Haptoglobin Binding to Apolipoprotein A-I Prevents Damage from Hydroxyl Radicals on Its Stimulatory Activity of the Enzyme Lecithin-Cholesterol Acyl-Transferase†

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ABSTRACT: Apolipoprotein A-I (ApoA-I), a major component of HDL, binds haptoglobin, a plasma protein transporting to liver or macrophages free Hb for preventing hydroxyl radical production. This work aimed to assess whether haptoglobin protects ApoA-I against this radical. Human ApoA-I structure, as analyzed by electrophoresis and MS, was found severely altered by hydroxyl radicals in vitro. Lower alteration of ApoA-I was found when HDL was oxidized in the presence of haptoglobin. ApoA-I oxidation was limited also when the complex of haptoglobin with both high-density lipoprotein and Hb, immobilized on resin beads, was exposed to hydroxyl radicals. ApoA-I function to stimulate cholesterol esterification was assayed in vitro by using ApoA-I-containing liposomes. Decreased stimulation was observed when liposomes oxidized without haptoglobin were used. Conversely, after oxidative stress in the presence of haptoglobin (0.5 μM monomer), the liposome activity did not change. Plasma of carrageenan-treated mice was analyzed by ELISA for the levels of haptoglobin and ApoA-I, and used to isolate HDL for MS analysis. Hydroxyproline-containing fragments of ApoA-I were found associated with low levels of haptoglobin (18 μM monomer), whereas they were not detected when the haptoglobin level increased (34−70 μM monomer). Therefore haptoglobin, when circulating at enhanced levels with free Hb during the acute phase of inflammation, might protect ApoA-I structure and function against hydroxyl radicals.

The process of removal and transport of cholesterol from peripheral cells to liver, known as reverse cholesterol transport, is mediated by high-density lipoprotein (HDL[1]) (*1*), and is of fundamental importance for prevention or reversal of atherosclerotic cardiovascular diseases (*2*). Free or lipid-poor forms of the apolipoprotein (Apo) A-I serve as acceptor targets for cholesterol and phospholipids from arterial wall macrophages. These particles, called nascent HDL, use ApoA-I to stimulate the enzyme lecithin-cholesterol acyl-transferase (LCAT, EC 2.3.1.45) for converting cell-derived cholesterol into ester forms, which are then placed into the lipoprotein core and change the particle shape and mass. Then HDL transports cholesterol and cholesteryl esters to liver, where these are selectively uptaken for production of bile acids, while ApoA-I is bound by specific cell receptors (*3*−*5*). Thus ApoA-I plays crucial roles in key steps of reverse cholesterol transport. In addition, ApoA-I binds also steroidogenic cells, which are thus provided with cholesterol for hormone synthesis (*6*). The function of this apolipoprotein may be severely altered by structure modifications, as those resulting from genetic factors (*7*) or oxidative damage (*8*−*12*). There is a growing belief that atherosclerotic cardiovascular diseases develop from vascular injury associated with inflammation (*13*, *14*). In particular, the immune response and the resulting oxidative stress severely contribute to monocyte infiltration into the arterial wall, where they differentiate into macrophages and, after cholesterol accumulation, foam cells (*14*). Therefore importance and efficiency of reverse cholesterol transport should be considered into the frame of inflammatory or oxidative processes. ApoA-I should be protected against oxidative stress, and several factors might be involved to prevent the attack of oxidants, thus saving the HDL function

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[1] Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); ApoA-I, apolipoprotein A-I; ApoD, apolipoprotein D; ApoC-II, apolipoprotein C-II; BSA, bovine serum albumin; DS, dextran sulfate; ELISA, enzyme-linked immunosorbent assay; Hb, hemoglobin; HDL, high-density lipoprotein; Hpt, haptoglobin; HSA, human serum albumin; GAR-HRP, goat anti-rabbit horseradish-linked peroxidase; LCAT, lecithin-cholesterol acyl-transferase; MS, mass spectrometry; PVDF, polyvinylidene difluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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in cholesterol transport. Antioxidant factors are mostly enzymes or free radical scavengers including nonenzyme proteins (*15*). Haptoglobin (Hpt), a polymorphic protein with enhanced plasma levels during the acute phase of inflammation (*16*), plays its antioxidant role by binding free Hb and forming a complex which is sequestered by liver or macrophages (*17*, *18*). Free or Hpt-bound Hb can trigger the Fenton reaction (*19*) from H_2O_2 originated by the vascular endothelium (*20*−*22*) or macrophages (*15*). This reaction oxidizes ferrous to ferric ion in Hb, producing the highly aggressive hydroxyl radical (*19*). The radical production can be reiterated as much as ascorbate molecules are available to recycle ferric ions back to the reduced state (*23*). Hpt also binds ApoA-I (*24*, *25*) by domain(s) different from those capturing Hb (*26*). The ApoA-I domain interacting with Hpt is also (partially) required for stimulating LCAT (*27*, *28*). Therefore the binding of Hpt to ApoA-I has been suggested to be an important regulatory factor for the LCAT activity (*27*). The amount of Hpt-bound ApoA-I depends on the ratio of Hpt with ApoA-I, and this ratio was found higher and negatively correlated with the LCAT activity in patients with rheumatoid arthritis (*29*), which is a pathologic condition associated with inflammation (*30*, *31*). Hpt might shield ApoA-I against harmful oxidants. In fact, Hpt has a molecular mass far greater than that of ApoA-I (*16*) and might scavenge oxidants by its reducing amino acid residues (*15*). On the other hand Hpt can form a complex with both Hb and ApoA-I (*25*, *26*), thus keeping ApoA-I very close to the source of hydroxyl radicals. In this case, Hpt might address the radical attack just to immobilized ApoA-I.

The purpose of our work was to study structure alteration of ApoA-I exposed to hydroxyl radicals, and ascertain whether Hpt, in conditions of oxidative stress similar to those present during inflammation, prevents or limits such an alteration. Correlation of Hpt level with ApoA-I hydroxylation was studied in mice treated with carrageenan. Information on the LCAT stimulatory function of ApoA-I, undergoing oxidative stress with or without Hpt, is also reported.

EXPERIMENTAL PROCEDURES

Materials. Chemicals of the highest purity, BSA, HSA, cholesterol, cholesteryl linoleate, human Hpt (mixed phenotypes), rabbit anti-human Hpt IgG, GAR-HRP IgG, ABTS, DS (Dextralip 50), carrageenan, and molecular mass markers, were purchased from Sigma-Aldrich (St. Louis, MO). The peptide P2a (SPLGEEMRDRARAHVDALRTHLA) was synthesized by INBIOS (Pozzuoli, Italy). Human ApoA-I and rabbit anti-human ApoA-I polyclonal IgG were from Calbiochem (La Jolla, CA). (1α,2α-^{3}H)Cholesterol (45 Ci/mmol) was obtained from Perkin-Elmer (Boston, MA). Modified porcine trypsin was from Promega (Madison, WI)-. Hi-Trap NHS-activated sepharose (Amersham Biosciences, Buckinghamshire, U.K.) and PVDF transfer membrane (Millipore, Bedford, MA) were used.

