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Assignment of the Binding Site for Haptoglobin on Apolipoprotein A-I*

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Haptoglobin (Hpt) was previously found to bind the high density lipoprotein (HDL) apolipoprotein A-I (ApoA-I) and able to inhibit the ApoA-I-dependent activity of the enzyme lecithin:cholesterol acyltransferase (LCAT), which plays a major role in the reverse cholesterol transport. The ApoA-I structure was analyzed to detect the site bound by Hpt. ApoA-I was treated by cyanogen bromide or hydroxylamine; the resulting fragments, separated by electrophoresis or gel filtration, were tested by Western blotting or enzyme-linked immunosorbent assay for their ability to bind Hpt. The ApoA-I sequence from Glu^{113} to Asn^{184} harbored the binding site for Hpt. Biotinylated peptides were synthesized overlapping such a sequence, and their Hpt binding activity was determined by avidin-linked peroxidase. The highest activity was exhibited by the peptide P2a, containing the ApoA-I sequence from Leu^{141} to Ala^{164}. Such a sequence contains an ApoA-I domain required for binding cells, promoting cholesterol efflux, and stimulating LCAT. The peptide P2a effectively prevented both binding of Hpt to HDL-coated plastic wells and Hpt-dependent inhibition of LCAT, measured by anti-Hpt antibodies and cholesterol esterification activity, respectively. The enzyme activity was not influenced, in the absence of Hpt, by P2a. Differently from ApoA-I or HDL, the peptide did not compete with hemoglobin for Hpt binding in enzyme-linked immunosorbent assay experiments. The results suggest that Hpt might mask the ApoA-I domain required for LCAT stimulation, thus impairing the HDL function. Synthetic peptides, able to displace Hpt from ApoA-I without altering its property of binding hemoglobin, might be used for treatment of diseases associated with defective LCAT function.

Apolipoprotein A-I (ApoA-I)[1] is a component of the high density lipoprotein (HDL) that plays a key role in the traffic of cholesterol between liver and peripheral cells. All the cells are supplied with cholesterol and cholesteryl esters by endocytosis of another major class of lipoproteins, namely the low density lipoprotein, but some cell types (steroidogenic cells) use docking receptors to bind just ApoA-I to allow the transfer of cholesteryl esters from HDL (1–4). In particular, ApoA-I is required for normal steroid production in adrenocortical cells (5), and HDL receptors are regulated by cholesterol level in luteal cells (6). ApoA-I is also required for removal of cholesterol excess from the plasma membrane (7, 8), an anti-atherogenic process called "reverse cholesterol transport" (9, 10) that prevents alteration of the membrane properties and cell death (7, 10–12). In particular, ApoA-I stimulates the efflux of cholesterol from cell toward HDL (13–16) and the enzyme LCAT (EC 2.3.1.43) to convert, on the HDL surface, cell-derived cholesterol into cholesteryl ester, which is then placed into the lipoprotein core and transported through circulation to liver for catabolism and bile production (9, 10). The ratio of cholesteryl ester to unesterified cholesterol in HDL, a value correlated with the total cholesterol amount in the lipoprotein (HDL-C), is therefore assumed to reflect the LCAT activity *in vivo* (17, 18). Mutations in the ApoA-I structure have been reported to be associated with low HDL-C and decreased stimulation of enzyme activity (18). On the other hand, it is conceivable that any factor interacting with ApoA-I might interfere with such activity or, more generally, the reverse cholesterol transport.

ApoA-I can bind haptoglobin (Hpt) in blood (19, 20) and follicular fluid (21). Hpt is a plasma oligomeric glycoprotein exhibiting enhanced levels during the acute phase of inflammation; it presents in humans three distinct phenotypes (determined by genetic polymorphism) with different prevalence in several diseases, including cardiovascular diseases (22). This binding was suggested to influence the role of HDL in cholesterol transport. Actually, Hpt was found to inhibit ApoA-I-dependent LCAT activity *in vitro* (23) and to associate with low reverse cholesterol transport in human ovarian follicular fluid (24). In addition, estradiol esterification in the follicle and ester delivery through HDL-mediated circulation to storage tissue (25, 26) for long acting hormonal and antioxidant function (27, 28) might be influenced by defective reverse cholesterol transport and reduced LCAT activity (24).

Hpt has long been known to capture and transport free hemoglobin (Hb) to the liver in the pathway of iron recycling for erythropoiesis (29). Hb has been reported to compete with ApoA-I for binding Hpt, although Hb interacts with an Hpt site

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[1] The abbreviations used are: ApoA-I, apolipoprotein A-I; HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; Hb, hemoglobin; Hpt, haptoglobin; BSA, bovine serum albumin; GAR-HRP IgG, goat anti-rabbit horseradish peroxidase-linked antibody; GAR-P IgG, GAR phosphatase-linked antibody; HA, hydroxylamine; PVDF, polyvinylidene fluoride; HATU, *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(7-azabenzotriazol-1-yl) uronium hexafluorophosphate; ELISA, enzyme-linked immunosorbent assay; CB, fragment from digestion by CNBr; aa, amino acid(s); DS, dextran sulfate.

