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Haptoglobin binds the antiatherogenic protein apolipoprotein E – impairment of apolipoprotein E stimulation of both lecithin:cholesterol acyltransferase activity and cholesterol uptake by hepatocytes

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Haptoglobin (Hpt) binds apolipoprotein A-I (ApoA-I), and impairs its stimulation of lecithin:cholesterol acyltransferase (LCAT). LCAT plays a major role in reverse cholesterol transport (RCT). Apolipoprotein E (ApoE), like ApoA-I, promotes different steps of RCT, including LCAT stimulation. ApoE contains amino acid sequences that are homologous with the ApoA-I region bound by Hpt and are involved in the interaction with LCAT. Therefore, Hpt was expected to also bind ApoE, and inhibit the ApoE stimulatory effect on LCAT. Western blotting and ELISA experiments demonstrated that the Hpt β-subunit binds ApoE. The affinity of Hpt for ApoE was higher than that for ApoA-I. High ratios of Hpt with either apolipoprotein, such as those associated with the acute phase of inflammation, inhibited, *in vitro*, the stimulatory effect of ApoE on the cholesterol esterification activity of LCAT. Hpt also impaired human hepatoblastoma-derived cell uptake of [^{3}H]cholesterol from proteoliposomes containing ApoE or ApoA-I. We suggest that the interaction between Hpt and ApoE represents a mechanism by which inflammation affects atherosclerosis progression. Hpt might influence ApoE function in processes other than RCT.

Structured digital abstract

- MINT-7258778: *Hpt beta chain* (uniprotkb:P00738) *binds* (MI:0407) to *APOE* (uniprotkb:P02649) by *filter binding* (MI:0049)
- MINT-7258829, MINT-7258868: *Hpt* (uniprotkb:P00738) *binds* (MI:0407) to *APOA1* (uniprotkb:P02647) by *competition binding* (MI:0405)
- MINT-7258848, MINT-7258819, MINT-7258877: *APOE* (uniprotkb:P02649) *binds* (MI:0407) to *Hpt* (uniprotkb:P00738) by *competition binding* (MI:0405)
- MINT-7258791: *Hpt* (uniprotkb:P00738) *binds* (MI:0407) to *APOE* (uniprotkb:P02649) by *pull down* (MI:0096)
- MINT-7258760: *Hpt* (uniprotkb:P00738) *physically interacts* (MI:0915) with *APOE* (uniprotkb:P02649) by *pull down* (MI:0096)
- MINT-7258811: *Hpt* (uniprotkb:P00738) *binds* (MI:0407) to *APOA1* (uniprotkb:P02647) by *enzyme linked immunosorbent assay* (MI:0411)

Abbreviations

ApoA-I, apolipoprotein A-I; ApoE, apolipoprotein E; ECL, enhancedchemiluminescence; HDL, high-density lipoprotein; Hpt, haptoglobin; HRP, horseradish peroxidase; HSA, human serum albumin; LCAT, lecithin:cholesterol acyltransferase; LDL, low-density lipoprotein; PVDF, poly(vinylidene difluoride); RCT, reverse cholesterol transport; SEM, standard error of the mean; VLDL, very low-density lipoprotein.

• MINT-7258801: *Hpt* (uniprotkb:P00738) *binds* (MI:0407) to *APOE* (uniprotkb:P02649) by *enzyme linked immunosorbent assay* (MI:0411)

Introduction

The fundamental role of inflammation in atherosclerosis, from onset through progression to, ultimately, the thrombotic complications of the disease, was recently reviewed [1–3]. The recognition of inflammation as a major cause of atherosclerosis has generated a sustained effort to investigate the roles of specific factors associated with alterations of critical pathways, such as reverse cholesterol transport (RCT). The maintenance of physiological levels of cholesterol, in both plasma and cells, is essential for cell function and survival. In fact, cholesterol is toxic when it accumulates in the plasma membrane or within the cell. Most peripheral cells and tissues are unable to catabolize cholesterol, which can thus be eliminated only by efflux to extracellular acceptors such as high-density lipoprotein (HDL). In RCT, excess cholesterol is removed from peripheral tissues, and is transported by HDL to the liver for excretion in the bile. Therefore, RCT is the major mechanism by which HDL protects against atherosclerosis and other cardiovascular diseases. Stimulation of RCT is a primary target for the development of drugs enhancing the level or reducing the catabolism of HDL [4,5]. Apolipoprotein A-I (ApoA-I), the major protein component of HDL, plays a key role in RCT, mainly by stimulating the efflux of cholesterol and activating another critical player, the enzyme lecithin:cholesterol acyltransferase (LCAT; EC 2.3.1.43). LCAT converts cholesterol into cholesteryl esters for HDL-mediated transport in the circulation [5]. ApoA-I can be bound by haptoglobin (Hpt) [6–8]. Hpt is a polymorphic glycoprotein that exhibits phenotype prevalence in cardiovascular diseases [9,10]. Hpt circulates at enhanced levels during the acute phase of inflammation [11,12], capturing free Hb and transporting this protein to the liver [12].

We previously demonstrated that binding of Hpt to ApoA-I is associated with reduced LCAT activity, and suggested that such binding decreases the amount of free ApoA-I available for enzyme stimulation, thus impairing cholesterol esterification [6,13,14]. A peptide with the ApoA-I amino acid sequence spanning from Leu141 to Ala164 and overlapping with the protein domain required for LCAT stimulation was able to displace Hpt from ApoA-I and restore the enzyme activity [6]. On the basis of the above information, high levels of Hpt were suggested to be a major cause of both poor cholesterol removal from peripheral cells and low levels of HDL cholesterol in the circulation [6,15]. In fact, an association of Hpt with an increased risk of developing cardiovascular disease or myocardial infarction was recently reported [9,16–18]. In this context, it is worth noting that high levels of Hpt might also limit ApoA-I stimulation of macrophage secretion of apolipoprotein E (ApoE), a major component of different classes of lipoproteins that plays a number of antiatherosclerotic and anti-inflammatory roles [19]. In particular, ApoE participates in cholesterol homeostasis in plasma by stimulating, like ApoA-I, different steps of RCT [19]. ApoE actually stimulates the release of excess cholesterol from peripheral cells, including macrophages and foam cells [19–21], activates LCAT for cholesterol esterification [22], and mediates lipoprotein binding to specific liver receptors for endocytosis and cholesterol elimination [19,23]. ApoE contains amino acid sequences that are homologous to ApoA-I sequences, including that bound by Hpt (see the Swiss-Prot database, entry P02647 versus entry P02649). It is therefore conceivable that Hpt might bind not only ApoA-I but also ApoE. This study aimed to evaluate this hypothesis experimentally. Furthermore, Hpt effects on the functions of both ApoE and ApoA-I in LCAT stimulation and lipoprotein-mediated delivery of cholesterol to hepatocytes were compared.