HDL Oxidation and LCAT Assay. HDL were isolated from pooled plasma of healthy donors as previously published (*32*). The oxidation system was constituted by a reaction mixture with 100 μM H_2O_2 and 100 μM ascorbate in TBS (TBS: 130 mM NaCl, 20 mM Tris-HCl, pH 7.4), supplemented with Hb as below described. The radical production was monitored by measuring the absorbance at 734 nm of reaction mixtures, containing 6 μM Hb and 450 μM ABTS, kept at 37 °C (*33*). Radicals were no more produced after 1 h of incubation as ABTS, when added to the reaction mixture at this time, was not converted into colored products. HDL oxidation was carried out by incubating the reaction mixture with 0.15 mg/mL lipoprotein and 6 μM Hb (15 min, 37 °C). This oxidation was performed, when required, in the presence of 12 μM HSA or Hpt. ApoA-I-containing proteoliposomes, prepared as below described, were oxidized with different concentrations of Hb (0.5, 1.5, 2.5, or 3.2 μM) in the presence of 0.5 μM Hpt, or with 0.5 μM Hb in the presence of different concentrations of Hpt (0.5, 1, 3, or 10 μM). Competition of ApoA-I with P2a for binding Hpt was carried out by a proteoliposome oxidation mixture containing 0.5 μM Hb and 0.5 μM Hpt, supplemented with different P2a concentrations (3, 9, 30, or 90 μM). The molarity of Hpt was calculated as concentration of monomers α-β (*27*). The LCAT assay was performed, with proteoliposomes untreated, or oxidized in different conditions, as previously published (*27*). The enzyme activity was determined as nanomoles of cholesterol incorporated per hour per milliliter of plasma, and expressed in units (U).

Electrophoresis and Immunoblotting. Electrophoresis was carried out in denaturing and reducing conditions on a 1.5 mm thick slab gel of 15% polyacrylamide, as previously reported (*34*). Molecular mass markers (BSA, 66 kDa; egg albumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; trypsin inhibitor, 20 kDa; α-lactalbumin, 14 kDa) were used as standards of the protein electrophoretic mobility. Gel fixing and staining were omitted when the gel was processed for immunoblotting analysis.

Western blotting onto PVDF membrane and staining by antibodies were carried out essentially as previously described (*35*). In particular, proteins were transferred to PVDF membrane, which was treated with anti-ApoA-I IgG, and then stained by GAR-HRP IgG using 4 mM 4-chloro-1-naphthol for detecting immunocomplexes.

Gels were processed for MS analysis as follows. Small slices of Coomassie-stained gels (1.5 mm thick, 10 mm wide, 1.5−2 mm long) were manually excised, and destained overnight with 40% ethanol in 25 mM ammonium bicarbonate. The gel pieces were washed twice with 25 mM ammonium bicarbonate and desiccated three times with acetonitrile. Each piece was then reswollen in 16 μL of 25 mM ammonium bicarbonate containing 0.8 μg trypsin. After overnight digestion at 37 °C, peptides were extracted by sonication in 25 mM ammonium bicarbonate and analyzed by MS.

Affinity Chromatography. Hi-Trap NHS-activated resin was used to prepare stationary phases for affinity chromatography. Hb was linked to the resin, according to the manufacturer's instructions. Hb (1 mg) was coupled with 0.5 mL of resin beads. After coupling, the Hb-coupled resin was divided into two equal aliquots, which were packed in separated small columns, and equilibrated with 10 volumes of 50 mM sodium phosphate at pH 7.4 (P buffer). One column was loaded with 100 μL of 58 μM Hpt at 0.2 mL/min, and then washed by P buffer at 1 mL/min (until the effluent absorbance at 280 nm was $<$0.01 OD/mL) to remove unbound protein. The column which was not treated with Hpt served as control. Both columns were loaded, at 0.2 mL/

min, with 100 μL of native HDL containing 28 μM Apo A-I in P buffer. Unbound HDL was washed out from the Hpt−Hb column by TBS. A solution (90 μL) of 100 μM H_2O_2 and 100 μM ascorbate in TBS was absorbed by each column, and the flow through was discarded. After 15 min at 37 °C, the columns were eluted with 0.1 M glycine-HCl at pH 2.8. The eluted proteins were pooled, concentrated by centrifugation under vacuum, and processed for electrophoresis.

Treatment of Mice and Analysis of Plasma and Isolated HDL. Male Swiss mice (CD-1; Charles River, Italy) were injected with 1% carrageenan in isotonic saline solution, according to a published procedure (*36*). The effect of inflammation was studied in animals sacrificed by carbon dioxide 6, 24, 48, 72, 96, and 144 h after injection. At each time length a group of animals ($N = 3$) was sacrificed, and their plasma was prepared. One group of untreated mice served as control. An aliquot of plasma (50 μL) from each mouse was sampled for titration of ApoA-I and Hpt. Another aliquot (350 μL) was pooled with equal aliquots from mice belonging to same group, and used for MS analysis. The level of ApoA-I and Hpt was determined by ELISA using mouse antigens, isolated by published procedures (*26*, *37*) and exhibiting over 98% purity by electrophoresis and densitometry analysis, as standards for calibration. The plasma pool was in part (50 μL) directly analyzed for the albumin level by MS, and in part (900 μL) treated with 0.1% DS and 50 mM $MnCl_2$ in TBS to precipitate lipoproteins other than HDL, which were removed by centrifugation. Supernatant HDL precipitated in 0.65% DS and 200 mM $MnCl_2$ in TBS, and were pelletted by centrifugation. The pellet was dissolved in 400 μL of TBS and then processed for MS analysis. The experiments on mice were conducted adhering to the requirements of the University of Naples Federico II for the care and use of laboratory animals.

NanoLC-Nanospray MS/MS Analysis. Peptides from human proteins fractionated by electrophoresis were prepared as above described. Peptides from mouse apolipoproteins were obtained by overnight incubation of isolated HDL with 47 μg/mL trypsin at 37 °C. Each peptide sample (1 μL) was loaded onto a ZORBAX 300 (75 μm × 150 mm, 3.5 μm particles) SB-C18 RP column (Agilent Italia, Milan, Italy) and eluted with a 50 min gradient of acetonitrile from 5% to 95% in 0.1% formic acid, at a 0.3 μL/min flow rate by a HP 1100 nanoHPLC system (Agilent Italia, Milan, Italy) coupled to a XCT-Plus nanospray-ion trap mass spectrometer (Agilent Italia, Milan, Italy). MS parameters were the following: 100−2200 *m*/*z* scan range, 8100 *m*/*z* s^{-1} scan speed, 5 L/min dry gas flow, 300 °C dry temperature, 1.8 kV capillary, 40 V skimmer, 125000 ion charge control (ICC) target, 300 ms maximum accumulation time. Positively charged peptide ions were automatically isolated and fragmented, and spectra were deconvoluted by the Data Analysis software (Bruker Daltonics, Bremen, Germany). For nanoLC-nanospray MS/MS experiments the NCBInr database and Mascot search algorithm (http://www.matrixscience.com/search_form_select.html) were chosen. Peptide tolerance ± 1.8 Da, MS/MS tolerance ± 0.8 Da, significance threshold $p < 0.05$ were used. Considered modifications were methionine oxidation, proline hydroxylation, and sodium adduct to aspartate, glutamate, and C-terminus.

