Haptoglobin Phenotypes Differ in Their Ability To Inhibit Heme Transfer from Hemoglobin to LDL†

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ABSTRACT: LDL oxidation plays a pivotal role in atherosclerosis. Excellular hemoglobin (Hb) is a trigger of LDL oxidation. By virtue of its ability to bind hemoglobin, haptoglobin (Hp) serves as an antioxidant. Oxidation of LDL by hemoglobin was analyzed to occur by heme displacement from methemoglobin lodged in LDL. The LDL-associated heme is disintegrated, and iron inserted this way in LDL triggers formation of lipid peroxides. The genetic polymorphism of haptoglobin was found to be a risk factor in the pathogenesis of atherosclerosis. Individuals with Hp2-2 have more vascular incidences as compared to those with Hp1-1. In the current study, oxidation of LDL by metHb was carried out at physiological pH without addition of external peroxides. Hb-derived oxidation of lipids and protein was found to be practically inhibited by Hp1-1 but only partially by Hp2-2. Heme transfer from metHb to LDL was almost completely omitted by Hp1-1 and only partially by Hp2-2. We concluded that partial heme transfer from the Hb−Hp2-2 complex to LDL is the reason for oxidation of LDL lipids as well as protein. These findings provide a molecular basis for Hp2-2 atherogenic properties.

Haptoglobin (Hp)[1] is one of the few acute phase reactive proteins conserved in all vertebrates (*1*). The strong association of Hp with Hb and the high conservation of the Hp gene across species indicate that recognition and binding affinity are key physiological roles of Hp. Inflammation, like other pathological states such as atherosclerosis, cancer, and certain inborn genetic error, is an example of a variety of conditions leading to increased oxidative stress (*2*). Atherosclerosis has drawn a lot of attention from biomedical research as the leading cause of death in industrial societies (*3*). A wealth of evidence indicates that oxidative damage to LDL plays a central role in atherosclerosis (*4*). One of the main causes of LDL oxidation in the vascular system is leakage of hemoglobin out of red blood cells (*5*, *6*). Once out of the cell, hemoglobin is no longer protected by cellular glutathione as well as by the specific enzyme methemoglobin reductase (*7*). Thus, its ferrous heme iron readily undergoes autoxidation to ferric state hemin to form methemoglobin (metHb). In metHb the bond between the globin and hemin is weakened, allowing hemin exchange with other proteins with high-affinity binding sites (*8*, *9*).

For over 30 years it has been known that Hp reduces loss of Hb and iron through the formation of an Hb−Hp complex which is not filtered through the glomeruli but transported to the liver (*10*). The Hb−Hp complex is cleared from circulation in the liver by recognition of a specific hepatic macrophage receptor (*11*, *12*). Thus, the in vivo function of haptoglobin was established as the plasma protein responsible for capture and clearance of excellular Hb from circulation, thereby preventing its toxicity to vasculature components (*13*). However, studies on Hp knockout mice demonstrated that lack of Hp does not impair clearance of Hb from plasma (*14*). Indication that uptake of free hemoglobin from circulation is faster than that of its complex with haptoglobin pointed to other, more urgent functions for this protein (*15*, *16*). It appears that, by binding excellular hemoglobin, Hp can serve as a vascular antioxidant. Haptoglobin was shown to completely inhibit the oxidative activity of Hb toward lipids as well as LDL protein (*17*, *18*). Because oxidative modification of LDL plays a critical role in the pathogenesis of atherosclerosis, Hp can be considered as a central antiatherosclerotic agent (*18*, *19*).

Humans have two different Hp alleles, designated as Hp1 and Hp2. Homozygotes for Hp1 and Hp2 alleles form Hp1-1 and Hp2-2 phenotypes, respectively, and individuals heterozygous for the alleles display a mixed Hp1-2 phenotype. In the past decade, a direct link was found between haptoglobin phenotype and susceptibility to cardiovascular

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[1] Abbreviations: ApoB, apolipoprotein B; APS, ammonium persulfate; BSA, bovine serum albumin; CD, conjugated diene; D-LDL, dansylated low-density lipoprotein; FRET, fluorescence resonance energy transfer; Hp, haptoglobin; Hb, hemoglobin; HO-1, heme oxygenase-1; Hx, hemopexin; HRP, horseradish peroxidase; LDL, low-density lipoprotein; Mb, myoglobin; MDA, malondialdehyde; metHb, methemoglobin; metMb, metmyoglobin; oxyHb, oxyhemoglobin; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TBARS, thiobarbituric acid reactive substances.