that is different from that involved in the ApoA-I binding (30). The inhibitory role of Hpt in the regulation of the HDL-dependent removal of cholesterol excess from peripheral cells, though of great interest in studies on diseases associated with cholesterol accumulation, has been poorly investigated. Hpt-dependent masking of the ApoA-I site involved in the LCAT stimulation was suggested to be responsible for decreased enzyme activity (23, 30). Thus, high Hpt levels, as present in the acute phase of inflammation, might impair cholesterol removal from peripheral cells, including vascular cells, and play an important role in worsening vascular endothelial dysfunction and accelerating atherosclerosis. Mapping the Hpt binding site of ApoA-I was therefore required to get more information about the biochemical mechanism(s) underlying the negative control of Hpt on reverse cholesterol transport. Major aims of this work were to identify the Hpt binding site in the amino acid sequence of ApoA-I by using protein chemical fragmentation and peptide synthesis techniques and to demonstrate that peptides sharing the sequence of this site are able to compete with ApoA-I or HDL for binding Hpt. The effect of such peptides on Hpt in LCAT inhibition and Hb binding experiments was also studied. Use of these peptides in therapy of diseases associated with defective reverse cholesterol transport is proposed.

EXPERIMENTAL PROCEDURES

Materials

Chemicals of the highest purity, BSA, human serum albumin, cholesterol, cholesteryl linoleate, human Hpt (mixed phenotypes: Hpt 1–1, Hpt 1–2, Hpt 2–2), rabbit anti-human Hpt IgG, GAR-HRP, GAR-P, *p*-nitrophenylphosphate, *o*-phenylenediamine, avidin-HRP, CNBr, HA, piperidine, 1,2-ethandithiol, triisopropylsilane, and molecular weight markers were purchased from Sigma. Human ApoA-I and rabbit anti-human ApoA-I IgG were from Calbiochem (La Jolla, CA). [1α,2α-^{3}H]cholesterol (45 Ci/mmol) was obtained from PerkinElmer Life Sciences. Sephadex G-50F (Amersham Biosciences), and PVDF transfer membrane (Millipore, Bedford, MA) were used. Amino acids and HATU were purchased from Novabiochem. Organic solvents from Romil (Cambridge, UK), polystyrene 96-well plates from Nunc (Roskilde, Denmark), and Sil-G plates for thin layer chromatography (0.25 mm thickness) (Macherey-Nagel, Düren, Germany) were used.

Methods

ApoA-I Cleavage and Chromatography of the Fragments—Cleavage of ApoA-I with CNBr or HA was performed as previously described (31, 32) using 13.5 or 7 nmol protein, respectively. The digestion products (100–150 μg) were loaded onto a column of Sephadex G-50F (0.6 × 24 cm) and eluted in TBS (20 mM Tris-HCl, 130 mM NaCl, pH 7.4) with a 3.5-ml/h flow rate at room temperature. Fractions of 90 μl were collected and 45-μl aliquots, as duplicates, were assayed by ELISA with anti-ApoA-I and GAR-HRP IgG.

Electrophoresis and Immunoblotting—The electrophoresis of peptides from ApoA-I cleavage by CNBr or HA was performed in denaturing and reducing conditions essentially as previously described (33), but 2-mm-thick gels (length, 10 cm) with 20 or 16.5% polyacrylamide, respectively, were used. Triosephosphate isomerase, myoglobin, α-lactalbumin, aprotinin, and insulin chain B (26.6, 17, 14.2, 6.5, and 3.5 kDa, respectively) were used as molecular mass markers. After electrophoresis (7 h, 100 V), the gels were fixed in 10% acetic acid containing 25% isopropanol, stained with Coomassie R-250 (0.05% in the fixing solution), and destained in 10% acetic acid. Fixing and staining were omitted when the gel was processed for immunoblotting.

Western blotting onto PVDF membrane and staining by antibodies were carried out essentially as previously described (21). In particular, proteins were transferred under electric field, using a semi-dry blot unit (Schleicher & Schuell, Dassel, Germany) for 3 h at 4 °C. The membrane was incubated overnight at 4 °C with 0.1 mg/ml Hpt in TBS containing 0.05% Tween 20 (T-TBS). After treatment (1 h, 37 °C) with rabbit anti-Hpt IgG (1:100 dilution in T-TBS), the membrane was incubated (1 h, 37 °C) with GAR-HRP IgG (1:300 dilution) and finally stained.

Peptide Synthesis—Peptides partially overlapping the ApoA-I sequence were synthesized by solid phase, using standard Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry (34) with acetylated or biotinylated N termini and amidated C termini. The synthesis of the amide peptides was performed in a model 348Ω Advanced Chemtech multiple peptide synthesizer using a PAL-PEG-PS resin (Perseptive Biosystem, Hamburg, Germany). The N-terminal residue was labeled using two equivalents of *N*-(+)-biotinyl-6-aminoexanoic acid (Fluka, Steinheim, Germany), two equivalents of HATU, and four equivalents of *N*,*N* diisopropylethylamine in *N*,*N* dimethylformamide overnight at room temperature. Cleavage of peptides from the resin was achieved by treatment with a mixture of trifluoroacetic acid, triisopropylsilane, water, 1,2-ethandithiol (90:2.5:2.5:5, v:v:v:v) at room temperature. Then all peptides were precipitated by ethyl ether, dissolved in appropriate mixtures of acetonitrile and water, and purified by high performance liquid chromatography on a C18 column (Juppiter; Phenomenex, Torrance, CA). In high performance liquid chromatography, linear increasing gradients of acetonitrile in water (in the presence of 0.1% trifluoroacetic acid) were used. All peptides were recovered by lyophilization. Peptide purity was checked by analytical high performance liquid chromatography. Matrix-assisted laser desorption ionization time-of-flight by a Voyager instrument (Perseptive Biosystems) was used to ascertain peptide identity.