Results

Binding of Hpt to ApoE

Hpt is usually purified from plasma by affinity chromatography, using Hb coupled with resin beads [7,24]. ApoA-I, as a result of forming a complex with Hpt, is positively selected by this technique [7,25]. ApoE, as a result of containing amino acid sequences homologous to the ApoA-I domain bound by Hpt, might be selected by bead-coupled Hb as well. In order to verify this hypothesis, we analysed the human plasma proteins that, after being loaded on a column of Sepharose coupled with Hb (Hb–Sepharose), were eluted together with Hpt. Elution was performed under mild acidic conditions (0.1 M glycine-HCl at pH 3.5). Electrophoretic analysis of the eluted material revealed that Hpt was released from the column together with a number of other proteins, including a protein of about 28 kDa, which was previously shown to be ApoA-I

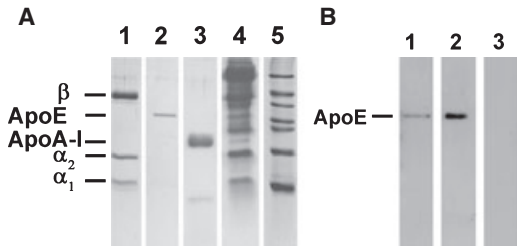

Fig. 1. Electrophoresis and western blotting of Hpt purified with Hb–Sepharose at pH 3.5. Hpt, partially purified from plasma with Hb–Sepharose, with elution at pH 3.5, was analysed by electrophoresis on 15% polyacrylamide gel in denaturing and reducing conditions, and by western blotting. (A) Coomassie-stained bands of isolated Hpt (lane 1), standard ApoE (lane 2), standard ApoA-I (lane 3), and partially purified Hpt from Hb–Sepharose (lane 4). Molecular mass markers (BSA, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; trypsin inhibitor, 20 kDa; α-lactalbumin, 14.2 kDa) are in lane 5. The migrations of the Hpt subunits (β, α2, and α1), ApoE and ApoA-I are indicated on the left. (B) Standard ApoE (lane 1) and antigens coeluted with Hpt from Hb–Sepharose at pH 3.5 (lane 2). The volume eluted at pH 3.5 from Hb–Sepharose, loaded with standard ApoE in a control experiment, was analysed (lane 3). After electrophoresis and western blotting, goat anti-ApoE IgG and rabbit anti-goat HRP-conjugated IgG were used for detecting immunocomplexes by ECL.

[7,24], and a protein of about 34 kDa (Fig. 1A, lane 4). The proteins fractionated by electrophoresis were processed by western blotting, and challenged with a polyclonal anti-ApoE IgG. The 34 kDa antigen reacted with the antibodies, thus confirming that ApoE effectively bound Hpt captured by the stationary phase, and was eluted together with this protein from Hb–Sepharose at pH 3.5 (Fig. 1B, lane 2). To rule out possible nonspecific interactions of ApoE with either the Sepharose beads or Hb during chromatography, purified ApoE was loaded on the Hb–Sepharose column. ApoE was not retained by the column in the absence of Hpt (Fig. 1B, lane 3). This result suggests that Hpt was required for ApoE retention, but did not exclude the possibility that ApoE was just trailed by Hpt-bound ApoA-I. Both of these apolipoproteins can actually be exposed by some HDL particles, and ApoA-I binding to Hb-captured Hpt might result from trapping of the whole lipoprotein cargo on the column. No HDL minor apolipoprotein (e.g. apolipoprotein C-I, apolipoprotein C-II, apolipoprotein A-II, or serum amyloid A) was detected by electrophoresis in the material eluted from Hb–Sepharose at pH 3.5 or 2.8 (data not shown). This finding alone, however, did not provide sufficient evidence that ApoE might be specifically bound by Hpt.

In order to further test whether ApoE interacts with Hpt and, in particular, to assess which Hpt chain (β or α) is involved in the binding, the material purified from Hb–Sepharose by a two-step elution (pH 3.5, followed by pH 2.8, as described in Experimental procedures) was analysed for ApoE binding. Purified Hpt was fractionated by SDS/PAGE, and blotted onto a poly(vinylidene difluoride) (PVDF) membrane that, after incubation with purified ApoE, was treated with anti-ApoE monoclonal IgG. Only the β-chain of Hpt reacted with the antibodies (Fig. 2, lane 2). Nonspecific interactions between blotted Hpt and antibodies were not detected when ApoE treatment was omitted. This result demonstrates that the Hpt β-subunit, which was previously found to bind ApoA-I [7], can also bind ApoE.

Hpt binding of ApoE was confirmed by further experiments using isolated Hpt. Commercial preparations of Hpt, in contrast to those of ApoE, are contaminated by a number of proteins, including ApoA-I. Therefore, a four-step procedure was set up to isolate Hpt from plasma. As described in Experimental procedures, plasma proteins obtained by salting out in 50% ammonium sulfate were processed by gel filtration and anion exchange chromatography. Finally, affinity chromatography with anti-Hpt IgG, coupled with Sepharose beads, was used to obtain Hpt with a purity of > 98% (Fig. 1A, lane 1). Isolated Hpt was then coupled with a column of NHS-activated resin for the binding of ApoE. Commercial

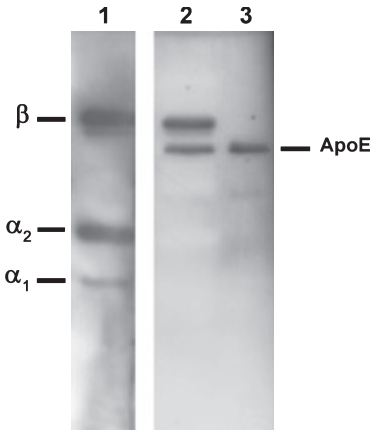

Fig. 2. Binding of ApoE to Hpt blotted on a membrane. Hpt, partially purified with Hb–Sepharose, with elution at pH 2.8, was processed for electrophoresis on 15% polyacrylamide gel in denaturing and reducing conditions, and blotted onto a PVDF membrane. The blotted material was detected with rabbit anti-Hpt IgG and goat anti-rabbit HRP-conjugated IgG (lane 1) or, after incubation with 0.1 mg·mL^{-1} ApoE, mouse anti-ApoE IgG and goat anti-mouse HRP-conjugated IgG (lane 2). Standard ApoE (lane 3) was processed in the same way as the sample in lane 2. The migrations of the Hpt subunits (β, α_2, and α_1) and ApoE are indicated.

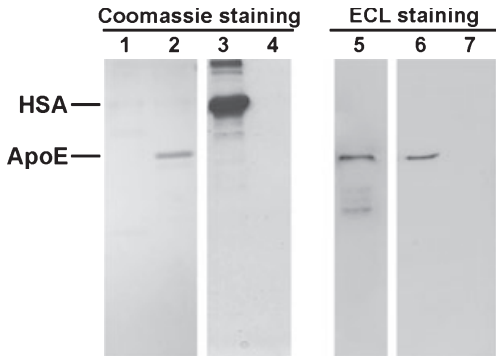

Fig. 3. Binding of ApoE or HSA to Hpt coupled with Sepharose. ApoE or HSA was separately processed with a column of Sepharose coupled with Hpt. Nonretained proteins (flowed through the column) and the fraction recovered by elution at pH 2.8 were analysed by electrophoresis on 15% polyacrylamide gel in denaturing and reducing conditions, and Coomassie staining or immunoblotting. The immunoblotting was performed, after protein transfer from gel to a PVDF membrane, with mouse anti-ApoE IgG and goat anti-mouse HRP-conjugated IgG, and ECL detection. Lane 1: nonretained proteins from ApoE-loaded column; Coomassie staining. Lane 2: proteins eluted from ApoE-loaded column; Coomassie staining. Lane 3: nonretained proteins from HSA-loaded column; Coomassie staining. Lane 4: proteins eluted from HSA-loaded column; Coomassie staining. Lane 5: standard ApoE; immunoblotting. Lane 6: proteins eluted from ApoE-loaded column; immunoblotting. Lane 7: proteins eluted from ApoE-loaded column of Sepharose coupled with ethanolamine (control); immunoblotting.