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diseases. The Hp1-1 phenotype has been associated with less frequent and less severe complications than the Hp2-2 phenotype (*13*, *20*, *21*). These differences are well expressed under oxidative conditions existing in diabetic patients (*22*, *23*). In healthy individuals haptoglobin polymorphism related changes were reported as well. Males (but not females) with the Hp2-2 phenotype have higher serum iron (*24*). Altogether, it seems established that individuals with the Hp2-2 phenotype have iron-related vascular pathologies. Thus, haptoglobin type can serve as a predictor of vascular complications under oxidative conditions such as hyperglycemia in diabetes (*23*).

Excellular hemoglobin is a trigger for LDL oxidation, and accumulation of the oxidized LDL in macrophages is involved in atherosclerosis (*25*). This information points to involvement of Hb-bound Hp in the antioxidant activity, but the molecular basis for this phenomenon is still unclear. Recent research from our and other laboratories indicated that the high reactivity of hemoglobin as a trigger of LDL oxidation is not the outcome of peroxidase or even pseudoperoxidase activity of the intact molecule as thought before (*5*, *18*) but relates to globin-free hemin, a highly potent oxidant (*19*). Most in vitro studies used hydrogen peroxide in addition to hemoglobin, on the premise that hemoglobin activity stems from peroxidase (or peroxidase-like) function, bestowed by the high-valence heme iron ($Fe^{IV}$and Fe^{V}) (*26*). Recent research led us to conclude that the role played by a peroxide, such as H_2O_2, is mainly conversion of oxy- to metHb, which then serves as a source of globin-free hemin (*6*, *19*). The idea that hemin anchored in metHb is the cause of vascular injury is not new. It was shown by previous studies that free Hb in plasma, when oxidized, can provide heme to endothelium as well as to LDL (*27*−*29*). It was concluded recently that "once heme is lodged within the LDL, spontaneous oxidative reactions involving small amounts of preformed LOOH will lead to oxidative lysis of the heme group and release of the heme iron within the LDL particle" (*19*). Thus, in the current study rather than oxyHb/peroxide, metHb, in which the heme−globin bond is weakened, was used to focus on the molecular basis of differences between Hp1-1 and Hp2-2 as inhibitors of LDL oxidation.

MATERIALS AND METHODS

Materials. Haptoglobins 1-1 and 2-2, 5-(dimethylamino)-naphthalene-1-sulfonyl (dansyl) chloride, and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co., St. Louis, MO. 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 (*30*) and eluted through two PD-10 columns to remove EDTA and KBr. In experiments where fast preparation was required, a single spin rate zonal density gradient was used (*31*). The concentration of LDL was determined as protein concentration by the Lowry method (*32*). To avoid oxygen in solution, argon was flashed above the freshly drawn plasma kept in a sealed container. All solutions added were presaturated with argon, and LDL stock preparations were kept under argon at 0 °C (ice/water mixtures) up to 7 days. Elevation of lipid peroxide beyond remnant level, available always in healthy donors (*19*), results in formation of LDL protein bityrosines. The specific fluorescence emission at 400 nm (excitation at 327 nm) typical to bityrosines was routinely used to monitor the freshness of stored LDL (*33*).

Isolation of Hemoglobin. Hemoglobin was isolated from red blood cell lysates by ion-exchange chromatography using CM-52 cellulose, followed by desalting on PD-10 columns (*6*). 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 (*6*). MetHb was prepared from the oxyHb (*34*), and before use it was purified by admixing with DE-52 cellulose for 5 min, followed by centrifugation to remove globin-free hemin contaminants (*18*). Concentrations of metHb are expressed in heme equivalents throughout this study (*34*).

Determination of Hp Concentrations. Hp concentrations were determined in stock solutions (in mg/mL) by repeated measurements using the Lowry method (*32*). Millimolar extinction coefficients at 278 nm of 53.90 for Hp1-1 and 58.65 for Hp2-2 (per $\alpha\beta$ unit of molecular masses 43 and 49.7 kDa, respectively) were calculated by us according to data in the literature (*13*). Several independent Sigma preparations were used throughout this study.