ELISA—ELISA was performed essentially as previously reported (30). In particular, microtiter plate wells were coated by incubation with a 45-μl aliquot from separated chromatography fractions or 1 μg of antigen (Hpt, HDL, or Hb) in 50 μl of 7 mM Na_2CO_3, 17 mM $NaHCO_3$, 1.5 mM NaN_3 (pH 9.6). Wells were incubated with 55 μl of primary antibody, 45 μl of ApoA-I cleavage products from G-50F, or 55 μl of biotinylated peptide (1, 3, 10, 30 μM). Anti-Hpt IgG (1:1500 dilution in T-TBS supplemented with 0.25% BSA) or anti-ApoA-I IgG (1:1000 dilution) was used as primary antibody. Bound immunocomplexes or peptides were incubated (1 h at 37 °C) with 60 μl of GAR-HRP IgG or avidin-HRP diluted as primary antibody, 1:3000 and 1:10000, respectively. Color development was monitored at 492 nm as previously described (21).

In experiments of competition of peptide with ApoA-I for Hpt binding, wells were coated with Hpt. Mixtures of 1.5 μM biotinylated peptide with different amounts of ApoA-I (0, 0.01, 0.05, 0.1, 0.5, 0.75, or 1.5 μM) in T-TBS supplemented with 0.25% BSA were incubated in the wells for 2 h at 37 °C. Peptide binding was detected by avidin-HRP as mentioned above.

In experiments of competition of peptide with HDL for Hpt binding, the wells were coated with HDL. Mixtures of Hpt (1 μM) with peptide (0, 1, 5, 10, or 20 μM) or ApoA-I (10 μM) in CB-TBS buffer (5 mM $CaCl_2$, 0.2% BSA, 130 mM NaCl, 20 mM Tris-HCl, pH 7.3) were kept for 2 h at 37 °C and then incubated in the wells (2 h, 37 °C). The binding of Hpt was detected by anti-Hpt IgG and GAR-HRP IgG as described above.

In experiments of competition of peptide with Hb for Hpt binding, mixtures of Hpt (1 μM) with different amounts (0, 3, 6, 10, or 20 μM) of peptide or ApoA-I or HDL in CB-TBS buffer were kept for 2 h at 37 °C and then incubated in Hb-coated wells (2 h, 37 °C). The binding of Hpt to Hb was detected by anti-Hpt IgG, GAR-P, and color development at 405 nm as previously described (30).

Commercial preparations of Hpt (mixed phenotypes) contain isoforms of different relative abundance. The protein molarity was expressed as concentration of monomer, that is the Hpt unit containing one subunit β (40 kDa) and one subunit α (α_1, 8.9 kDa, or α_2, 16 kDa) (22). The protein mixture was analyzed, and a molecular mass of 53.5 kDa was assigned to the unit $\alpha\beta$. In particular, the Hpt subunits were fractionated by electrophoresis in denaturing conditions, and the intensities of the Coomassie-stained bands were measured by densitometry (35). Therefore, the ratio of α_1 (present in phenotypes Hpt 1–1 and Hpt 1–2) to α_2 (present in phenotypes Hpt 1–2 and Hpt 2–2) was determined to calculate the molar contribution of $\alpha_1\beta$ and $\alpha_2\beta$ to the amount of used Hpt.

LCAT Assay—A pool of plasma samples, treated with 0.08% DS (50-kDa molecular mass) in 0.16 M $CaCl_2$ to remove low density and very low density lipoprotein, was used as source of LCAT (DS-treated plasma). The enzyme activity was measured using a proteoliposome (ApoA-I:lecithin:cholesterol = 1.5:200:18 molar contribution) as substrate, essentially according to published procedures (36, 37). In detail, 8 μl of 50 mg/ml egg lecithin in ethanol were mixed with 18 μl of 1 mg/ml cholesterol in ethanol, 40 μl of [1,2-^{3}H]cholesterol (1 μCi/ml) into a glass vial. The solvent was carefully evaporated under nitrogen stream at room temperature, and 170 μl of a suspension medium (85 mM sodium cholate, 150 mM NaCl, 10 mM Tris-HCl, pH 8) were added to the dried lipids. After vigorous whirling (3 min, room temperature), the micelle suspension was incubated (90 min, 37 °C) and repeatedly shaken every 10 min until clear. 90 μl of 1.21 mg/ml ApoA-I were added to the lipid suspension, which was further incubated for 1 h at 37 °C. The resulting proteoliposome suspension was extensively dialyzed against TBE (140 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.3), at