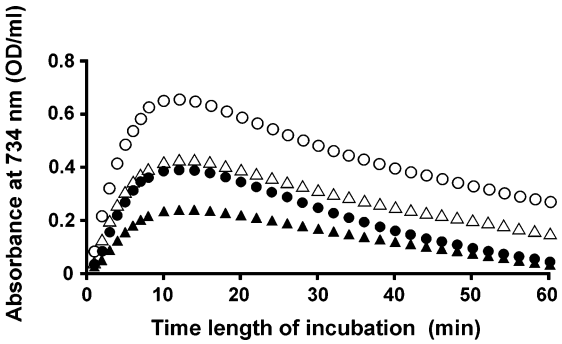

FIGURE 1: Kinetics of hydroxyl radical production. Hydroxyl radicals were produced by incubating 6 μM Hb with 100 or 50 μM H_2O_2 in the presence of 100 μM ascorbate at 31 °C. The reaction mixture contained ABTS, as radical target. Formation and fading of the colored $ABTS^{\bullet+}$ cation with the radical was monitored by measuring the absorbance of the mixture at 734 nm. Mixtures supplemented with HDL were analyzed. Open circles: 450 μM ABTS + 100 μM H_2O_2. Open triangles: 450 μM ABTS + 50 μM H_2O_2. Filled circles: 450 μM ABTS + 100 μM H_2O_2 + 1 mg/mL HDL. Filled triangles: 450 μM ABTS + 50 μM H_2O_2 + 1 mg/mL HDL.

Statistical Analysis. Each sample in all the experiments, except those from mice, was processed at least in triplicate, and the datum was expressed as mean value ± SEM. Samples in experiments with mice were processed in duplicate to obtain averages and deviations; interassay coefficients of variation, for signal intensity at each time length of treatment, from three separate experiments were calculated. The program "Graph Pad Prism 3" (Graph Pad Software, San Diego, CA) was used to perform *t*-test analysis.

RESULTS

ApoA-I Oxidation by Hydroxyl Radicals and Hpt Protective Effect. A system for producing hydroxyl radicals by Fenton chemistry was set up, using the conversion of Hb ferrous into ferric ions to feed the reaction $H_2O_2 \rightarrow HO^- + HO^{\bullet}$. Recycling of oxidized iron back to its reduced form, a mandatory step to extend this process when H_2O_2 is in stoichiometric excess, was obtained by supplementing the reaction mixture with ascorbate. Radical production was monitored by measuring the amount of colored $ABTS^{\bullet+}$ cations originating from the reaction of $HO^{\bullet}$ with ABTS. As shown in Figure 1, the adduct level increased in less than 15 min from the reaction start, but the formed color was not stable, as fading was observed in extended incubation. This system of radical production was used to oxidize HDL. The lipoprotein, when added to the reaction mixture, effectively competed with ABTS for hydroxyl radicals, as formation of colored cations resulted remarkably lowered (Figure 1). These data show that HDL were oxidized by H_2O_2 concentration similar to that produced in pathological conditions (*15*).

After oxidative treatment, the HDL was analyzed by SDS−PAGE. The pattern of Coomassie-stained proteins from oxidized HDL, as compared with that from native HDL, exhibited additional or more intense bands (Figure 2; lane 4 versus lane 2). In particular, the band of oxidized ApoA-I migrated broader than that of native ApoA-I, with apparent molecular mass slightly lower than 24 kDa, and several additional bands were detected all over the protein fraction-

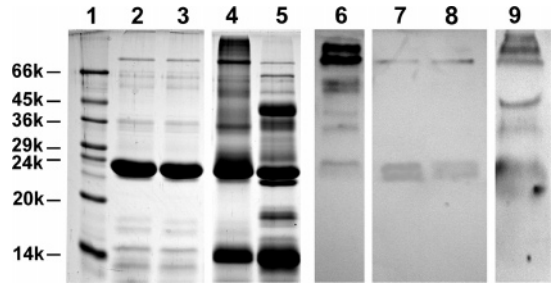

FIGURE 2: Electrophoretic analysis of HDL treated by oxidative stress. Aliquots of HDL solution containing 6 μM ApoA-I were used. The proteins were fractionated by SDS−PAGE and stained by Coomassie R250, or blotted onto PVDF membrane for immunostaining with rabbit anti-Apo A-I IgG and HRP-GAR. Samples of 40 μL were loaded on the gel. Lane 1: Molecular mass standards, Coomassie-staining. Lane 2: Mixture of native HDL with 6 μM Hb, Coomassie-staining. Lane 3: HDL incubated with H_2O_2, Coomassie-staining. Lane 4: HDL oxidized by Hb/H_2O_2/ascorbate (6/100/100 μM respectively), Coomassie-staining. Lane 5: HDL oxidized by Hb/H_2O_2/ascorbate in the presence of 12 μM Hpt, Coomassie-staining. Lane 6: HDL oxidized by Hb/H_2O_2/ascorbate, immunoblotting. Lane 7: native HDL, immunoblotting. Lane 8: HDL oxidized by Hb/H_2O_2/ascorbate in the presence of 12 μM Hpt, immunoblotting. Lane 9: HDL oxidized by Hb/H_2O_2/ascorbate in the presence of 12 μM HSA, immunoblotting.

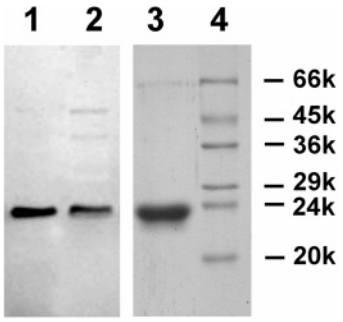

FIGURE 3: Electrophoretic analysis of HDL oxidized by bead-coupled Hb. Columns of sepharose (0.25 mL) were coupled with Hb. One column was loaded with 0.5 mL of solution containing 1.3 μg/mL HDL (28 μM Apo A-I) and 58 μM Hpt, washed by TBS, and then loaded with Hb/H_2O_2/ascorbate. The other column was loaded with the same HDL amount, and directly loaded with the oxidation mixture. The retained material was eluted by 0.1 M glycine−HCl at pH 2.8, and analyzed by SDS−PAGE and immunoblotting. Lane 1: antigens from the treatment with Hpt. Lane 2: antigens from the treatment without Hpt. Coomassie staining of purified ApoA-I and molecular mass markers, fractionated by SDS−PAGE, are shown in lanes 3 and 4 respectively.