ApoE, which was partly oxidized (Fig. 3, lane 5), was loaded on the column and, after being washed, the retained material was eluted at pH 2.8. The column flowthrough and the elution fractions were analysed by electrophoresis, and native form(s) of ApoE were recovered in the fractions eluted in acidic conditions, but not in the flowthrough, as assessed by Coomassie staining (Fig. 3, lanes 2 and 1, respectively). The presence of ApoE in the elution fractions was also demonstrated by immunoblotting with monoclonal antibodies against ApoE (Fig. 3, lane 6), thus confirming that resin-linked Hpt was able to bind the apolipoprotein. In a control experiment, ApoE was not retained by a column of ethanolamine-coupled Sepharose (Fig. 3, lane 7). In a further control experiment, human serum albumin (HSA) was loaded on the Hpt-coupled column of Sepharose, and both the flowthrough and the fractions, collected by acidic elution following extensive washing, were analysed by electrophoresis and Coomassie staining. As shown in Fig. 3, HSA was recovered only in the column flowthrough (lane 3), but not in the eluted fractions (lane 4). These results indicate that ApoE is specifically retained by Hpt in the stationary phase.

Hpt binding to low-density lipoprotein (LDL) or very low-density lipoprotein (VLDL)

Possible binding of Hpt to LDL or VLDL apolipoproteins other than ApoE was investigated as follows. VLDL and LDL were purified from a pool of plasma samples ($N = 5$) by sequential flotation ultracentrifugation [26], and processed by SDS/PAGE. Proteins were stained with Coomassie (Fig. 4, lanes 1 and 2) or blotted onto PVDF membranes. The membrane was incubated with biotinylated Hpt (0.1 mg·mL^{-1}), and then treated with horseradish peroxidase (HRP)-conjugated avidin for detection of protein-bound Hpt. Hpt was found to be bound to a 34 kDa protein that turned out to be ApoE, as it reacted with polyclonal anti-ApoE IgG (data not shown), and to an unknown protein of about 50 kDa (Fig. 4, lanes 3 and 4). No Hpt binding to other lipoprotein-bound proteins, such as albumin, was observed. Similar results were obtained by using two other preparations of these lipoproteins from two different pools.

Hpt was previously found to be associated with lipoproteins containing ApoA-I [8,27]. Moreover, Hpt was identified as an abundant component in the

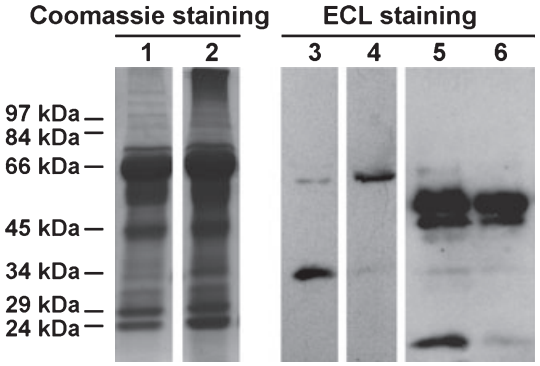

Fig. 4. Hpt binding to VLDL and LDL proteins. The proteins of isolated VLDL and LDL were processed by electrophoresis on 10% polyacrylamide gel in denaturing and reducing conditions, and detected by Coomassie staining or with biotinylated Hpt. Biotinylated Hpt was used, after protein transfer from gel to the PVDF membrane, with HRP-conjugated avidin and ECL. Coomassie-stained bands of VLDL and LDL proteins are shown in lanes 1 and 2, respectively. VLDL and LDL proteins, blotted onto the PVDF membrane and incubated with biotinylated Hpt, are shown in lanes 3 and 4, respectively. VLDL and LDL proteins, after blotting and reaction with biotinylated Hpt (i.e. the same as for lanes 3 and 4), were treated for alkaline stripping of biotinylated Hpt, and this was followed by immunostaining with rabbit anti-Hpt IgG and goat anti-(rabbit HRP-conjugated IgG) (lanes 5 and 6, respectively). The migrations of phosphorylase b (97 kDa), fructose-6-phosphate kinase (84 kDa), BSA (66 kDa), ovalbumin (45 kDa), ApoE (34 kDa), carbonic anhydrase (29 kDa) and trypsinogen (24 kDa) are indicated on the left.

protein preparation from isolated LDL [28]. In order to check whether Hpt is purified together with ApoE-containing lipoproteins, the same membrane with blotted proteins from the VLDL or LDL preparations was processed to strip off biotinylated Hpt. Then, the membrane was incubated with rabbit anti-Hpt IgG and rabbit anti-goat HRP-conjugated IgG. Two bands, reacting with the antibodies and with the molecular masses of β and α_2 (41 and 21 kDa, respectively), were detected (Fig. 4, lanes 5 and 6). Furthermore, Hpt concentrations in the VLDL or LDL preparations from pooled plasma were measured by ELISA, and found to be 0.18 ± 0.008 or 0.01 ± 0.006 mg Hpt·mg^{-1} of protein, respectively.

Individual plasma samples ($N = 5$) from healthy subjects were used to purify VLDL and LDL, and to investigate whether free Hpt correlates with lipoprotein-bound Hpt. Plasma levels of Hpt, expressed as mg Hpt per mg total protein, were found to be positively correlated with both Hpt levels in VLDL preparations, expressed as mg Hpt per mg VLDL protein, and with Hpt levels in LDL preparations, expressed as mg Hpt per mg LDL protein ($r = 0.94$, $P = 0.016$, and $r = 0.96$, $P = 0.008$, respectively).

Hpt affinities for ApoE and ApoA-I

In order to compare the affinities of ApoA-I and ApoE for Hpt, ELISA experiments were performed. Hb-coated wells were first incubated with isolated Hpt (0.25 μM), and then with different concentrations of ApoA-I or ApoE (0–0.3 μM). The binding of ApoE or ApoA-I to Hb-linked Hpt was measured by using antibodies. In particular, goat anti-ApoE IgG or rabbit anti-ApoA-I IgG was used, respectively, to form immunocomplexes, which were detected by rabbit anti-goat HRP-conjugated IgG or goat anti-rabbit HRP-conjugated IgG. The higher the concentration of apolipoprotein in the incubation medium, the higher the level of Hpt-bound immunocomplexes (with ApoE exhibiting higher binding than ApoA-I at any assayed concentration) (Fig. 5A).