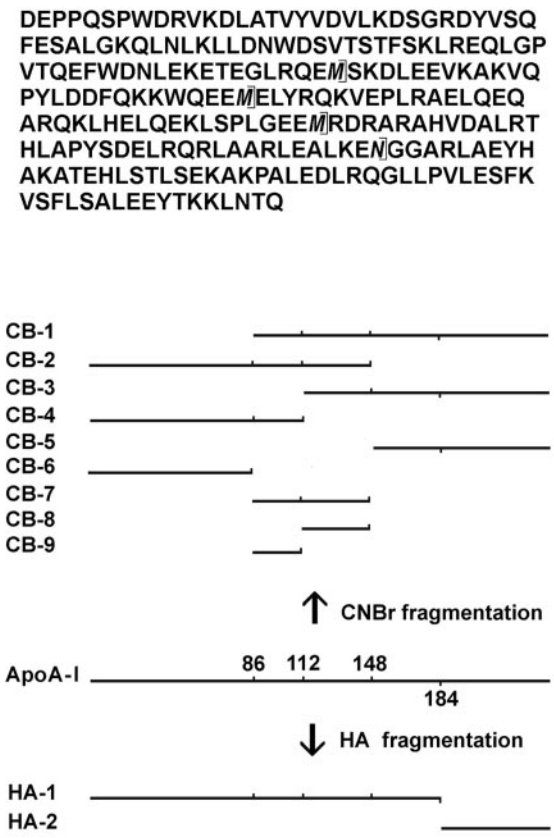

FIG. 1. **Fragmentation of ApoA-I.** The amino acid sequence of ApoA-I is reported. The *brackets* embedded in the sequence refer to the predicted sites of fragmentation by CNBr (Met-*X*) or HA (Asn-Gly). The segments represent ApoA-I and all the peptides that may originate from its fragmentation. On the ApoA-I segment, the *upper numbers* indicate the Met residues; the *lower number* indicates the Asn residue in the amino acid sequence.

4 °C to remove cholate. The volume of the dialyzed suspension was adjusted to 285 μl using TBE. The reaction mixture (1 ml final volume) was prepared by putting 697 μl of TBE containing 5 mM $CaCl_2$, 83 μl of 6% human serum albumin, and 160 μl of proteoliposome suspension (diluted 1:20 in TBE) into a screw-capped tube and heating at 38 °C for 30 min. The assay was carried out by addition of 2.5 μl of 2 mM β-mercaptoethanol and 3.5 μl of DS-treated plasma to 100 μl of reaction mixture, which was rapidly divided into three aliquots of 32 μl and incubated (1 h, 37 °C). The reaction was stopped by addition of 130 μl of ethanol to each aliquot. Lipids were extracted in 600 μl of hexane containing 10 μg/ml cholesterol and 10 μg/ml cholesteryl linoleate. After recovering the organic phase, the aqueous phase was twice treated with 500 μl of the extraction solution, and the three extracts were pooled. Hexane was removed under nitrogen stream, and the dried lipids were dissolved in 50 μl of chloroform. Cholesteryl esters were separated from cholesterol by thin layer chromatography, using petroleum ether, diethyl ether, and acetic acid (90:30:1, v:v:v) as mobile phase. The lipid spots were visualized under iodine vapor and recovered for scintillation analysis. The enzyme activity was expressed as nmol of cholesterol esterified/h/ml of plasma.

Statistical Analysis—ELISA was carried out with single aliquots from chromatography fractions, whereas at least three replicates were processed in all other cases. Samples in the LCAT assay were analyzed in triplicate. The program "Graph Pad Prism 3" (Graph Pad Software, San Diego, CA) was used to obtain trend curves and perform regression analysis or *t* test.

RESULTS

Fragmentation of ApoA-I and Identification of the Fragments Binding Hpt—To map the ApoA-I region interacting with Hpt, the apolipoprotein was fragmented with CNBr or HA and the resulting peptides were analyzed for their binding to Hpt. Four peptides were predicted to result from the ApoA-I fragmentation by CNBr (Fig. 1), but more molecular species including the undigested protein were observed by SDS-PAGE (Fig. 2, *lane c*). This finding indicates that incomplete cleavage occurred at the residues Met^{86}, Met^{112}, and Met^{148}, producing nine fragments (CB-1: aa 87–243, 18.1 kDa; CB-2: aa 1–148, 17.2 kDa; CB-3: aa 113–243, 14.9 kDa; CB-4: aa 1–112, 12.9 kDa; CB-5: aa 149–243, 10.5 kDa; CB-6: aa 1–86, 9.8 kDa; CB-7: aa 87–148, 7.5 kDa; CB-8: aa 113–148, 4.3 kDa; CB-9: aa 87–112, 3.2 kDa) (Fig. 1).

After peptide transfer on PVDF membrane, the blotted material was incubated with Hpt and then treated with rabbit anti-Hpt IgG. The immunocomplexes, detected by GAR-HRP IgG, stained two bands with the same electrophoretic mobilities as CB-1 and CB-3 (Fig. 2, *lane b*). This means that the sequence of CB-9, as present in CB-1 but absent in CB-3, might not be required for binding Hpt. After another limited digestion by CNBr, the produced fragments were fractionated by gel filtration on Sephadex G-50F and analyzed by ELISA for their ability to bind Hpt. In detail, aliquots of the elution fractions were incubated in Hpt-coated wells and the complexes detected by rabbit anti-ApoA-I IgG and GAR-HRP IgG. Again, CB-1 was found to bind Hpt (Fig. 3*A*).