Heme Transfer from Hemoglobin to LDL. Transfer of heme from globin to LDL was assessed by independent methods. (1) Size-based separation: Ultrafiltration was carried out using XM300 ultrafiltration membranes allowing filtration of the Hb or its complexes with Hp1-1 and Hp 2-2. Ultrafiltration cells with stirring (3 mL) were used (Amicon, Inc.). The amount of heme in protein complex was determined as hemoglobin Soret absorption. (2) Density-based separation: In this method separation takes advantage of the differences in density between the LDL lipoprotein and hemoglobin (alone or as a complex with Hp). LDL was separated from reaction mixtures by addition of KBr for the concentration used in the final step of LDL separation by sequential ultracentrifugation for 18 h at 150000*g* (*27*). (3) FRET (fluorescence resonance energy transfer): This method exploits fluorescence quenching due to energy transfer from LDL dansyl to heme transferred from Hb (*18*, *33*). As an extrinsic fluorophore, free amino and alcohol groups of LDL were labeled by dansyl (*18*). It should be emphasized that LDL was labeled with dansyl groups 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 conjugated diene (CD) formation within 4 h of preparation.

Dansylation of LDL. 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 (*35*). The fluorescence intensity of dansylated LDL, λ_{ex} 350 nm and λ_{em} 550 nm, was measured using a Jasko FP-6200 spectrofluorometer. The instrument was

modified by the supplier according to our request to allow a sequential (2 min intervals) four-cuvette continuous measure of fluorescence intensity in thermostated solutions. The term "fluorescence intensity" in the *y*-axis of Figure 5 stands for $100 - 100(F_0 - F_t)/(F_0 - F_\infty)$, where F_0 is the fluorescence intensity of LDL following addition of hemin or metHb (to ignore any trivial quenching contribution, the actual F_0 was considered as the fluorescence intensity after the mixing time of 2 min), F_∞ is fluorescence intensity of LDL 3.5 h after addition of free hemin, and F_t is fluorescence intensity of LDL at any time after addition of metHb.

LDL Oxidation Experiments. All reactions were carried out at 37 °C in PBS, pH 7.4, under air to allow the presence of a minimal amount of lipid hydroperoxide (LOOH) to start the cascade of hemin disintegration and LDL oxidation (*36*). Since the aim of the current study was to analyze differences between Hp phenotypes in protecting LDL from heme−Hb-derived oxidation, care was taken to repeat the experiments with materials from different preparations. This included LDL prepared from different donors and Hp from different Sigma lots. Oxidation products of ApoB were followed by SDS−PAGE with β-mercaptoethanol using 4.5−12% acrylamide bilayer slabs. Gels were stained with Coomassie Brilliant Blue R-250. Oxidatively modified ApoB emits light in the wavelength range of 440−460 nm due to formation of lipid−protein conjugates (*37*) and at 400−410 nm due to formation of intra- and interbityrosines (*33*). To monitor all changes related to ApoB protein modifications, samples were excited at 327 nm. Lipid oxidation kinetics in LDL at 268 nm and deterioration of hemin at 405 nm were measured by monitoring time-dependent changes, respectively (*6*, *31*, *36*), using a UV/vis 920 spectrophotometer (GBC, Dandenong, Australia). This spectrophotometer allows measurement of full-spectrum kinetics simultaneously in six thermostated mixtures.

RESULTS

Effect of Haptoglobin Phenotypes on Kinetics of UV and Soret Absorption Changes in metHb/LDL Reaction Mixtures. In Hb−heme-derived LDL oxidation, absorption changes in the UV region (UVA) reflect both formation of CD, with typical 234 and 268 nm absorption (*38*), and light scattered from LDL aggregation (*6*). Figure 1 shows time-dependent UVA changes in reaction mixtures containing Hb, alone or as a complex with one of the Hp phenotypes. In Hp-devoid reaction mixtures, a phase of slow increase in UVA was succeeded by a second phase of a much faster rate. Replacing metHb by its complex with Hp1-1 completely abolished the fast phase. In case the metHb was replaced by its complex with Hp2-2, the fast phase still could be observed, albeit with a smaller amplitude. Addition of equimolar H_2O_2 (3 μM) as an external peroxide resulted in acceleration of the UVA changes (data not shown).

Previous studies indicated that hemin- and Hb-derived LDL oxidation involves decreased Soret absorption (*6*, *19*, *27*, *36*). Accordingly, a followup of changes in absorbance at 405 nm in the same reaction mixtures was carried out, and the results are presented in Figure 2. The results indicate parallel differences in the effect of the two Hp phenotypes. In the reaction including Hp2-2, a fast phase was observed although to a smaller degree as compared to the Hp-deficient