ation range. When Hpt was added to the oxidation system, the HDL pattern was found changed. Actually, in this case, the heaviest oxidation products were not found and new bands, not present in the patterns of "untreated" or "treated without Hpt" HDL, were observed (Figure 2; lane 5 versus lane 2 or 4 respectively). The proteins separated by electrophoresis were also analyzed by immunoblotting. After protein transfer onto PVDF membrane, rabbit anti-ApoA-I and HRP-GAR IgG was used to stain ApoA-I and ApoA-I-like antigens. Native ApoA-I was found migrated as two bands, accompanied with a band having apparent molecular mass of 73 kDa (Figure 2; lane 7). This slow migrating band of ApoA-I-like antigens might be a protein constituted by ApoA-I cross-linked with other apolipoprotein(s). Samples of oxidized HDL mostly contained such a band, more intense than from native HDL, and a slower band (over 80 kDa) of comparable intensity, accompanied with a number of additional antigens migrated in the range of apparent molecular mass from 66 to 29 kDa (Figure 2; lanes 6). It cannot be excluded from this experiment that minor oxidative modifications, not influencing the electrophoretic mobility, are present also in Apo A-I of treated samples. When Hpt was present during oxidative stress, the antigens detected in the sample of oxidized HDL seemed just the same observed in the pattern of untreated HDL (Figure 2; lane 8 versus 7). A control experiment was carried out by incubating HDL with HSA in the oxidation mixture. The electrophoretic pattern of ApoA-I antigens, as detected by immunoblotting, was similar to that observed from samples of native HDL incubated without HSA (Figure 2; lane 6 versus lane 9). It is worth noting that all the analyzed samples contained the same amounts of HDL, but anti-ApoA-I IgGs reacted with some ApoA-I-like antigens much better than with ApoA-I.

The data indicate that oxidation alters the electrophoretic mobility of several HDL proteins, and suggest that antigens containing ApoA-I epitopes originate from attack of hydroxyl radicals to ApoA-I structure. The finding that Hpt limits the production of several oxidation products from HDL, particularly those with lower electrophoretic mobility, and prevents the conversion of ApoA-I into antigens with higher molecular mass suggests that bound Hpt protects ApoA-I against hydroxyl radicals.

Complex of Hpt with Both Hb and ApoA-I: Hpt-Mediated Defense of ApoA-I against Radicals from Hb. A large part of Hpt circulates in the form of a complex with Hb (*38*). The Hpt property to bind ApoA-I has above been mentioned. Since Hpt is a polymorphic protein, present as dimer or oligomer forms, it is conceivable that it might be simultaneously associated with both Hb and ApoA-I. In this case, it might join the important hydroxyl source Hb with the hydroxyl target ApoA-I. We therefore wondered whether Hb-bound Hpt would still be able to protect HDL ApoA-I from Hb-triggered oxidative damage. Hb was coupled to resin beads, which were then used to pack two small columns. One of these columns was loaded with Hpt excess to form stable Hb−Hpt complexes. After extensive washing for removing free Hpt, the resin was loaded with HDL, containing ApoA-I in an amount lower than that of Hb-bound Hpt. After this treatment, the resin was extensively washed to remove free HDL. Then, a solution containing ascorbate and H_2O_2 was added for stimulating resin-linked Hb to produce radicals. After incubation, HDL was eluted and analyzed by SDS−PAGE and immunoblotting. The other column, also packed with resin-linked Hb, was used as control, as it was not treated with Hpt, but directly loaded with HDL. This column was not washed, to avoid HDL release in the effluent, and incubation with ascorbate and H_2O_2 was carried out. The results indicate that ApoA-I, when exposed to radicals, was partly converted into minor forms with altered electrophoretic mobility only in the absence of Hpt (Figure 3). This experiment indicates that Hb-bound Hpt maintains the property to capture ApoA-I, and suggests that ApoA-I is protected from oxidative attack even though kept by Hpt close to the source of hydroxyl radicals.

MS Analysis of the ApoA-I Oxidation Forms. In order to characterize the degradation pattern of oxidized ApoA-I, we performed MS analysis of proteins from gels reported in Figure 2. The tracks of untreated HDL, oxidized HDL, and

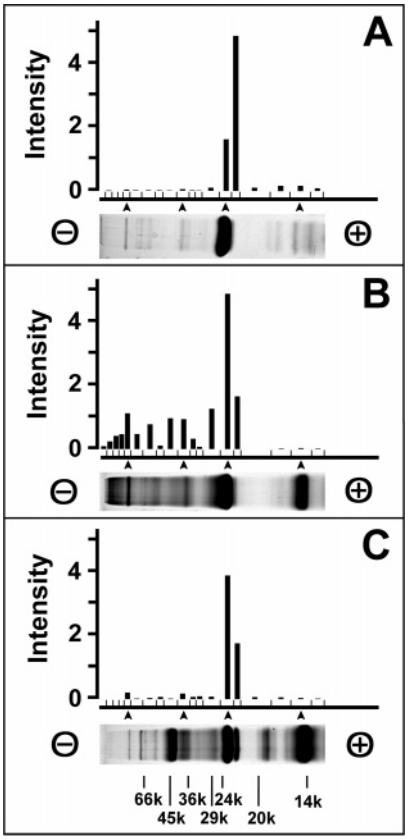

FIGURE 4: MS analysis of ApoA-I, oxidized in the presence or absence of Hpt, after electrophoresis. Tracks 3, 4, and 5 of the gel reported in Figure 2 were used. Panel A shows the migration of native HDL. Panels B and C show the migration of HDL oxidized in Hpt absence or presence, respectively. Each track was sliced into 19 fragments, destained, and digested by trypsin. The arrowheads indicate the slice numbers 5, 10, 14, and 18. The digestion mixture obtained from each slice was processed for nano-ESI analysis. The total intensity of peaks from Apo A-I fragments is reported as a vertical bar in correspondence of each slice, and expressed as arbitrary units from peak integration. The bars indicate how ApoA-I (GI:82571472, precursor, 267 amino acids) is distributed all over the gel pattern. A given bar, when compared with other bars, roughly expresses the relative amount of ApoA-I in the pattern, because different ionization efficiency of each tryptic peptide, different detection sensitivity at *m/z*, and simultaneous acquisition of MS and MS/MS are all averaged by considering the total mixture of ApoA-I fragments.

HDL oxidized in the presence of Hpt (Figure 2; lanes 2, 4, and 5, respectively) were cut each into 19 slices (Figure 4). Each slice was destained and, then, digested by trypsin for MS fingerprinting. As a first approach, we characterized the peptide pool from each gel slice, by nano-ESI MS. ApoA-I fragments from untreated HDL were mostly localized in slices 14−15 (Figure 4; panel A). These slices contained peptides accounting for about 75% of ApoA-I aminoacid sequence. Slice 5, corresponding to a protein band migrated with apparent molecular mass of 73 kDa, also contained some ApoA-I fragments, while all over the rest of the gel track ApoA-I was barely detected. After HDL oxidation (Figure 4; panel B), ApoA-I fragments were markedly detected in gel slices corresponding to molecular mass higher than expected. Increased content of ApoA-I fragments in slices 1 to 7 and 9 to 10 was observed, and a smear of ApoA-I fragments all over the track from slices 1 to 15 was detected. In particular, ApoA-I fragments containing the sulfone forms of Met-112 and Met-148 were detected in slice 5 (data not shown). The same ApoA-I fragmentation pattern was detected when HSA was used during HDL oxidation. Conversely, when Hpt was used during HDL oxidation (Figure 4; panel C), high molecular mass ApoA-I-containing proteins did not form, as indicated by the distribution pattern of ApoA-I fragments, which mostly matched that of untreated HDL (see panel A). It is worth noting that sulfones from Met-112 or Met-148 were not found. Since these residues are very close or within, respectively, the ApoA-I sequence interacting with Hpt (*27*), we argue that Hpt effectively protected its binding site on HDL.

