb-haptoglobin is one of the strongest noncovalent protein-protein associations known. The globin binding sites include regions of both α and β chains containing aromatic amino acids as well as histidines, all of which are good candidates for the formation of globin radical. Nevertheless, none of the haptoglobin binding regions seems to be in direct contact with the heme crevice (Nagel & Gibson, 1971; Hwang & Greer, 1980; McCormic & Atassi, 1990; Bunn & Forget, 1986). This information correlates with the ability of haptoglobin to inhibit oxidative reactivity of the globin radical by initiating propagation of radicals in other macromolecules, without affecting the heme oxidized ferryl state. Haptoglobin may inhibit formation of the globin radical on one hand, and on the other hand may prevent its collision with other radicals without inhibition of the radical formation. The actual fate of the globin radical in a Hb-haptoglobin complex has yet to be determined.

It should be mentioned that while globin-derived oxidative reactivity of Hb and myoglobin (proteins designed to transfer oxygen) is a pathological event, some heme-peroxidases, which participate in normal metabolic pathways, also operate via a protein radical rather than via heme. An example is cytochrome *c* peroxidase (CcP), which oxidizes small molecules slowly but is extremely efficient toward the macromolecule, cytochrome *c*, its natural substrate. The first electron to reduce H_2O_2 arrives from the heme iron in both HRP and CcP. The second oxidation equivalent, which is responsible for the difference in their catalytic activity, comes from the porphyrin ring in HRP and leads to a porphyrin radical cation. In CcP, however, it is stored in the protein as an unpaired electron on a tryptophan and partially on tyrosine residues (Ortiz de Montellano, 1992). As in Hb and myoglobin, cross-linking of CcP tyrosine residues upon its interaction with H_2O_2 has been reported (Spangler & Erman, 1986). The information on CcP, Hb and myoglobin points to the need for an oxidizable residue in the vicinity of the heme group in order to form a protein oxidative equivalent rather than a porphyrin-centered one.

The present study not only reveals the mechanism by which Hb exerts its highly efficient pathological oxidative reactivity, but also sheds light on the metabolic functions of haptoglobin. Traditionally, haptoglobin is considered to be synthesized in the liver and to function in the plasma by binding extracorpuscular Hb and delivering the complex to the liver to be catabolized there. In this way, haptoglobin allows both iron recycling and protection of the kidneys from Hb damage (Bowman, 1992). Various studies suggested other functions for haptoglobin. Haptoglobin was suggested to act as a natural bacteriostat by preventing the utilization of Hb iron by pathogenic bacteria (Eaton et al., 1982). Extracellular Hb has been shown to be responsible for vasospasm resulting from arterial damage (Macdonald & Weir, 1991). In correlation with the role of haptoglobin in inhibition of Hb-derived lipid oxidation (Gutteridge, 1987), hypohaptoglobinemia was found to be a significant factor in the pathogenesis of epilepsy (Panter et al., 1985).

Among the acute phase proteins, haptoglobin is one of the few proteins in which increased synthesis during inflammation is conserved in all vertebrate species (Bowman, 1992). A common feature of many acute phase proteins is their involvement in host defense against infection and repair of damaged tissues. Nevertheless, as with other acute phase proteins the potential benefits deriving from its increased level are not quite clear. The present study demonstrated that haptoglobin prevents Hb-derived oxidative vascular damage, and thus plays a major role as an antioxidant. The fact that the potential peroxidase reactivity of Hb is implemented in the presence of oxidants, that are elevated during inflammation, explains the need for increased haptoglobin under acute phase conditions. It should be mentioned in this regard that in previous studies similar conclusions have been drawn for hemopexin, which also provides antioxidant reactivity against Hb-mediated LDL and is known as an acute phase plasma protein (Hunt et al., 1996; Miller et al., 1996b). Recent studies have indicated that human peripheral blood neutrophils and monocytes can bind haptoglobin and can take it up and actively exocytoze it upon activation of the cells (Oh et al., 1990; Wagner et al., 1996). Although acute phase proteins are synthesized mainly in the liver, extrahepatic expression of some of these proteins was recently reported (Hunt et al., 1996). Expression and inflammatory regulation of a haptoglobin gene was clearly demonstrated in adipocytes (Friedrichs et al., 1995). These data, taken together with the data from the present study, suggest that local enhancement of haptoglobin levels in both intra- and extravascular sites provides efficient protection for the vascular system from Hb-derived oxidative pathogenesis.

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