The binding affinities of ApoA-I and ApoE for Hpt were also analysed in a competition assay with Hb. Different concentrations of ApoE or ApoA-I (0–3 μM) were preincubated with 0.3 μM Hpt. The mixtures were then loaded into Hb-coated wells. Hb-bound Hpt was detected with rabbit anti-Hpt IgG and goat anti-rabbit HRP-conjugated IgG. The binding of Hpt to immobilized Hb decreased as the concentration of either apolipoprotein was increased (Fig. 5B). The concentrations of ApoE and ApoA-I producing half-maximal inhibition (IC_{50}) of Hpt binding to Hb were calculated from

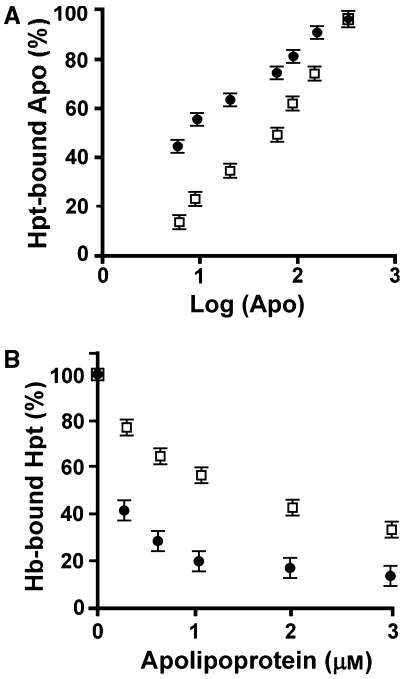

Fig. 5. Hpt binding to ApoA-I and ApoE. ApoA-I and ApoE were separately processed for ELISA, using wells coated with Hpt and Hb. Hpt was attached to the wells before binding of the apolipoproteins (A), or preincubated with the apolipoproteins before loading into the wells (B). Different concentrations of ApoE (solid circles) or ApoA-I (open squares) were used in triplicate. The amount of bound antigens was measured as absorbance at 492 nm, with an antibody-based detection system using *o*-phenylenediamine and H_2O_2. (A) Aliquots (50 μL) of 0.25 μM Hpt were loaded into the wells to form immobilized Hpt–Hb complexes; goat anti-ApoE IgG and rabbit anti-goat HRP-conjugated IgG, or rabbit anti-ApoA-I IgG and goat anti-rabbit HRP-conjugated IgG, were used to detect Hpt-bound ApoE or ApoA-I, respectively; the data are expressed as mean ± SEM versus log nanomolar concentration, and reported as percentage of the value obtained with 300 nM apolipoprotein. (B) Mixtures (50 μL) containing 0.3 μM Hpt and different amounts of apolipoprotein were loaded into the wells; rabbit anti-Hpt IgG and goat anti-rabbit HRP-conjugated IgG were used to detect Hb-bound Hpt; the data, reported as mean ± SEM, are expressed as percentage of the value obtained with incubation of Hpt alone. In each panel, a single representative of at least three independent experiments is shown. The interassay coefficient of variation from three independent experiments was 7.5%.

nonlinear regressions, and were 0.17 and 1.054 μM, respectively. These data confirm that the affinity of Hpt for ApoE is higher than that for ApoA-I.

Displacement of Hpt from ApoE by Hb or P2a

Competition assays were carried out to investigate whether Hb or an ApoA-I mimetic peptide displaces Hpt from ApoE. Hpt (0.3 μM) was preincubated with different concentrations of Hb (0–10 μM), and the

mixtures were then loaded into ApoE-coated wells. After incubation, the bound Hpt was detected with rabbit anti-Hpt IgG and goat anti-rabbit HRP-conjugated IgG. The data obtained indicate that Hb, at concentrations lower than 0.7 μM, did not affect the binding of Hpt to ApoE. Conversely, at higher concentrations, Hb effectively competed with ApoE for binding to Hpt (Fig. 6).

A similar competition assay was performed by using the peptide P2a, which has an amino acid sequence homologous with a region of ApoE [6]. Hpt was preincubated with different concentrations of P2a (0–30 μM), and the mixtures were then loaded into ApoE-coated wells. Hpt binding to ApoE decreased as the P2a amount used in the incubation mixture was increased (Fig. 6). In control experiments, Hb or P2a did not bind to ApoE-coated wells in the absence of Hpt, as demonstrated by failure of rabbit anti-Hb IgG or anti-ApoA-I IgG, respectively, to form immunocomplexes with goat anti-rabbit HRP-conjugated IgG. It is worth mentioning that the anti-ApoA-I IgG used is able to bind P2a-coated wells.

Competition between ApoA-I and ApoE for binding to Hpt

As both ApoA-I and ApoE interact with the same subunit of Hpt, they should be expected to compete for binding with Hpt. Two experiments were designed to test this hypothesis. In the first experiment, fixed amounts of ApoA-I (56 nM) were incubated with different concentrations of ApoE (1.4–280 nM) in Hpt-coated wells of microtiter plates. The binding of ApoA-I was evaluated by using rabbit anti-ApoA-I IgG and goat anti-rabbit HRP-conjugated IgG. The higher the amount of ApoE in the incubation mixture, the lower the binding of ApoA-I (Fig. 7A). In particular, ApoA-I binding to Hpt was halved in the presence

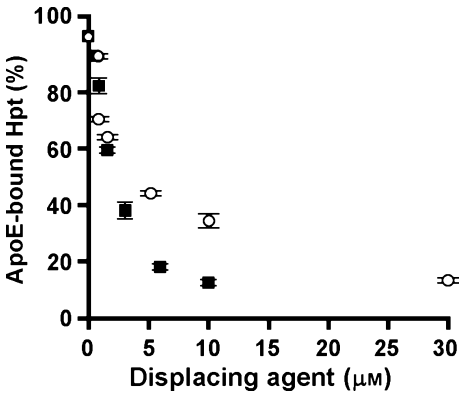

Fig. 6. Competition of P2a or Hb with ApoE for binding to Hpt. Hpt (0.3 μM) was incubated with different concentrations of P2a (open circles) or Hb (solid squares). Aliquots (50 μL) of the mixtures were separately loaded into ApoE-coated wells for ELISA. The amount of ApoE-bound Hpt was determined by using rabbit anti-Hpt IgG and goat anti-rabbit HRP-conjugated IgG, and measuring the absorbance at 492 nm, with the *o*-phenylenediamine and H_2O_2 system. The samples were analysed in triplicate. The data are reported as percentage of the value obtained by incubation of Hpt alone, and expressed as mean ± SEM. A single representative of at least three independent experiments is shown. The interassay coefficient of variation from three independent experiments was 8.3%.

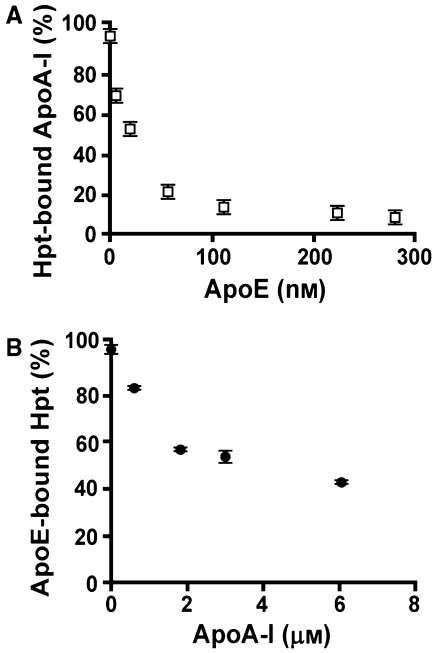

Fig. 7. Competition between ApoA-I and ApoE for binding to Hpt. (A) Competition of ApoA-I with ApoE for binding to immobilized Hpt is shown. ApoA-I (0.056 μM) was incubated with different concentrations of ApoE, and aliquots (50 μL) of the mixtures were then separately loaded into Hpt-coated wells for ELISA. The amount of Hpt-bound ApoA-I was determined by using rabbit anti-ApoA-I IgG and goat anti-rabbit HRP-conjugated IgG, and measuring the absorbance at 492 nm, with the *o*-phenylenediamine and H_2O_2 system. The samples were analysed in triplicate. The data are reported as percentage of the value obtained by incubation of ApoA-I alone, and expressed as mean ± SEM. (B) Competition of ApoA-I with immobilized ApoE for binding Hpt is shown. Hpt (0.114 μM) was incubated with different concentrations of ApoA-I, and aliquots (50 μL) of the mixtures were then separately loaded into ApoE-coated wells. The amount of ApoE-bound Hpt was determined by using rabbit anti-Hpt IgG and goat anti-rabbit HRP-conjugated IgG, and measuring the absorbance at 492 nm, with the *o*-phenylenediamine and H_2O_2 system. The samples were analysed in triplicate. The data are reported as percentage of the value obtained by incubation of Hpt alone, and expressed as mean ± SEM. In each panel, a single representative of at least three independent experiments is shown. The interassay coefficient of variation from three independent experiments was 6.7%.