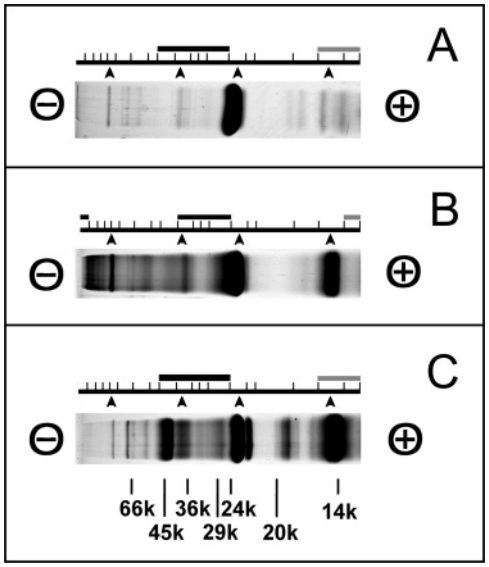

FIGURE 5: Electrophoretic analysis of oxidized ApoD or ApoC-II. Tracks 3, 4, and 5 of the gel reported in Figure 2 were used. Panel A shows the migration of native HDL. Panels B and C show the migration of HDL oxidized in Hpt absence or presence, respectively. Each track was sliced into 19 fragments, destained, and digested by trypsin. The arrowheads indicate the slice numbers 5, 10, 14, and 18. The digestion mixture obtained from each slice was processed by nano-ESI analysis for detecting ApoD (black horizontal bars) or ApoC-II (gray horizontal bars).

In addition to studying the Hpt protective effect on the structure of ApoA-I, we wondered whether such an effect was extended to minor HDL apolipoproteins. Consistently with previous data (*39*), ApoD amino acid sequences were mostly detected in slices 8 to 13 from the gel track loaded with untreated HDL, while ApoC-II was found only in slices 18 and 19 (Figure 5; panel A). After processing of oxidized HDL, ApoC-II fragments were found migrated similarly to those in the pattern of untreated HDL, while ApoD peptide fragments were detected in two track regions, one corresponding to slice 1 and the other to slices 10 to 13 (Figure 5; panel B). The feature that ApoD and ApoC-II structures are differently modified by oxidation suggests that not all the HDL apolipoproteins are equally exposed to oxidative stress, and only some of them react with other proteins to form heavy complexes on the HDL surface. When Hpt was present during the HDL oxidation, the formation of high molecular mass complexes was prevented (Figure 5; panel C), as already observed for ApoA-I (see Figure 4). In this case, the ApoD pattern in panel C was similar to that in panel A, and that of ApoC-II resulted unaffected. Details on the number or length of the peptides detected are reported in Tables 1 and 2. These data suggest that Hpt specifically protects some HDL apolipoproteins from oxidative damage.

Table 1: Tryptic Fragments of ApoD in Gel Slices[a]

slice	coverage (%)	peptides
A-9	9	152−164
A-10	21	76−92, 152−164, 165−175
A-11	17	52−60, 152−164, 165−175
A-12	25	46−51, 52−60, 76−82, 152−164, 165−175
A-13	33	8−21, 26−31, 32−40, 56−62, 132−144, 145−155
B-1	6	165−176
B-10	5	152−164
B-11	5	52−60
B-12	17	52−60, 152−164, 165−175
B-13	25	46−51, 52−60, 76−82, 152−164, 165−175
C-9	18	152−164, 165−185
C-10	21	52−60, 76−82, 152−164, 165−175
C-11	25	46−51, 52−60, 76−82, 152−164, 165−175
C-12	19	52−60, 76−82, 152−164, 165−175
C-13	24%	46−51, 52−60, 76−82, 152−164, 165−175

[a] Proteins from native (A) or oxidized HDL were fractionated by electrophoresis on polyacrylamide gels. HDL oxidation was performed in the absence (B) or presence (C) of Hpt. Each gel was cut into 19 slices, which were processed for ESI analysis as described in Experimental Procedures. Only slices containing ApoD peptides are shown. The protein sequence coverage was calculated from the sequence length of the detected peptides. Each peptide sequence is expressed as amino acid positions in the primary structure of ApoD (GI:13938509; protein precursor; 189 residues).

Table 2: Tryptic Fragments of ApoC-II in Gel Slices[a]

slice	coverage (%)	peptides
A-18	38	1−19, 20−30
A-19	87	1−19, 20−30, 31−39, 40−48, 56−76
B-19	35	1−19, 31−39
C-18	87	1−19, 20−30, 31−39, 40−48, 56−76
C-19	23	31−39, 40−48

[a] Proteins from native (A) or oxidized HDL were fractionated by electrophoresis on polyacrylamide gels. HDL oxidation was performed in the absence (B) or presence (C) of Hpt. Each gel was cut into 19 slices, which were processed for ESI analysis as under Experimental Procedures. Only slices containing ApoC-II peptides are shown. The protein sequence coverage was calculated from the sequence length of the detected peptides. Each peptide sequence is expressed as amino acid positions in the primary structure of ApoC-II (GI:14277770, 79 residues).

Other proteins, like Hb which was added to HDL for radical production, form high molecular mass complexes as much as ApoA-I during oxidative stress, but they conversely were not protected by Hpt (data not shown).