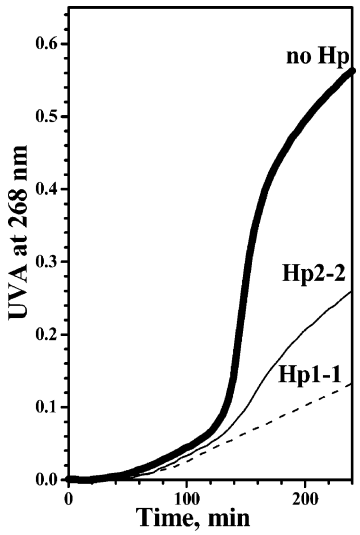


FIGURE 1: Dependence of LDL oxidation rate on Hp phenotype. UVA kinetics measured at 268 nm is shown (details in ref *6*). Shown is a representative of three independent experiments. Reactions were carried out in PBS, pH 7.4 at 37 °C. Time-dependent evolution of the reaction mixtures was monitored. Reaction mixtures contained 100 μg/mL LDL and 3 μM metHb (heme base) plus Hp, when present, 1.5 μM ($\alpha\beta$ unit base). Key: solid line, no Hp; dashed line, includes Hp1-1; solid bold line, includes Hp2-2. Absorbance was monitored at 268 nm rather than 234 nm to decrease the light scatter contribution.

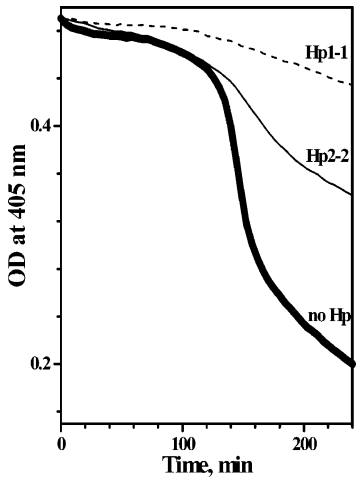


FIGURE 2: Time-dependent heme disintegration in the process of LDL oxidation. Shown is a representative of three independent experiments. The kinetics of absorbance at 405 nm, Soret absorption maximum of hemin bound to globin or ApoB (*6*). The conditions and reagents were similar to those of Figure 1: no Hp (solid line); Hp1-1 (dashed line); Hp2-2 (solid bold line).

reaction. In contrast, in the Hp1-1-containing reaction, the fast phase was not observed at all.

Analysis of Time-Dependent Changes in the Reaction Mixtures by Absorption Spectra. Since analysis of single wavelength kinetics where both absorption and light scatter are involved is complicated, additional information could be drawn from kinetics of absorption spectra monitored (every 5 min) in both visible and UV regions of the reaction mixtures provided in Figures 1 and 2. Spectra monitored at key time points are shown in Figure 3. Time-dependent changes were observed in three spectral regions: UV indicating CD and LDL aggregate; Soret for heme; 450−550 nm region corresponding to antioxidants such as β-carotene. In the absence of Hp, few changes were observed

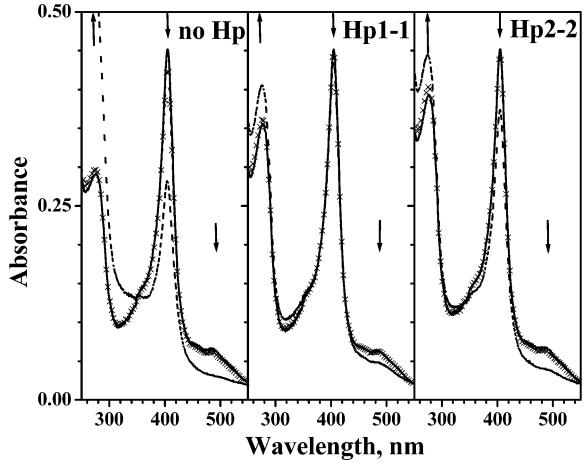


FIGURE 3: Time course of light absorption spectra of metHb/LDL mixtures. Shown is a representative of three independent experiments. In each window are shown spectra in the 245−550 nm range: spectra at time zero (solid line); following 60 min of reaction (crosses); following 150 min of reaction (dashed line). The conditions and reagents were similar to those of Figure 1. The arrows show the direction of time-dependent changes. Note that any left-shifted UVA peak results from formation of the 268 nm peak (conjugated diene absorption) added to the original 280 nm peak (protein absorption).