of 20 nM ApoE, and was reduced to 20% when the two apolipoproteins were incubated at the same concentration (i.e. 56 nM). In the second experiment, the wells were coated with ApoE, and then incubated with mixtures of 0.114 μM Hpt containing different amounts of ApoA-I (0.6, 1.8, 3 or 6 μM). Hpt binding to ApoE was measured by using anti-Hpt IgG and goat anti-rabbit HRP-conjugated IgG. Hpt binding to ApoE decreased as the ApoA-I amount used in the incubation mixture was increased (Fig. 7B). ApoA-I, even at the highest concentration used (presumed 12-fold or 14-fold excess over immobilized ApoE, and 53-fold excess over Hpt), could not impair Hpt binding to ApoE. In control experiments, ApoA-I (or anti-Hpt IgG) did not bind to ApoE-coated wells. The results from the two above experiments demonstrate that Hpt binds ApoE better than ApoA-I, and ApoE effectively competes with ApoA-I as a target of Hpt.

Hpt influence on ApoE stimulation of LCAT

High levels of Hpt were previously found to impair ApoA-I stimulation of LCAT activity [6,15]. According to the results of Hpt binding to ApoE, it was expected also that Hpt might inhibit this apolipoprotein in stimulating the enzyme. LCAT activity was assayed in reaction mixtures containing liposomes with 0.05 μM ApoE or ApoA-I, and the effects of different amounts of Hpt (0.5, 1.5, 3 and 4 μM; Hpt/apolipoprotein ratios of 10, 30, 60, and 80) were evaluated.

As previously reported, Hpt inhibited the stimulatory function of ApoA-I on LCAT *in vitro* (Fig. 8). For the first time, we report here that Hpt also inhibited ApoE stimulation of LCAT *in vitro* (Fig. 8). In particular, as the Hpt/ApoE ratios used were similar to those occurring during the acute phase of inflammation, the results suggest that this pathological condition promotes formation of the Hpt–ApoE complex on the basis of the mass action law, and Hpt-bound ApoE does not stimulate LCAT for cholesterol esterification *in vivo*.

Effect of Hpt on ApoA-I-mediated and ApoE-mediated uptake of reconstituted lipoproteins by hepatocytes

ApoA-I and ApoE induce hepatocytes to take up cholesterol from circulating lipoproteins [19,23,29,30]. To investigate whether Hpt can influence this function of ApoA-I and ApoE, reconstituted lipoproteins containing cholesterol and phosphatidylcholine with either apolipoprotein were incubated with HepG2 cells in culture. In particular, the cells were incubated with the

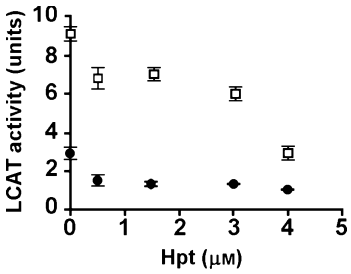

Fig. 8. Effect of Hpt on ApoA-I or ApoE stimulation of LCAT activity. The LCAT activity was assayed by incubating dextran sulfate-treated plasma with a proteoliposome suspension containing [^{3}H]cholesterol, phosphatidylcholine, and 0.05 μM ApoA-I (open squares) or ApoE (solid circles). The enzyme activity was measured in the presence of different concentrations of Hpt. The Hpt/apolipoprotein molar ratio in the assay ranged from 10 to 80. As a control, a sample without Hpt was processed. The LCAT activity was expressed as nanomoles of cholesterol esterified per hour per millilitre of plasma (units). The samples were analysed in triplicate, and the data are expressed as mean ± SEM. A single representative of at least three independent experiments is shown. The interassay coefficient of variation from three independent experiments was 4.7%.

proteoliposomes, in the absence or presence of Hpt. Labelled cholesterol was used as tracer. As shown in Fig. 9, Hpt significantly inhibited the cholesterol uptake mediated by both ApoA-I and ApoE ($P = 0.0004$ and $P = 0.0353$, respectively). Uptake inhibition was not observed when albumin, instead of Hpt, was present in the culture medium. Moreover, the uptake was fully restored when Hb was present during incubation with Hpt. These findings indicate that Hpt specifically impaired both the apolipoproteins in promoting cholesterol uptake by the cells, and suggest that Hb displaced Hpt from the apolipoproteins, which were therefore free to interact with their cell receptors for cholesterol internalization. Incubation of proteoliposomes with Hb alone in the culture medium did not affect their cholesterol delivery to the cells.

Discussion

The capacity of HDL to protect against atherosclerosis has already been reported [4]. However, the protective effect of HDL is recognized to be modified by interacting proteins, e.g. serum amyloid A and paraoxonase. In this article, we report, for the first time, that the binding between Hpt and ApoE may influence this apolipoprotein in stimulating LCAT and mediating HDL cholesterol delivery to the liver. This finding suggests that Hpt is a ligand not only for small HDL, whose major protein is ApoA-I, but also for other classes of ApoE-containing lipoproteins, such as

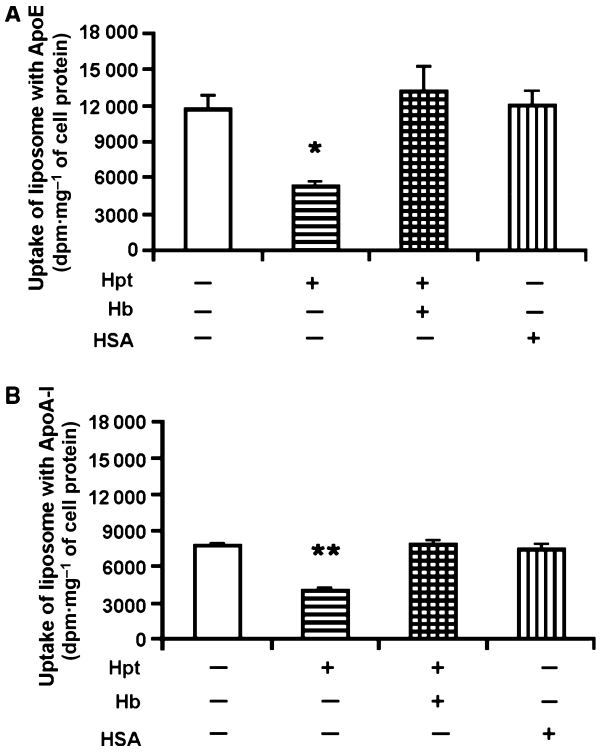

Fig. 9. Effect of Hpt on the uptake of ApoE-containing or ApoA-I-containing liposomes by HepG2 cells. HepG2 cells were incubated with proteoliposome suspensions containing [^{3}H]cholesterol, phosphatidylcholine, and 8 nM ApoE (A) or 15 nM ApoA-I (B). The assay was performed in the absence (open bar, control) or presence (bar with horizontal lines) of 3 μM Hpt. Hb (6 μM, bar with grid) or HSA (5 μM, bar with vertical lines) were added to Hpt-supplemented or Hpt-free culture, respectively. After incubation, the cell were lysed for measurement of their radioactivity and protein concentration. The amount of cholesterol internalized by the cells is expressed as dpm per mg of cell protein. Significant differences from control are indicated (*$P < 0.05$; **$P < 0.01$). The samples were analysed in triplicate, and the data are expressed as means ± SEM. A single representative of at least three independent experiments is shown. The interassay coefficient of variation from three independent experiments was 8.3%.