Oxidation of ApoA-I in HDL from Mice with Different Hpt Levels. Inflammation in mice was induced by carrageenan. At different time lengths following the antigen administration (0, 6, 24, 48, 72, 96, and 144 h), the animals were sacrificed and their blood was collected. Plasma aliquots were used for titration of ApoA-I and Hpt by ELISA, and for preparation of HDL. This lipoprotein fraction and other plasma aliquots were processed for nano-ESI-nanospray MS/MS analysis. ApoA-I levels in plasma, as detected by antibodies, did not significantly change until 96 h following carrageenan administration but were found decreased at 96 and 144 h, while Hpt levels increased in the time range from 6 to 48 but dropped down to normal values later on (Figure 6). In particular, the molar ratios of Hpt with ApoA-I were 0.68 ± 0.10, 0.75 ± 0.06, 2.02 ± 0.17, 4.00 ± 0.32, 1.5 ± 0.30, 1.6 ± 0.10, and 1.60 ± 0.21 after 0, 6, 24, 48, 72, 96, and 144 h respectively. A fragment of plasma albumin (439−452: APQVSTPTLVEAAR; from GI:5915682, precursor), containing amino acids which were not affected by oxidation and therefore representing the total protein amount (i.e., oxidized plus native forms), were detected by MS analysis as signals with intensity decreased during inflammation, as shown in Figure 7 (panel A). Albumin was also used as marker of circulating proteins which are exposed to oxidative stress, as its fragment 199−212 (ESCLT**P**KLDGVKEK) was found harboring hydroxylated Pro-204 from 6−24 to 144 h (Figure 7; panel B). Hydroxylation of Pro was assessed by the finding of at least five peptide ions overlapping the fragment amino acid sequence and displaying 16 Da higher mass, corresponding to conversion of one proline residue into 4-hydroxyproline, 5-hydroxyproline, or glutamic semialdheyde. The plasma concentration of ApoA-I was too low to be detected by MS. Therefore HDL, precipitated from plasma by DS and then dissolved in small volume, was used for MS analysis of ApoA-I. The fragment 142−154 (VAPLGAELQESAR; from GI:6753096, precursor), which was never found oxidized, was used as marker of the ApoA-I amount. The MS signal for this fragment was found decreased in samples from blood collected during inflammation (Figure 7; panel A). It is worth noting that the decrease in the ApoA-I levels, as observed by MS analysis, seems similar to that found by ELISA (Figure 7 panel A versus Figure 6). Also for ApoA-I, as in the analysis of albumin oxidation, hydroxylated Pro was chosen as marker of damage by hydroxyl radical attack. The fragment 237−247 (HSLM**P**MLETLK) was detected with hydroxylated Pro-241 in apolipoprotein samples prepared from blood collected 72 to144 h after carrageenan administration (Figure 7; panel B). In particular five peptide cations (three Y-type and two B-type, i.e., starting at Asp-234 or ending at Glu-244 respectively), having different length but all overlapping part of the fragment sequence, were detected. This result indicates that ApoA-I was hydroxylated only late (i.e., after 72 h) during inflammation, when the Hpt level dropped down, and

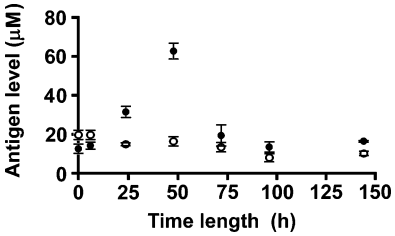

FIGURE 6: Titration of ApoA-I and Hpt in plasma from mice treated with carrageenan. Mice were injected with 50 μL of 1% carrageenan. After 6, 24, 48, 72, 96, and 144 h groups of three animals were sacrificed. One group was not treated and served as control (time 0). Blood was collected, and plasma from each animal was prepared. Samples ($N = 3$) were analyzed by ELISA for measuring the concentration of ApoA-I and Hpt. Rabbit anti-ApoA-I or anti-Hpt IgG was used to form immunocomplexes, which were incubated with GAR-HRP and, then, OPD and H_2O_2 for color development. Absorbance at 492 nm was measured, and the level of ApoA-I (open circles) or Hpt (filled circles) was determined using mouse Apo A-I and Hpt for calibration. The data are expressed as means ± SEM. In this figure a typical experiment is shown. Comparable data (no significant difference between values at each time point) from other two separate experiments were obtained.

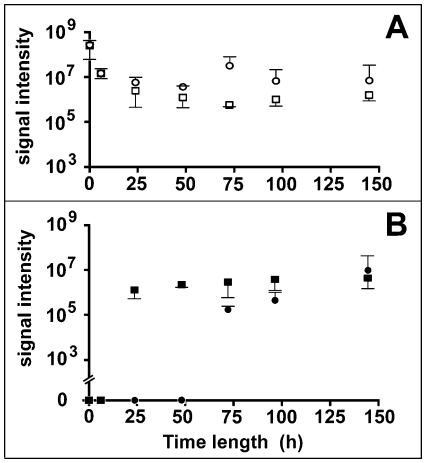

FIGURE 7: MS analysis of ApoA-I and albumin from mice treated with carrageenan. Mice were injected with 50 μL of 1% carrageenan. After 6, 24, 48, 72, 96, and 144 h groups of three animals were sacrificed. One group was not treated and served as control (time 0). Blood was collected, and plasma from each group was pooled. Samples from each pool were processed as such for albumin analysis, or treated with DS for isolating HDL. Untreated samples and isolated HDL were digested by trypsin, and the resulting polypeptide fragments were analyzed by nano-ESI MS for detection of albumin and ApoA-I amino acid sequences respectively. In panel A, the relative amounts of fragment 439−452 of albumin (open squares) and fragment 142−154 of Apo A-I (open circles), which were never found oxidized and therefore originated from both oxidized and native protein, are shown. In panel B, signal intensities for fragment 199−212 of albumin (filled squares) and fragment 237−247 of ApoA-I (filled circles), which harbor hydroxylated proline residues, are shown. GI:5915682 and GI:6753096 were used for identification of albumin and ApoA-I peptides respectively. Hydroxyproline was identified by at least three Y-type and two B-type ions originated from these fragments ($\Delta m/z = +16$). The intensities of the spectrometer signals are expressed in arbitrary units. Samples from each time length of treatment were processed in duplicate, and averages and deviations were calculated. Averages and deviations (ApoA-I, positive deviations; albumin, negative deviations) from one experiment are shown. Three separate experiments were carried out. Interassay coefficients of variation for signal intensity, at each time length, were never found higher than 8%.

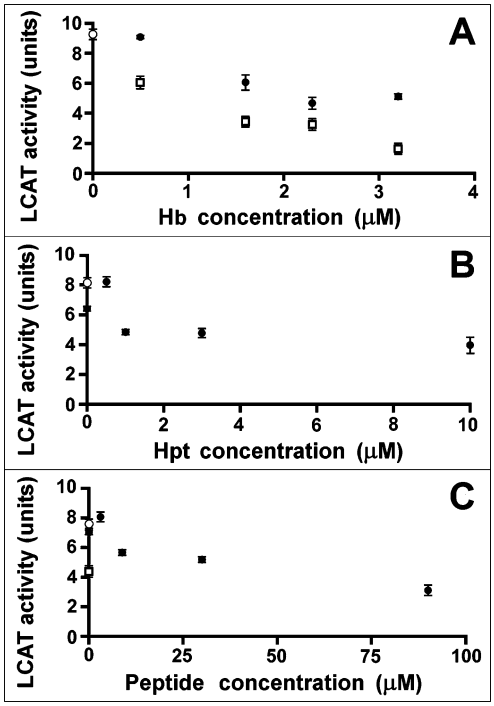

FIGURE 8: LCAT assay with oxidized proteoliposomes. The LCAT activity was assayed by incubating a pool of dextran sulfate-treated plasma with a reaction mixture, containing a labeled proteoliposome (ApoA-I:lecithin:^{3}H-cholesterol, 1.5:200:18 molar ratio) as substrate. Before incubation, the proteoliposome was kept in TBS (open circle, in all the panels), or TBS supplemented with oxidizing mixtures. Panel A shows the activity measured by using proteoliposomes oxidized with different Hb concentrations in the presence (filled circles) or absence (open squares) of 0.5 μM Hpt. Panel B shows the activity measured by using proteoliposomes oxidized with 0.5 μM Hb in the presence of different concentrations of Hpt (filled circles). Panel C shows the activity measured by using proteoliposomes oxidized with 0.5 μM Hb and 0.5 μM Hpt, in the presence of different concentrations of the peptide P2a (filled circles); a control assay was performed with proteoliposomes oxidized in the absence of Hpt (open square). The LCAT activity was expressed as units (1 U = one nanomole of cholesterol incorporated per hour per milliliter of plasma). The samples were analyzed in triplicate: the data are expressed as means ± SEM.

the Hpt/ApoA-I ratio decreased to 1.5. Conversely, ApoA-I was not oxidized on its Pro-241 when Hpt circulated at higher levels (Hpt/ApoA-I ratio = 2−4), even though albumin was attacked by hydroxyl radicals.