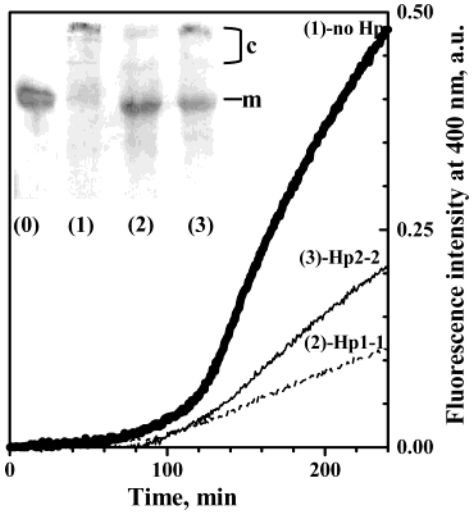


FIGURE 4: Effect of Hp phenotypes on oxidative modification of LDL protein (ApoB) by metHb. Shown is a representative of three independent experiments. The kinetic patterns of emission at 405 nm (upon excitation at 327 nm) of oxidized LDL's fluorescent products are shown. Reactions were carried out in PBS, pH 7.4 at 37 °C, and contained 100 μg/mL LDL, 3 μM metHb (solid line), and 1.5 μM Hp1-1 (dashed line) or 1.5 μM Hp2-2 (solid bold line). Insert: The protein pattern was visualized by SDS−PAGE. 600 μg/mL LDL (lane 0) was incubated with 10 μM metHb (lane 1) and 5 μM Hp1-1 (lane 2) or 5 μM Hp2-2 (lane 3) in PBS, pH 7.4 at 37 °C, for 240 min, and the samples from each reaction mixture were subjected to gel electrophoresis. Letters m and c indicate monomer and cross-linked ApoB, respectively.

in all regions within the first 60 min. After the lapse of 150 min, however, the absorbance of components in the 450−550 nm region had completely disappeared, the Soret absorption was reduced to 50%, and in the UVA region scattered light completely masked any other absorption. In the Hp2-2-containing reaction mixture, no changes were observed by 60 min. After 150 min, the Soret absorption was reduced to 75%, part of the β-carotene absorbance vanished, and in the UVA region minimal light scatter developed, allowing observation of CD formed. In the Hp1-1-containing reaction mixture, minimal reduction in the antioxidants and in the Soret region was observed within 150 min and some CD formed.

Modification of LDL Protein. To follow the fate of ApoB, we used two techniques: SDS−PAGE, which allows estimation of ApoB modification from monomer to covalently cross-linked protein, and fluorescence emission, which measures bityrosine formation resulting from termination of tyrosine radicals (*6*). Unlike the former, the latter technique has the advantage of allowing continuous monitoring. Typical results are demonstrated in Figure 4. In the absence of Hp, no bityrosine emission was observed in the first 60 min, little up to 110 min, and, in the following, a fast phase of bityrosine formation. A similar pattern appeared in the Hp-containing reaction mixtures except that the rate of fluorescence increase following 110 min was much reduced. Inhibition by Hp1-1 was more pronounced as compared to Hp2-2.

The protein pattern of reaction mixtures was compared at 240 min corresponding to the end of continuous monitoring period parameters as shown in the SDS−PAGE of Figure 4. In the absence of Hp, all ApoB monomers turned to high molecular mass aggregates, part of which can be seen in the gel origin (compare lane 1 with lane 0). In the Hp2-2-containing reaction mixture only part of ApoB remained as monomers while the rest formed aggregates as concluded from appearance of high molecular mass material in the gel origin (lane 3). In the presence of Hp1-1, only minimal protein could be observed as aggregates while a major part stayed as monomers (lane 2).

Effect of Haptoglobin Phenotypes on Heme Transfer from Hemoglobin to LDL. By all parameters and in different LDL samples, we observed up to this point poorer protection of metHb-derived LDL oxidation by Hp2-2 as compared to Hp1-1. In previous literature, metHb oxidative pathology in the vascular system has been assigned to globin-free hemin (*19*, *31*). We therefore devoted the final section of this study to find out differences in heme transfer from globin to LDL when hemoglobin is haptoglobin bound (*18*, *19*). The amount of hemin transferred from Hb to LDL can best be assessed by separation of LDL and the hemin-containing proteins followed by determination of the amount of the globin-bound hemin. LDL can be separated from Hb or its Hp complexes on the basis of size or density differences. Size-based separation was performed using ultrafiltration, which allows fast separation. XM300 membranes were found suitable because the Hb−Hp complexes were washed out while these membranes were impermeable to LDL. Reactions of LDL and Hb−Hp complexes were carried out under identical conditions to those presented in Figures 1−3. At time intervals samples were filtered (see Materials and Methods), and the concentration of hemin in the LDL-lacking filtrate was measured. Filtrates from Hb−Hp1-1-containing reactions retained higher hemin concentrations as compared to Hb−Hp2-2-containing reactions. However, in all cases part of the hemin remained membrane associated (data not shown). Since recovery of the amphipathic hemin molecule from any other matrix is subject to the same pitfalls, to allow measurement of location of all hemin in the reaction mixtures, density-based separation of LDL and proteins in