The data shown above strongly suggested that the amino acid sequence from Ser^{87} to Gln^{243} (*i.e.* CB-1) harbors the binding site for Hpt. It is worth noting that neither CB-5 nor CB-7, which are joined together in the primary structure of CB-1, was able to bind Hpt. In a further experiment, ApoA-I was cleaved by HA at its unique Asn^{184}-Gly^{185} bond (Fig. 1), and the reaction products were analyzed as the CNBr fragments. A slight band, migrating in electrophoresis like the N-terminal fragment (HA-1: aa 1–184, 21.8 kDa) was bound by Hpt and detected by anti-Hpt IgG (Fig. 2, *lane g*). After gel filtration and ELISA, ApoA-I was detected by anti-ApoA-I IgG together with HA-1 and the C-terminal fragment (HA-2: aa 185–243, 6.4 kDa) (Fig. 3*B*). Binding of HA-1 to Hpt was comparable with that of ApoA-I, whereas complexes of HA-2 with Hpt were not found (Fig. 3*B*). Analysis of ApoA-I digestion by HA indicated that the sequence from Gly^{185} to Gln^{243} is not required for binding ApoA-I to Hpt. These results, together with the data from the protein digestion by CNBr, strongly suggested that the Hpt binding domain of ApoA-I contains the CNBr cleavage site at Met^{148} and spans from Glu^{113} to Asn^{184}.

Use of Synthetic Peptides to Identify the Hpt Binding Site of ApoA-I—To localize the Hpt binding site within the ApoA-I amino acid sequence from Glu^{113} to Asn^{184}, three biotinylated peptides with partial ApoA-I amino acid sequences, namely P1 (Glu^{113}-Gln^{133}), P2 (Leu^{134}-Ala^{164}), and P3 (Pro^{165}-Asn^{184}), were synthesized and incubated in Hpt-coated wells. Binding of P2 was significantly higher ($p < 0.0001$) than that of P1 or P3 (Fig. 4, *upper panel*).

Shorter biotinylated peptides, partially overlapping the P2 sequence, were synthesized to further define the Hpt binding site. These peptides, namely P2a (Leu^{141}-Ala^{164}), P2b (Met^{148}-Ala^{164}), and P2c (Val^{156}-Ala^{164}), were processed for ELISA as above. Peptide P2a exhibited the highest level of Hpt binding ($p < 0.01$), and such a level was comparable with that of P2 (Fig. 4, *lower panel*). This result suggested that the difference in sequence length between P2 and P2a, *i.e.* residues 134–140, corresponds to a region of ApoA-I that is not required in the interaction with Hpt.

To compare the binding affinities of P2a and ApoA-I for Hpt, the following experiment was carried out. Different amounts of ApoA-I were incubated with 1.5 μM biotinylated P2a in Hpt-coated wells, and avidin-HRP was used to detect the bound peptide. Data obtained allowed us to calculate that the binding of P2a could be halved in the presence of about 0.2 μM ApoA-I (Fig. 5). This result suggested that, in our assay conditions, the