Radical Attack to Free or Hpt-Bound ApoA-I: Effect on Stimulation of LCAT. Oxidative damage of ApoA-I structure might affect the protein property of stimulating the enzyme LCAT for production of cholesteryl esters. The LCAT activity is commonly measured by using ApoA-I embedded in micelles containing, as enzyme substrates, phospholipids and ^{3}H-labeled cholesterol. These proteoliposomes were incubated at first with the oxidizing mixture of Hb, ascorbate, and H_2O_2 (0.5, 100, and 100 μM in TBS, respectively), and then with LCAT. The second incubation was done 1 h after the end of the first one, when radicals were no more produced (see Experimental Procedures). Not oxidized samples (i.e., proteoliposomes with only TBS in the first incubation) were used as positive controls to confirm the ApoA-I effect on LCAT. The enzyme activity was found significantly impaired by oxidized proteoliposomes (4.71 ± 0.31 versus 9.49 ± 0.16 U in controls, $P < 0.003$). Absence of either ascorbate or Hb during the proteoliposome treatment did not alter the enzyme activity (8.95 ± 0.42 or 10.12 ± 0.45 U respectively).

LCAT assays with proteoliposomes oxidized in the presence of Hpt were performed in order to evaluate whether this protein can protect not only the structure but also the function of ApoA-I. The effect of 0.5 μM Hpt, during oxidative treatment of the proteoliposome with different amounts of Hb, was clearly protective for ApoA-I function (Figure 8; panel A). In a further experiment, the radical source was kept constant (0.5 μM Hb in the oxidizing mixture), and the effect of higher Hpt concentrations in the LCAT assay was studied (Figure 8; panel B). The data obtained indicate that the proteoliposomes oxidized in the presence of 0.5 μM Hpt (Hpt/ApoA-I ratio = 2.5) stimulated the enzyme as those not oxidized. Conversely, the proteoliposomes oxidized in the presence of increasing amounts of Hpt (Hpt/ApoA-I ratio from 5 to 50) severely impaired the cholesterol esterification. Finally, experiments with proteoliposomes oxidized in the presence of the peptide P2a were carried out. This peptide contains (part of) the ApoA-I sequence interacting with Hpt, and can therefore displace Hpt from HDL (*27*). Use of such a peptide, during proteo-

liposome oxidation in the presence of 0.5 μM Hpt, was expected to impair the interaction of Hpt with ApoA-I, which should thus be exposed to the hydroxyl attack. Increased amounts of peptide were actually found associated with decreased LCAT activity (Figure 8; panel C).

These results, taken together, indicate that Hpt might effectively prevent Hb-triggered oxidative damage of ApoA-I function when the Hpt/ApoA-I molar ratio is comparable with that present in physiological conditions (*29*). Furthermore the data suggest that Hpt, at high concentration or when bound by P2a, is no more able to save the ApoA-I role in the LCAT stimulation.

DISCUSSION

During inflammation, the plasma level of Hpt increases (*16*) as well as the release of free Hb from erythrocytes (*40*), and the production of reactive oxygen species from the endothelium (*15*). ApoA-I is known to be a target of oxidative stress (*8*−*12*), but poor information is available on the effect of the attack of hydroxyl radicals on its structure and function (*8*, *41*, *42*). This attack may be a major cause of ApoA-I modification, not only because HDL are exposed to circulating free Hb, but also owing to the binding of ApoA-I to Hpt and therefore complexes Hpt−Hb, which are potential sources of hydroxyl radicals. In this work, we set up a system of oxidative stress based on levels of Hb, H_2O_2 and ascorbate in the range from physiological to inflammatory conditions, and demonstrated that the hydroxyl attack modified the ApoA-I structure. ApoA-I molecules might be converted into homodimers or heterodimers with other HDL apolipoproteins. Hydroxyl radicals can actually produce tyrosyl radicals on Tyr residues of ApoA-I, thus cross-linking two ApoA-I molecules or ApoA-I with ApoA-II (*8*). These adducts were previously found in mouse HDL following oxidation by hydroxyl radicals (*43*). Antibodies are a powerful tool to identify or isolate cross-linked apolipoproteins. We used anti-ApoA-I antibodies to detect, after SDS−PAGE and Western blotting, ApoA-I-containing epitopes among the products originated from HDL by oxidative stress. Antigens with electrophoretic mobility lower than ApoA-I, namely, molecules containing ApoA-I or part of it cross-linked with other apolipoproteins or peptide fragments, displayed higher immunoreactivity, which was likely dependent on epitopes exposed by oxidation (*44*). Poor immunoreactivity of ApoA-I, following oxidation by Fenton chemistry, was already described (*42*). It cannot be excluded that peptide fragments originated from ApoA-I, in the oxidation system we used, but were not detected by the antibodies, likely because they were very short or presenting epitopes altered by oxidation. It is worth noting that the production of high molecular mass ApoA-I-containing antigens was greatly limited by Hpt, whereas it was not impaired by albumin. This result clearly indicates that albumin, known as the most abundant plasma protein and an effective radical scavenger, does not prevent ApoA-I oxidation in the assay conditions, and strongly suggests that the antioxidant role of Hpt is specifically protective for ApoA-I. This is a major result from our work, and enlightens a new property of Hpt.

Recently, LDL and HDL proteins were intensively studied by the rapidly expanding use of MS, and some information on lipoproteomics associated with different diseases is now available (*39*, *45*). Even though the protein composition of HDL from different conditions was examined with some detail by MS analysis, this technology has never been used, to the best of our knowledge, for studying the effect of oxidation on HDL and, in particular, on ApoA-I. Nano-ESI, given its high resolving power, due to chromatographic separation of tryptic peptides before MS, is a suitable technique for analyzing complex protein mixtures like those of HDL. In this lipoprotein environment, the ApoA-I oxidation was studied. MS analysis allowed us to ascertain that the protective effect of Hpt on ApoA-I is mostly displayed by preventing the formation of covalent adducts of this apolipoprotein with other proteins. As a consequence of the radical attack, also ApoD or ApoD fragments cross-linked or reacted with other HDL proteins, and formed high molecular mass adducts. It is worth noting that Hpt prevented the formation of such adducts. Thus the importance of Hpt might be extended over the antioxidant defense of ApoA-I. In particular, we present here the hypothesis that HDL-bound Hpt might save structure and function also of apolipoproteins other than (and, possibly, neighboring to) ApoA-I. Differently from ApoA-I and ApoD, ApoC-II did not seem to form adducts with other apolipoproteins. This means that reactive fragments of ApoC-II, if produced in our oxidation system, were under the detection threshold in MS analysis. It cannot be excluded that ApoC-II is a poor target for hydroxyl radicals, or is shielded by some other apolipoprotein on the HDL surface.