VLDL or LDL. According to our data, the affinity of Hpt for ApoE is higher than for ApoA-I. This could be necessary to improve Hpt binding to ApoE, whose circulating levels are lower than those of ApoA-I, and it might result in effective regulation by Hpt of functions shared by both apolipoproteins, including stimulation of LCAT and promotion of cholesterol elimination.

The inhibitory effect of high Hpt levels on ApoA-I function in stimulating LCAT was already well known, and was supposed to depend on the Hpt structure overlapping with the ApoA-I domain required for the enzyme activation [6]. We also proposed the hypothesis that this inhibition might be limited to the acute phase and aimed at protecting the stimulatory domain of ApoA-I from oxidative damage [15]. It cannot be excluded that Hpt masks by steric hindrance or overlaps with the ApoE domain involved in the interaction with LCAT. Whether Hpt binding to ApoE also results in protection of the stimulatory domain of this apolipoprotein against oxidative attack by reactive oxygen species remains to be investigated.

We found that a peptide with the amino acid sequence 131–150 of ApoE is bound by Hpt, and can displace this protein from ApoE. This sequence contains the binding sites of ApoE for heparin (142–147) and LDL receptor (136–150) [31]. This result suggests that Hpt should impair or limit the ApoE interaction with these targets.

The binding and the consequent shielding of ApoA-I and ApoE by Hpt are expected to influence these apolipoproteins in their interaction not only with LCAT but also with other protein targets. Both ApoA-I and ApoE actually mediate the uptake and degradation of lipoproteins through their ability to bind different receptors on liver cells [19,23,29,30]. Our results from the cholesterol internalization assay show that Hpt compromises the cholesterol delivery mediated by ApoA-I or ApoE from reconstituted lipoproteins to hepatic cells in culture. The increase in Hpt concentration, occurring during the acute phase of inflammation, might impair the hepatocytes' ability to recognize ApoE-containing and ApoA-I-containing lipoproteins, and therefore could unbalance the concentration of circulating lipoproteins. The link between lipoprotein accumulation and cardiovascular disease is well known [1–3]. In particular, the importance of the final step of RCT is also demonstrated by massive accumulation of lipoproteins and lipoprotein remnants in patients with cardiovascular disease associated with defective ApoE binding to LDL receptors. Enhanced Hpt levels might represent a further way by which inflammation worsens the onset and the rate of progression of atherosclerosis.

We evaluated the negative effects of Hpt on LCAT activity and cholesterol uptake by hepatocytes by using molar ratios of Hpt with ApoE or ApoA-I similar to those that are detectable during the acute phase of inflammation. On the other hand, Hpt, in physiological conditions, might play a protective role for ApoE, as was reported for ApoA-I [15]. On the basis of our results for Hpt binding to either apolipoprotein, it is not clear whether the positive effects of Hpt outweigh the negative effects, or vice versa. It cannot be excluded that Hpt might be a protective factor for ApoA-I and ApoE function or a proatherogenic agent during the acute phase.

Could Hpt promote apolipoprotein shedding from lipoproteins, thus remodelling the size and shape of these particles? Does Hpt influence the catabolism of (some) lipoproteins? A further question is whether Hpt, upon binding ApoA-I and/or ApoE, directs the lipoproteins to specific extravascular compartments, where they dissociate, allowing the apolipoprotein function to be restored. These and other questions are raised by our work, and answers may be expected from further experiments. It also remains to be investigated whether each Hpt haplotype binds the three ApoE isoforms with different affinities. Genetic polymorphism might influence the role of Hpt not only in RCT, but also in some other ApoE-dependent process, e.g. the regulation of cholesterol homeostasis [23,32,33] or β-amyloid accumulation in the brain [34–36], where Hpt has recently been suggested to be synthesized [37,38].

In conclusion, we provide here new information on Hpt and ApoE, suggesting that their interaction represents a novel link between the acute phase of inflammation and ApoE function that should be considered when the effects of either protein are investigated.

Experimental procedures

Materials

Chemicals of the highest purity, BSA, HSA, *N*-hydroxysuccinimidobiotin, cholesterol, human Hpt (mixed phenotypes: Hpt 1-1, Hpt 1-2, and Hpt 2-2), Hb, rabbit anti-(human Hpt IgG), goat anti-rabbit HRP-conjugated IgG, goat anti-mouse HRP-conjugated IgG, HRP-conjugated avidin and molecular weight markers were purchased from Sigma-Aldrich (St Louis, MO, USA). DMEM and fetal bovine serum were from BioWhittaker (Verseviers, Belgium); L-glutamine, penicillin and streptomycin were from Gibco (Milano, Italy). ApoA-I, ApoE (from human plasma VLDL) and rabbit anti-human ApoA-I IgG were from Calbiochem (La Jolla, CA, USA). Recombinant human ApoE3 was from RELIA Tech (Mascheroder, Germany). Goat polyclonal anti-human ApoE IgG and rabbit anti-goat HRP-conjugated IgG were obtained from Chemicon (Millipore, Billerica, MA, USA). Monoclonal mouse anti-human ApoE IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The ApoA-I mimetic peptide P2a was synthesized by INBIOS (Naples, Italy), using standard Fmoc chemistry with amidated C-end, and was over 98% pure as evaluated by HPLC and MS analysis. PVDF transfer membrane, and Amicon centrifugal filters from Millipore (Billerica, MA, USA) were used. The dye reagent of Bio-Rad (Bio-Rad, Hercules, CA, USA) was used for protein titration. StartingBlock blocking buffer was from Thermo Fisher Scientific (Rockford, IL, USA). Polystyrene 96-well ELISA plates were purchased from Nunc (Roskilde, Denmark), and Hi-Trap NHS-activated columns, enhanced chemiluminescence (ECL) reagents and Kodak Biomax light film from GE-Healthcare (Milano, Italy).

Purification of Hpt

Hpt was partially purified by affinity chromatography for some experiments on its binding to ApoE. Plasma samples from different subjects ($N = 5$) were pooled, and the resulting mixture was processed in two steps. In the first step, Hi-Trap NHS-activated Sepharose (in a 1 mL prepacked column) was used to bind 10 mg of Hb, according to the manufacturer's instructions. The column was equilibrated with 10 volumes of P-buffer (50 mM Na_2HPO_4/NaH_2PO_4, pH 7.4), and then loaded with 2 mL of plasma at a flow rate of 0.2 mL·min^{-1}. After washing with P-buffer at a flow rate of 1 mL·min^{-1}, a proportion of Hpt and loosely bound proteins were recovered with 15 mL of 0.1 M glycine-HCl at pH 3.5. More tightly retained Hpt was then eluted with 0.1 M glycine-HCl at pH 2.8, and fractions of 0.5 mL were collected into tubes containing 10 μL of 1 M Tris. $A_{280\ nm}$ in the effluent volume allowed the detection of Hpt-containing fractions. Hpt purity was over 90%, as assessed by SDS/PAGE and densitometric analysis of the Coomassie-stained bands. This Hpt preparation contained small amounts of ApoA-I and ApoE, and was free of albumin and other protein contaminants.