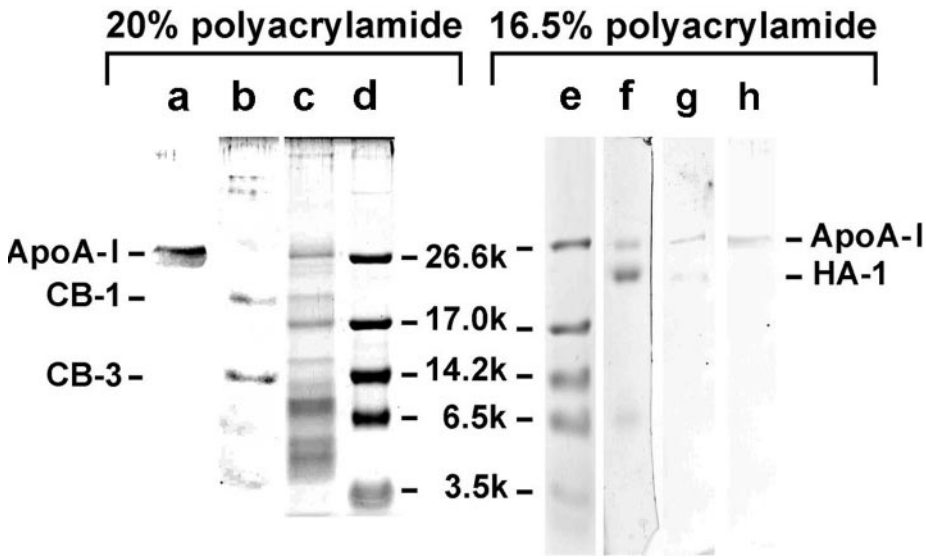

FIG. 2. **Electrophoresis of the ApoA-I fragments produced by cleavage with CNBr or HA.** ApoA-I was fragmented by CNBr or HA, and the reaction products were fractionated by electrophoresis in denaturing and reducing conditions on 20 or 16.5% polyacrylamide gel, respectively. The fragments from CNBr digestion were stained by Coomassie (*lane c)* or, after blotting onto PVDF membrane and incubation with 0.1 mg/ml Hpt, by rabbit anti-Hpt IgG and GAR-HRP IgG (*lane b*). The fragments from HA digestion were stained by Coomassie (*lane f)* or, after blotting onto PVDF membrane and incubation with 0.1 mg/ml Hpt, by rabbit anti-Hpt IgG and GAR-HRP IgG (*lane g*). *Lanes d* and *e* show the Coomassie-stained pattern of electrophoresis markers. *Lanes a* and *h* show undigested ApoA-I from 20 or 16.5% gel, respectively, following Hpt treatment and antibody staining.

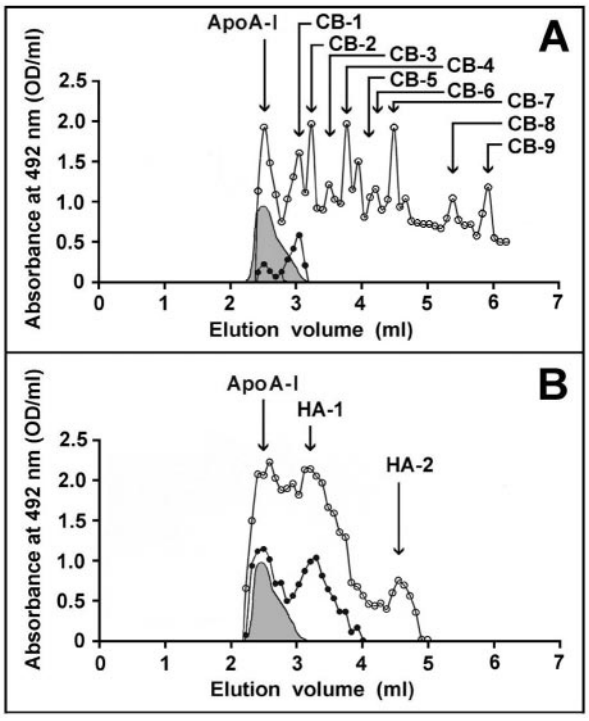

FIG. 3. **Gel filtration of the ApoA-I fragments obtained by cleavage with CNBr or HA.** 150 or 100 μg of ApoA-I were treated with CNBr (*A*) or HA (*B*), respectively. The digestion products were separated by Sephadex G50F and detected in the elution fractions by anti-ApoA-I IgG (*open circles*). Hpt-coated wells were used to bind fragments that were detected by anti-ApoA-I IgG (*solid circles*). The molecular mass of the fragments was assessed on the basis of their elution volume, using standard proteins in a calibration chromatography (myoglobin: 17 kDa; insulin trimer: 16 kDa; cytochrome C: 12.3 kDa; insulin dimer: 10.7 kDa; insulin: 5.3 kDa; glucagone: 3.5 kDa). Intact ApoA-I (10 μg) was used in a control experiment, and its elution (*gray peak*) is shown overlapping the patterns of digested protein.

affinity of ApoA-I for Hpt was at least 7-fold higher than that of P2a.

Competition of P2a with HDL or Hb for Binding Hpt—The peptide P2a was analyzed for its ability to influence Hpt binding to HDL *in vitro*. HDL-coated wells were incubated with Hpt in the absence or presence of different amounts of acetylated P2a. The peptide displaced over 75% of Hpt from binding HDL ($p < 0.001$; Fig. 6). No binding was detected when Hpt was incubated with a 10-fold excess of ApoA-I. This result confirmed previous data on the Hpt property to interact with both free and lipid-embedded ApoA-I (19–21, 30) and demonstrated that the ApoA-I sequence in P2a can effectively prevent Hpt binding to HDL.

Acetylated P2a was also assayed for its ability to compete with Hb for binding Hpt. Hpt was incubated with different amounts of P2a or ApoA-I or HDL in Hb-coated wells. Incubation of Hpt alone was used as control. The Hpt binding was analyzed by *p*-nitrophenol production from immunocomplexes with anti-Hpt IgG and GAR-P IgG. Such a binding was not significantly influenced by treatment with P2a, whereas it was heavily decreased by ApoA-I (1 μM: $p < 0.02$; 3–20 μM: $p < 0.001$) or HDL ($p < 0.001$ for all the concentrations used) (Fig. 7). The results suggested that the Hpt function of capturing Hb for catabolism in the liver (29) might be retained in the presence of P2a.

LCAT Activity in the Presence of Hpt: Effect of P2a Addition—The property of P2a to compete with ApoA-I for binding Hpt was also tested in the LCAT assay. DS-treated plasma and proteoliposomes (LCAT and cholesterol sources, respectively) were incubated with 0.3 μM Hpt in the absence or presence of 0.9 μM P2a. The enzyme activity was inhibited by Hpt ($p = 0.016$) but fully restored when the peptide was present during incubation (Fig. 8). In particular, the enzyme stimulation by ApoA-I was rescued by only 3-fold molar excess of P2a over Hpt. The peptide, when incubated without Hpt, did not significantly affect cholesterol esterification.