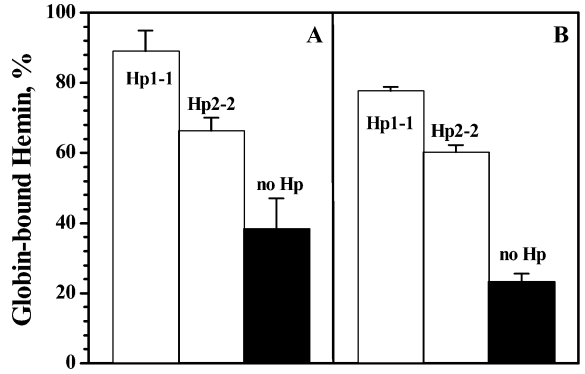


FIGURE 5: Separation of LDL and globin-bound hemin. Reaction mixtures (3 mL) contained 100 μg/mL LDL and 3 μM metHb either with no Hp, with 1.5 μM Hp1-1, or with 1.5 μM Hp2-2. Reactions were carried out in PBS, pH 7.4 at 37 °C. At specific time intervals KBr was added, and the mixtures were subjected to ultracentrifugation (see Materials and Methods for details). Following 18 h the upper and lower parts of the solutions were separated. Absorption spectra of reaction mixtures prior to centrifugation and of each fraction after centrifugation at 250−700 nm were measured. The amount of globin-bound hemin prior to reaction (time zero) was set as 100%. The relative amounts of hemin in each fraction were assessed according to Soret absorption. Shown are % of globin-bound hemin in the LDL-lacking fractions of the reaction mixtures applied to separation in the time zero (A) or after incubation for 240 min (B). Data shown present the mean ± SD of four independent experiments.

the reaction mixtures was performed. Since in these separations KBr is used, we first tested whether KBr affects the reactions showed in Figures 1−4. We found slight kinetic differences in the oxidation pattern of reactions, but the differences between Hp1-1- and Hp2-2-containing reactions were retained (data not shown). Thus, at time points KBr was added to reaction mixtures followed by ultracentrifugation (see Methods). The upper (LDL-containing) and lower (LDL-free) protein fractions were separated. As judged by absorption spectra, the upper fractions contained protein but no hemin (data not shown), while in the lower fractions both protein and hemin were present. As seen in the results presented in Figure 5, partial hemin transfer occurs during separation (Figure 5A), but preincubation of reaction mixtures at 37 °C for 240 min resulted in propagation of the hemin transfer (Figure 5B). Importantly, under all conditions the fraction of globin-bound hemin varied in the following order: Hp1-1-containing reaction > Hp2-2 > hemoglobin alone containing one.

To assess continued kinetics of hemin transfer to LDL, we used the FRET method (see Materials and Methods), allowing followup of molecule translocation. This method was recently used by us successfully to analyze heme transfer from Hb (*6*). To this end, experiments were performed with dansylated LDL, and the time-dependent changes of fluorescence intensity of the dansyl probe were followed. In the absence of Hp, we observed time-dependent reduction in the fluorescence intensity. As seen from representative results (Figure 6), the kinetic pattern consists of a slow phase followed by a fast one. The biphasic pattern of the fluorescence quenching kinetics was not evident in our previous study (Figure 5A in ref *6*) because the data were obtained using a technology with a lower signal-to-noise ratio and, in addition, did not allow continuous monitoring of several samples. These data indicate that both Hp phenotypes slowed the fluorescence reduction but inhibition of fluorescence