Isolation of Hpt for *in vitro* assays and cell culture was carried out as follows. Plasma proteins were fractionated by salting out in ammonium sulfate, and three chromatography steps. In detail, 24.3 g of solid ammonium sulfate was added to 100 mL of plasma and, after shaking for 1 h at 18 °C, the insoluble material was removed by centrifugation (75 min at 12 000 ***g***). Ammonium sulfate was added to the supernatant up to a concentration of 30.6% (w/v, 50% of saturation). The solution was stirred for 1 h at 18 °C and, after centrifugation (75 min at 12 000 ***g***), the pellet was dissolved with 10 mM NaCl in 20 mM Tris/HCl at pH 7.4. This protein solution was freed of salts by gel filtration with a column of Sephacryl S-200 (3 × 42 cm), previously equilibrated with 10 mM NaCl in 20 mM Tris/HCl at pH 7.4. Specifically, the column was loaded with 1.5 mL of sample (about 130 mg of proteins), and the elution was carried out with 10 mM NaCl in 20 mM Tris/HCl (pH 7.4), with a 10 mL·h^{-1} flow rate at room temperature. Fractions of 1.5 mL were collected and, after measurement of $A_{280\ nm}$ to monitor protein elution, analysed by electrophoresis in denaturing and reducing conditions. Fractions containing Hpt were pooled and further processed by anion exchange chromatography with a column of DEAE–Sepharose (1.5 × 12.5 cm) previously equilibrated with 10 mM NaCl in 20 mM Tris/HCl at pH 7.4. The chromatography was performed at a flow rate of 12 mL·h^{-1} and room temperature. The column was washed with 50 mM NaCl in 20 mM

Tris/HCl at pH 7.4 until $A_{280\ nm}$ was not detected in the effluent volume. Then, Hpt was eluted with a linear gradient of NaCl, from 100 to 250 mM, in 20 mM Tris/HCl (pH 7.4). Fractions of 1.2 mL were collected and analysed, as above, to select the Hpt-containing volume for further purification by affinity chromatography. Anti-Hpt IgG was coupled with CNBr-activated Sepharose, according to the manufacturer's instructions, and the resulting affinity resin was used to pack a column (1 × 5 cm) in P-buffer. The column was loaded with the protein solution at 0.2 mL·min^{-1}, and then washed at a flow rate of 1 mL·min^{-1} with P-buffer. Hpt was eluted with 20 mL of 0.1 M glycine-HCl at pH 3.0. Fractions of 1 mL were collected into tubes containing 10 μL of 1 M Tris, and analysed by electrophoresis as above. Hpt obtained by this procedure was over 98% pure, as assessed by SDS/PAGE and densitometric analysis of Coomassie-stained bands. Fractions containing purified Hpt were pooled, concentrated, and dialysed against NaCl/P_i (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4), using an Amicon ultracentrifugal filter device with 50 000 M_r cut-off.

The molarity of isolated Hpt was determined by measuring the protein concentration as mg·mL^{-1}, and calculating the M_r of the monomer αβ as previously described [39]. Therefore, Hpt molarity refers to monomer molarity.

Binding of ApoE or HSA to Hpt coupled with Sepharose

Approximately 10 mg of Hpt, purified with Hb–Sepharose with elution at pH 2.8, were coupled with Hi-Trap NHS-activated Sepharose (1 mL prepacked column), according to the manufacturer's instructions. The column was equilibrated with 10 volumes of P-buffer, and then loaded with 0.5 mL of 0.6 mg·mL^{-1} ApoE at a flow rate of 0.2 mL·min^{-1}. Extensive washing with P-buffer (1 mL·min^{-1} flow rate) was performed to remove unbound material. When protein was no longer detected by $A_{280\ nm}$ in the effluent volume, ApoE was eluted with 0.1 M glycine-HCl at pH 2.8 (0.8 mL·min^{-1} flow rate), and fractions of 0.5 mL were collected into tubes containing 10 μL of 1 M Tris. ApoE-containing fractions were detected by measuring $A_{280\ nm}$, and were analysed by electrophoresis and western blotting. In a control experiment, NHS-activated Sepharose was coupled with ethanolamine, and used to process ApoE.

In order to evaluate the ability of Hpt to bind HSA, purified Hpt was coupled with Hi-Trap NHS-activated Sepharose, as described above. The column was equilibrated with P-buffer, and then loaded with 1.5 mL of 0.9 mg·mL^{-1} HSA at a flow rate of 0.2 mL·min^{-1}. After washing, to remove unbound material, the elution was carried out with 0.1 M glycine-HCl at pH 2.8 (0.8 mL·min^{-1} flow rate). Fractions of 0.5 mL were collected into tubes containing 10 μL of 1 M Tris, and analysed by electrophoresis.

Electrophoresis and immunoblotting

Electrophoresis in denaturing and reducing conditions was carried out on 15% polyacrylamide gel, as previously reported [7]. Samples containing 3–5 μg of protein were analysed. Protein staining with Coomassie R-250, or western blotting onto PVDF membranes, was performed as previously described [25,39]. After protein blotting, the membrane was rinsed in NaCl/Tris (130 mM NaCl, 20 mM Tris/HCl, pH 7.4) containing 0.05% (v/v) Tween-20 (T-NaCl/Tris), and treated with 5% nonfat milk for 1 h at 37 °C. ApoE, after blotting from the gel or following incubation (10 μg·mL^{-1} in NaCl/Tris; 1 h at 37 °C) with blotted Hpt, was detected as follows. The membrane was incubated at 37 °C with the primary antibody for 1 h, and then with the secondary antibody for 1 h. Goat anti-ApoE IgG followed by rabbit anti-goat HRP-conjugated IgG was used for detection of blotted ApoE, and mouse anti-ApoE IgG followed by goat anti-mouse HRP-conjugated IgG for detection of ApoE bound to blotted Hpt. Each antibody was diluted 1 : 1000 in NaCl/Tris containing 5% nonfat milk. The immune complexes were detected with the ECL detection system, using luminol as substrate, according to the manufacturer's protocol. As a control, blotted Hpt was incubated, omitting treatment with ApoE, with mouse anti-ApoE IgG followed by goat anti-mouse HRP-conjugated IgG.

In experiments on Hpt binding to VLDL and LDL proteins, the lipoproteins were purified from a pool of human plasma samples ($N = 5$) by sequential flotation ultracentrifugation [26], and processed by electrophoresis on 10% polyacrylamide gel in denaturing and reducing conditions. Proteins were stained with Coomassie, or blotted onto PVDF membrane as above. After protein blotting, the membrane was rinsed in NaCl/Tris containing 0.4% Tween-20, and then treated with NaCl/Tris containing 5% BSA for 1 h at 37 °C. The membrane was incubated (2 h, 37 °C) with biotinylated Hpt (0.1 mg·mL^{-1} in NaCl/Tris containing 1% BSA) and, after extensive washing with T-NaCl/Tris, treated with HRP-conjugated avidin (diluted 1 : 10 000 in NaCl/Tris containing 1% BSA) for 1 h at 37 °C. Isolated Hpt was biotinylated by using *N*-hydroxy-succinimidobiotin, according to the manufacturer's protocol. The ECL system was used for detection. Controls were performed by omitting the treatment with biotinylated Hpt. In order to check whether Hpt was associated with lipoproteins purified from human plasma, the same membrane used for staining with biotinylated Hpt was processed as follows. The stained membrane was extensively washed with NaCl/Tris containing 0.4% (v/v) Tween-20, and then rinsed in 0.2 M NaOH (10 min, room temperature) to strip biotinylated Hpt. After being washed with H_2O and 0.4%

(v/v) Tween-20 in NaCl/Tris, the membrane was incubated (1 h, 37 °C) with rabbit anti-Hpt IgG (1 : 3000 dilution in NaCl/Tris containing 1% BSA), and then with goat anti-rabbit HRP-conjugated IgG (1 : 6000 dilution in NaCl/Tris containing 1% BSA). The immunocomplexes were detected with the ECL detection system.