The Hpt protective effect on the ApoA-I structure during in vitro oxidation was supported by the results obtained from the experiment with carrageenan-treated mice. High Hpt levels were actually found associated with no detectable hydroxyproline in the structure of ApoA-I from HDL collected in inflammatory conditions. Conversely, hydroxyproline in ApoA-I was formed only when the Hpt level dropped down in inflamed mice. This finding suggests that Hpt, when circulating at a level sufficiently high for binding HDL, prevents hydroxylation of ApoA-I. Detection of hydroxyproline-containing fragments was used to study ApoA-I oxidative damage because hydroxylation of proline residues can be considered a footprint of hydroxyl radical attack to polypeptide chains (*15*, *46*), which are not cleaved by such an attack (*47*, *48*) and result therefore labeled for further analysis. The analysis was performed by MS, which is to date the best tool not only for identifying hydroxylation targets but also for assigning the attached hydroxyl to specific residues in a given amino acid sequence. Hydroxylated aliphatic amino acids are also suitable footprints for studying metal ion-catalyzed Fenton chemistry with proteins (*49*), but most of these amino acid residues of ApoA-I might be hydroxylation targets poorer than proline. In fact, they are not exposed to water solution as embedded in the HDL lipid fraction, which is more susceptible than apolipoproteins to oxidation (*8*).

Sulfoxides of Met-86 and Met-112 were previously found not decreasing the ApoA-I activity in LCAT stimulation in vitro (*41*). It cannot be excluded that sulfone but not sulfoxide forms of the oxidized Met residues change the function of ApoA-I. We found that sulfone forms of Met-112 and Met-148 were produced by our oxidation system in the presence of 6 μM Hb, and that the enzyme activity resulted heavily

impaired also at lower Hb concentration. It should be noted that Met-148 lies just in the ApoA-I domain interacting with the enzyme (*28*), and its conversion into the sulfone form might be responsible for decreased cholesterol esterification in the in vitro assay. However, oxidation of residues other than methionine might be involved in loss of ApoA-I function. The LCAT assay confirmed the hypothesis that Hpt, as interacting with ApoA-I, prevents this apolipoprotein from losing its function of stimulating the enzyme. This interaction is based on chemical equilibrium between free and Hpt-bound ApoA-I, and such equilibrium is regulated by the Hpt concentration, according to the mass action law. It is therefore conceivable that the higher the Hpt concentration, the lower the amount of free ApoA-I, and the lower the stimulation on LCAT. As a matter of fact, we found full stimulation of the enzyme activity with 0.5 μM Hpt. This Hpt concentration was about 2-fold that of ApoA-I in the assay, in stoichiometric ratio which is comparable with that present in physiological conditions (*16*, *29*). The ApoA-I stimulation decreased as much as higher Hpt concentration, resembling the raising of Hpt levels during the acute phase of inflammation (*16*, *29*), was used in the LCAT assay (see Figure 8, panel B). This means that, in these conditions, the amount of free ApoA-I decreased, owing to competition of Hpt with LCAT for the same binding site on the apolipoprotein structure. Inhibition of LCAT in vitro by increasing levels of Hpt was already reported (*27*, *50*), and the stoichiometric ratio of Hpt with ApoA-I was previously found negatively correlated with the enzyme activity in vivo (*29*). The enzyme assay was carried out also to confirm the hypothesis that Hpt effectively prevents the oxidative damage of the ApoA-I function in stimulating LCAT. In fact, the protective Hpt property against hydroxyl radicals was lost when the synthetic peptide P2a, sharing amino acid sequence with the ApoA-I binding domain for Hpt (*27*), was used during the oxidative stress. The results suggest that such a peptide competed with ApoA-I for the Hpt binding, therefore keeping Hpt separated from ApoA-I, and leaving the apolipoprotein exposed to hydroxyl radicals. We want to point out that the here presented results were obtained by using Hpt preparations which were mixtures of the three protein phenotypes. Hpt should generally behave as scavenger of reactive oxygen species, and the role of Hpt polymorphism in protecting ascorbate (*51*) or LDL (*52*) against hydroxyl radicals was previously reported. The Hpt isoforms, as having different molecular mass or conformation, are expected to differently protect also ApoA-I from oxidative damage. In fact, it seems conceivable that the ApoA-I structure be protected from hydroxyl attack by Hpt steric hindrance. Therefore, the efficiency of different isoforms in masking HDL oxidation targets is expected to change, as depending on how large is the shield resulting from Hpt isoform structure. In this frame, a link between Hpt polymorphism and antioxidant defense of ApoA-I function can also be hypothesized.

It remains to be discussed why Hpt would limit the reverse cholesterol transport during the acute phase of the inflammation, just when oxidative stress reaches its highest level, and the resulting oxidized LDL promotes cholesterol accumulation in the cells of the vascular wall. A possible interpretation of our data is that a regulatory mechanism for the ApoA-I function might exist, based on a balance of advantages and disadvantages. It is worth noting that ApoA-I, in addition to the property of stimulating LCAT, displays other important activities (*51*−*53*) which might be saved by Hpt during oxidative stress as well. In this frame, it is conceivable that Hpt might compete with the heme protein myeloperoxidase for binding ApoA-I, thus limiting the enzyme-catalyzed oxidation which impairs the HDL function as observed in subjects with cardiovascular disease (*12*). Therefore Hpt might act as a defense for ApoA-I against oxidation and loss of function both in physiological conditions, when significant though low levels of radicals are continuously produced (*15*), and during the acute phase of inflammation, when severe oxidative stress occurs. In the former case, ApoA-I protection by Hpt would not impair the stimulation of LCAT, the amount of Hpt-free ApoA-I being sufficient for such an activity: this protection might be addressed to prolong the HDL life. In the latter case, the enhanced levels of Hpt would bind so many ApoA-I molecules that LCAT activity would suffer by lack of stimulation. This condition should not be prolonged, otherwise the reverse cholesterol transport would be severely limited. We suggest that, so far as the acute phase and the associated enhanced Hpt levels last a short time, ApoA-I protection represents an advantage because this apolipoprotein would be ready to work as soon as inflammation goes out and Hpt returns to normal levels.

Although further experiments, including epidemiologic studies and clinical trials, are required to validate our model on the importance of the ApoA-I binding of Hpt, our data strongly suggest that therapeutic interventions for treatment of cardiovascular disease might target Hpt with drugs competing for the ApoA-I binding only if combinatorial antioxidant administration is provided.

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