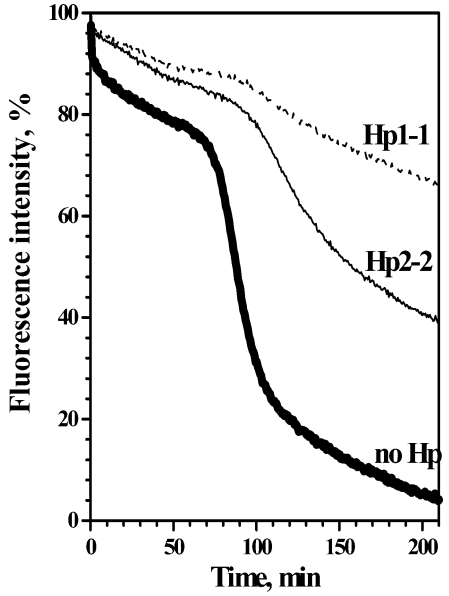


FIGURE 6: Time-dependent quenching of dansyl fluorescence intensity in LDL. Shown is a representative of three independent experiments. Reactions were carried out in PBS, pH 7.4 at 37 °C, and contained 100 μg/mL dansylated LDL (details concerning dansylation are found in Materials and Methods), 3 μM metHb (solid line), and 1.5 μM Hp1-1 (dashed line) or 1.5 μM Hp2-2 (solid bold line). To minimize quenching of the fluorophores by light absorbance of the hemoglobin (trivial quenching), low (micromolar) concentrations were used, and their contribution to quenching was subtracted. For details of analysis, see Materials and Methods. Note that the kinetic curve of dansyl quenching in the control (designated no Hp) appears different from a curve of similar experiments in ref *6* (Figure 5). The current curve provided with improved equipment should be considered updated.

intensity quenching by Hp1-1 was much more pronounced than inhibition by Hp2-2 (Figure 6).

DISCUSSION

As stated in the introduction, the literature provides extensive evidence that individuals with the Hp2-2 phenotype are less protected against vascular oxidative pathology as compared to those with Hp1-1 (*13*). These differences have been well documented in pathologies of oxidative conditions such as diabetes mellitus, where the tendency for atherosclerosis is high (*23*). Oxidative stress triggers excellular Hb transformation to metHb, which can be bound by both haptoglobin phenotypes (*39*). It has been established that preincubation of metHb with haptoglobin inhibits oxidative modification of LDL and its cytotoxicity to vascular endothelium (*19*). Previous results and the current study indicate the oxidative role of hemin lodged within the LDL (*6*, *19*, *36*). To minimize the damage induced to hemoglobin as a pseudoperoxidase only (*18*), hydrogen peroxide was omitted from the reaction mixtures of the current study. Thus any hemoglobin-triggered oxidation should rely on LDL lipid peroxides (*19*, *40*).

To allow a record of any minute differences between the two Hp phenotypes in protecting LDL against Hb-derived oxidation, experiments relied on identical reaction composition besides Hp. By all parameters used to continuously monitor oxidation kinetics, two main phases were observed: in the first time window small and slow changes in the lag time took place; in the second, high-rate oxidative

activity occurred (Figures 1, 2, and 4). Both Hp phenotypes inhibited oxidation of LDL components, albeit to a different degree, Hp1-1 being a more potent inhibitor than Hp2-2. The kinetics of the UVA (Figure 1) shows that while the Hp-lacking and the Hp2-2-containing reactions are characterized by a fast burst phase, in the reaction containing Hp1-1, only a slow linear kinetics occurred. The decrease of the Soret peak indicates a complementary kinetic pattern (Figure 2), namely, a linear, burst-devoid, kinetics in the Hb−Hp1-1-containing reaction only. The small reduction in the Soret absorption relates to changes in the molar extinction coefficient upon hemin translocation from metHb to ApoB of LDL (*6*). Such a linear UVA kinetics of LDL peroxidation has been shown as typical for reactivity of intact hemoproteins such as horseradish peroxidase (*40*). Thus, the linear pattern of oxidation in the Hb−Hp1-1-containing reaction should be attributed to the activity of the intact metHb, and the burst kinetics should relate to hemin breakdown within the LDL providing free iron. The latter is in correlation with the consequences of previous studies related to hemin-derived LDL oxidation (*6*, *19*, *36*). The fact that hemoglobin bound to Hp1-1 tends to hold its hemin can be best seen from the results of Figure 5. Experiments where LDL was separated from the hemoglobin clearly show that (i) hemin transferred to LDL was completely destroyed and (ii) Hp2-2 fails under all conditions to hold the globin-bound hemin as strong as Hp1-1.

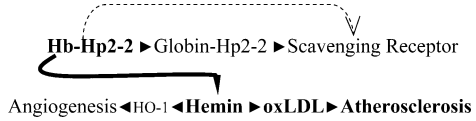
Of specific interest are kinetic results of bityrosine formation. Although, as by other oxidation parameters, Hp1-1 is a more potent inhibitor than Hp2-2, both Hp phenotypes diminished most of the bityrosine signal intensity (Figure 4). Potentially, bityrosines in the metHb/LDL can result from termination of ApoB inter- and intratyrosine radicals as well as hetero-cross-linking of globin and ApoB tyrosine radicals (*18*, *41*). Since no external peroxide was used in the current study, globin radicals could only be formed by LDL peroxides (*42*). To form hetero-cross-linking, globin radicals should be able to contact ApoB radicals. However, attachment of Hp to globin might prevent contact of globin and ApoB radicals, independent of hemin release. It thus appears that a large fraction of bityrosines in the Hp-lacking reaction results from hetero-cross-linking as noted by other methodologies (*41*).