DISCUSSION

The previously observed binding of Hpt to ApoA-I (19–21) was suggested to reduce the amount of ApoA-I available for LCAT stimulation, thus impairing enzyme activity (23). Here we have reported data from chemical fragmentation of ApoA-I suggesting that a domain, localized in the amino acid sequence

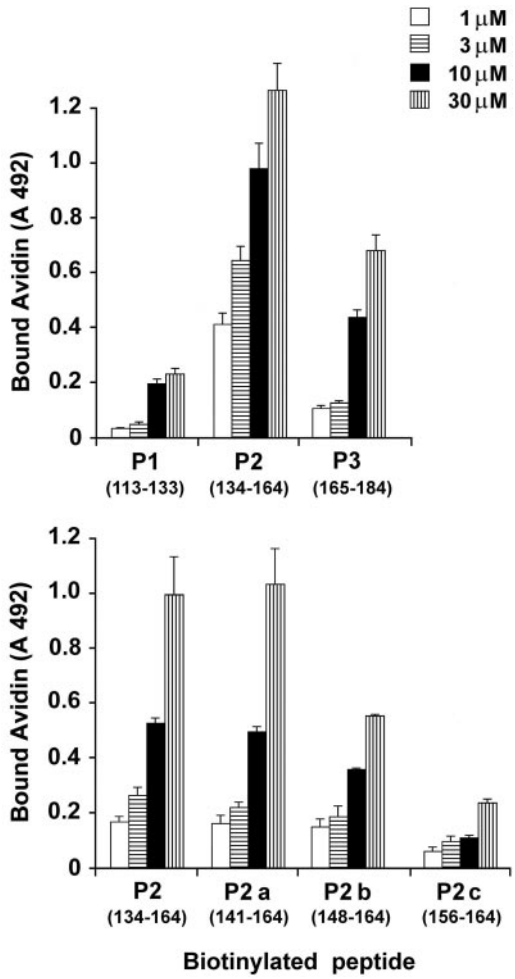

FIG. 4. **Binding of synthetic peptides to Hpt.** Biotinylated peptides, sharing parts of the ApoA-I sequence putatively involved in the Hpt binding (aa 113–184), were synthesized. The amino acid sequences of ApoA-I, matched by the different peptides, are indicated in *parentheses*. Four solutions of each peptide were prepared (1, 3, 10, and 30 μM), and samples from each solution were incubated in Hpt-coated wells. Avidin-HRP was used to detect bound peptides. The two panels refer to distinct experiments, performed with different batches of commercial Hpt. The samples were analyzed in triplicate; the data are expressed as means ± S.E.

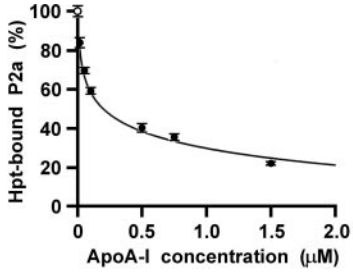

FIG. 5. **Competition of P2a with ApoA-I for binding Hpt.** Intact ApoA-I and P2a (aa 141–164) were compared for their affinities to Hpt. Biotinylated P2a (1.5 μM) was mixed with different amounts of ApoA-I (0, 0.01, 0.05, 0.1, 0.5, 0.75, or 1.5 μM). Aliquots (50 μl) from each mixture were incubated in Hpt-coated wells. Avidin-HRP was used to detect bound peptides. The samples were analyzed in triplicate. The data are reported as percent of the value obtained by incubation of P2a alone (*open circle*) and expressed as means ± S.E.

spanning from Glu[113] to Asn[184], is able to bind Hpt. By using synthetic peptides, we found that such a domain is essentially restricted to the ApoA-I sequence from Leu[141] to Ala[164], which contains helix 6 of the protein. It has been reported that most of the ApoA-I structure is comprised of ten tandem repeating

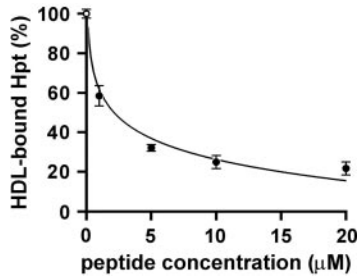

FIG. 6. **Competition of P2a with HDL for binding Hpt.** Acetylated P2a (aa 141–164) was analyzed for its ability to interfere with Hpt binding to HDL. Aliquots of 1 μM Hpt (50 μl), previously incubated with different amounts of P2a (1, 5, 10, or 20 μM) or 10 μM ApoA-I, were loaded onto HDL-coated wells. Rabbit anti-Hpt IgG and GAR-HRP IgG were used to detect the Hpt binding to HDL. The amount of immunocomplexes was determined by measuring the absorbance at 492 nm. The samples were analyzed in triplicate. Hpt binding was not detected in the presence of ApoA-I. The data are reported as percent of the value obtained by incubation of Hpt alone (*open circle*) and expressed as means ± S.E.