The kinetics of LDL fluorescence quenching in both the absence and presence of Hp (Figure 6) revealed two phases as well. In the control (absence of Hp) reaction, the first phase (lasting ∼60 min) can definitely be attributed only to heme transfer from globin to ApoB high-affinity sites (*6*). In the second phase (70−110 min from reaction start), accelerated reduction of the probe fluorescence was observed. This time period overlaps the burst phase presented by all parameters used to observe LDL oxidation. Therefore, this phase seems to include, in addition to fluorescence quenching by heme transfer, bleaching of the fluorescent probe by the free iron triggered lipid peroxides formed within the LDL. Both Hp phenotypes decreased the rate and amount of heme transferred to LDL. Logically, under conditions allowing a larger amount of heme and thus formation of more lipid peroxides, the bleaching of the probe was faster as well. Independent of detailed kinetic analysis, these results indicate that Hp1-1 is much more efficient than Hp2-2 as an inhibitor of heme transfer from metHb to LDL.

Continuous monitoring of full spectra kinetics assists the analysis of the differences between the Hp phenotypes, indicating that LDL in the Hp2-2-containing reaction is under a higher oxidative stress as compared to the Hp1-1-containing one (Figure 3). This is well demonstrated by abolishment of the absorption typical to antioxidants such as β-carotene (∼490 nm). The UV region indicates a higher light scatter contribution, resulting from aggregation of LDL, in the Hp2-2-containing reaction as compared to the Hp1-1-containing one. Still, the partial protection ability of Hp2-2 is indicated by a massive light scatter contribution in the UV region of the Hp-devoid reaction. The oxidative modification of the LDL protein (SDS−PAGE, insert to Figure 4) is consistent with the relative LDL oxidizability as demonstrated by the above parameters. Loss of the ApoB monomer in the SDS−PAGE protein patterns to form protein, thereby LDL, aggregates (*18*) indicates that, while Hp1-1 inhibited ApoB covalent cross-linking almost completely, Hp2-2 was only partially effective.

Although many studies pointed out the potential oxidizability in individuals with the Hp2-2 phenotype (*13*, *45*), the molecular basis for this phenomenon is still unclear. Sorting out the literature indicates apparently contradicting information. On the one hand, the Hp−Hb complex was reported to have an elevated peroxidase activity relative to Hb (*43*), and on the other hand, Hp has been shown to be an antioxidant (*17*−*19*). This apparent contradiction is explained by understanding that intact metHb can oxidize small substrates while the hazard to macromolecules and cells is inherited in the detached hemin (*18*, *27*, *40*). It appears that, despite all protection afforded by the high avidity of all Hp phenotypes for Hb (*46*, *47*) and the uptake of the complex by the macrophage receptor (*11*, *12*), translocation of hemin into LDL suffices as a trigger for vascular pathology (*19*, *31*, *36*). It appears that at least some of Hb−Hp units of the oligomeric Hp2-2 (*13*) fail to strengthen the hemin to globin bond, allowing heme release to LDL.

Previous extensive studies indicated the complexity of the role played by hemin in the vasculature. On one hand, Hb-detached hemin is toxic to the endothelium integrity, while on the other hand, the hemin-induced expression of HO-1 in endothelial cells provides protection against oxidation (*19*, *27*−*29*). Apparently, HO-1 expression protects not only through the enzymatic cleavage of cytotoxic hemin but also by facilitating angiogenesis (*48*, *49*). The fact that Hp2-2 was found to be a more potent angiogenesis activator than Hp1-1 (*44*) probably relates to our findings that heme escapes easily from Hb−Hp2-2 as compared to Hb−Hp1-1. This suggests that the heme which has leaked from circulating Hb−Hp complexes and lodged in the endothelial cells is the trigger of angiogenesis. The following scheme of reactions summarizes the Hb−Hp2-2 triggered vasculature events (information of the current study in bold):



It thus appears that the human body has developed a way of defense allowing increased angiogenesis in individuals with Hp2-2. However, the negative effect of increased

angiogenesis in cancer should also be considered. Our results suggest that Hp2-2 does not provide efficient protection to the heme-threatened vascular pathology. The answer as to why evolution did not yet get rid of the Hp2-2 gene probably resides in the fact that atherosclerosis is a relatively "new" disease of the modern industrial society.

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