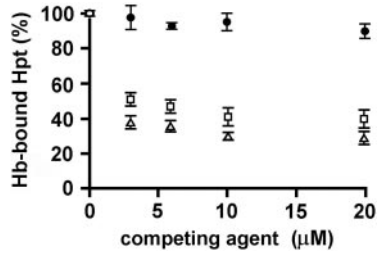

FIG. 7. **Competition of P2a with Hb for binding Hpt.** Acetylated P2a (aa 141–164) was assayed for its ability to compete with Hb for Hpt binding. Aliquots of 1 μM Hpt (50 μl), previously incubated with different amounts (3, 6, 10, or 20 μM) of P2a (*solid circles*) or ApoA-I (*open squares*) or HDL (*open triangles*), were loaded onto Hb-coated wells. Hpt binding to Hb was detected using rabbit anti-Hpt IgG and GAR-P IgG and monitoring the color development at 405 nm. The samples were analyzed in triplicate. The data are reported as percent of the value obtained by incubation of Hpt alone (*open circle*) and expressed as means ± S.E.

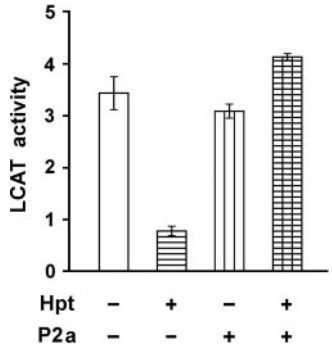

FIG. 8. **Effect of P2a on Hpt inhibition in the LCAT assay.** LCAT activity was assayed by incubating a pool of DS-treated plasma with a standard reaction mixture containing a proteoliposome (ApoA-I:lecithin:[3H]cholesterol, 1.5:200:18 molar ratio) as substrate. The enzyme activity was measured in the presence of either Hpt (0.3 μM) or P2a (0.9 μM) or both. A control assay was performed without Hpt and P2a. LCAT activity was expressed as nmol of cholesterol incorporated/h/ml of plasma. The samples were analyzed in triplicate; the data are expressed as means ± S.E.

α-helices (38), and the ApoA-I property to stimulate LCAT was assigned to helix 6 (aa 143–164) (39–43), although the adjacent helix 7 (aa 165–186) seemed to partially contribute to activation (42, 44). In particular, deletion of repeats 6 and 7, as well as the conformational changes accompanying mutations within these helices, dramatically reduces the ability of ApoA-I to activate the LCAT-catalyzed esterification of cholesterol (38,

45–48). Our data demonstrated that, in the ApoA-I sequence, the Hpt binding site overlaps the LCAT activating site (39–43). The results strongly suggested that the observed inhibitory role of Hpt on LCAT activity can be explained by competition of Hpt with the enzyme for the same ApoA-I region, that is helix 6. Masking this region by Hpt, a circumstance depending on the Hpt concentration, might therefore be associated with reduced LCAT stimulation. Competition of the synthetic peptide P2a (aa 141–164) with HDL for binding Hpt supported this hypothesis. This binding, while preventing the interaction of Hpt with ApoA-I on the HDL surface, did not affect the well known function of Hpt of capturing free circulating Hb to prevent iron loss (49) and oxidative damage (50). On the contrary, ApoA-I (free or HDL-linked) was found to interfere with Hpt binding to Hb, according to previous data obtained by an experimental approach similar to that described here (30). These results suggest that ApoA-I competition with Hb for Hpt binding is based on steric hindrance by protein region(s) different from helix 6 rather than on involvement of this helix.

The *in vitro* activity of LCAT, although strongly reduced in the presence of Hpt, was found restored when the mixture was supplemented with P2a. These findings suggested that the ApoA-I site stimulating LCAT works poorly if bound by Hpt but this inhibitory ligand can be displaced by the peptide. Thus P2a, as able to engage Hpt and save ApoA-I function, might be used to prevent the inhibitory effect of Hpt or rescue the enzyme activity in the presence of Hpt.

Hpt, being capable of binding ApoA-I and reducing LCAT activity, might be a major cause of poor cholesterol removal from peripheral cells and, therefore, low HDL cholesterol levels in the circulation. Enhanced Hpt concentration, as detected during the acute phase of inflammation (51, 52), might promote cholesterol accumulation in endothelial cells and arterial wall, thus representing a risk factor for ethiogenesis or progression of endothelial dysfunction and atherosclerosis. As a matter of fact, a fundamental role for inflammation in mediating all stages of this pathology was recently established (53), and association of Hpt with cardiovascular disease was previously demonstrated (54, 55). In this context, it is worth noting that ApoA-I helix 6 is also involved in stimulation of apolipoprotein E (ApoE) secretion by macrophage foam cells (56, 57), which are well known to play a crucial role in formation of atheromatous plaque. As ApoE is addressed to the HDL surface for recognition and uptake by hepatocyte (58–61), it cannot be excluded that Hpt interaction with ApoA-I might also negatively affect HDL in cholesterol delivery to the liver.

In conclusion, our results have provided novel information on the role of Hpt in the regulation of HDL function and reverse cholesterol transport. Synthetic peptides, competing with ApoA-I as a target of Hpt binding, might be used to prevent the inflammation-dependent rising of cardiovascular disease or, more generally, in therapy of pathologies associated with defective LCAT function.

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