ELISA

ELISA was performed essentially as previously reported [6]. In experiments on ApoE or ApoA-I binding to Hpt, each well of the microtiter plate was incubated (overnight at 4 °C) with 1 mg of Hb in 100 μL of coating buffer (7 mM Na_2CO_3, 17 mM $NaHCO_3$, 1.5 mM NaN_3, pH 9.6). After four washes with T-NaCl/Tris, and four washes with high-salt NaCl/Tris (500 mM NaCl in 20 mM Tris/HCl at pH 7.4), a solution of 0.25 μM Hpt was loaded into the wells and left for 2 h at 37 °C. The extensive washing was repeated, and different amounts of ApoA-I or ApoE (0.006, 0.009, 0.02, 0.06, 0.09, 0.15 or 0.3 μM in 50 μL of NaCl/Tris) were incubated for 2 h at 37 °C. The wells were then incubated for 1 h at 37 °C with 60 μL of T-NaCl/Tris supplemented with 0.25% BSA containing rabbit anti-ApoA-I IgG (1 : 4000 dilution) or goat anti-ApoE IgG (1 : 15 000 dilution), respectively. The immunocomplexes were incubated (1 h at 37 °C) with 60 μL of goat anti-rabbit HRP-conjugated IgG (1 : 5000 dilution) or rabbit anti-goat HRP-conjugated IgG (1 : 20 000 dilution) for detection of ApoA-I or ApoE, respectively, by measuring (at 492 nm) peroxidase-catalysed colour development from *o*-phenylenediamine as previously described [7]. Nonspecific binding was determined by omitting the Hpt loading in the wells.

Inhibition of Hpt binding to Hb-coated wells, in the presence of ApoA-I or ApoE, was analysed as follows. Hpt (0.3 μM in NaCl/Tris) was preincubated with different concentrations of ApoA-I or ApoE (0, 0.3, 0.6, 1, 2 or 3 μM in NaCl/Tris) for 2 h at 37 °C. The mixtures were added to Hb-coated wells and, after 3 h of further incubation, the microtiter plate was extensively washed. Bound Hpt was detected by treatment with anti-Hpt IgG (1 : 6000 dilution in T-NaCl/Tris supplemented with 0.25% BSA; 1 h at 37 °C) followed by goat anti-rabbit HRP-conjugated IgG (diluted 1 : 12 000) as the primary antibody (1 h at 37 °C) and colour development at 492 nm, as described above. Absorbance values were converted to percentage of Hpt binding in the absence of apolipoprotein.

The competition of P2a or Hb with ApoE for binding to Hpt was evaluated as follows. The wells were coated with 50 μL of 0.004 $mg{\cdot}mL^{-1}$ ApoE, as described above. Hpt (0.3 μM in NaCl/Tris) was preincubated with different concentrations of P2a (0, 0.1, 0.7, 1.5, 5, 10 or 30 μM in NaCl/Tris) or Hb (0, 0.05, 0.1, 1.5, 3, 6 or 10 μM in NaCl/Tris). After blocking with StartingBlock, the wells were extensively washed, and loaded with aliquots from each mixture. After incubation (2 h at 37 °C), the bound Hpt was detected by treatment with anti-Hpt IgG (1 : 4000 dilution in StartingBlock supplemented with 0.05% Tween-20; 1 h at 37 °C) followed by goat anti-rabbit HRP-conjugated IgG (diluted 1 : 12 000) as the primary antibody (1 h at 37 °C), and colour development at 492 nm as above. Nonspecific binding of Hpt was determined by omitting ApoE from the coating step. Absorbance values were converted to percentage of Hpt binding in the absence of P2a or Hb, respectively.

In experiments on competition of ApoA-I with ApoE for binding to Hpt, the wells were coated with 50 μL of 0.01 $mg{\cdot}mL^{-1}$ Hpt, as described for Hb coating. After blocking with NaCl/Tris containing 1% BSA, the wells were extensively washed. Mixtures of 0.056 μM ApoA-I with different amounts of ApoE (0.0014, 0.02, 0.056, 0.112, 0.224 or 0.280 μM) in NaCl/Tris were incubated for 2 h at 37 °C. The wells were again washed, and bound ApoA-I was detected with anti-ApoA-I IgG (1 : 4000; 1 h at 37 °C) and goat anti-rabbit HRP-conjugated IgG (1 : 5000; 1 h at 37 °C), as described above. Background values (i.e. nonspecific binding of ApoA-I) were measured in wells processed without Hpt coating. These values were less than 8% of those obtained with Hpt-coated wells. Absorbance values were converted to percentage of ApoA-I binding in the absence of ApoE.

A different experiment on competition between the two apolipoproteins was carried out as follows. The wells were coated with 50 μL of 0.002 $mg{\cdot}mL^{-1}$ ApoE in NaCl/Tris, and blocked and washed as described above. Mixtures of Hpt (0.114 μM) with different amounts of ApoA-I (0.6, 1.8, 3 or 6 μM) in NaCl/Tris were first kept for 2 h at 37 °C, and then incubated in the wells (2 h, 37 °C). After washing, bound Hpt was detected with anti-Hpt IgG (1 : 3000; 1 h at 37 °C) and goat anti-rabbit HRP-conjugated IgG (1 : 3000; 1 h at 37 °C), as described above. Background values (i.e. nonspecific binding of Hpt to BSA) were measured in wells processed without ApoE coating. These values were less than 10% of those obtained with ApoE-coated wells. Absorbance values were converted to percentage of Hpt binding in the absence of ApoA-I.

Hpt concentrations in plasma, VLDL and LDL obtained from healthy subjects ($N = 5$) were measured as previously described [7], using rabbit anti-Hpt IgG (1 : 4000 dilution in T-NaCl/Tris supplemented with 0.25% BSA) followed by goat anti-rabbit HRP-conjugated IgG (diluted 1 : 12 000 as the primary antibody). Measures were obtained using a calibration curve, obtained by determining the immunoreactivity of 0.1, 0.25, 0.50, 0.75, 1.0 and 2.0 ng of standard protein.

In each experiment, controls were performed by omitting incubation with the primary antibody (i.e. rabbit anti-Hpt IgG or rabbit anti-ApoA-I IgG or goat anti-ApoE IgG). No signal was found when primary antibody was omitted from the immunodetection system.

LCAT assay

A pool of plasma samples ($N = 5$) was treated with 0.65% dextran sulfate (molecular mass of 50 kDa) in 0.2 M $CaCl_2$, and then used as a source of LCAT. The enzyme activity was measured using proteoliposomes (ApoA-I/lecithin/cholesterol molar ratio of 1.5 : 200 : 18, or ApoE/lecithin/cholesterol molar ratio of 1.5 : 200 : 18) as substrate, as previously reported [6,40,41]. The apolipoprotein concentration in the assay was 0.05 μM.

Cell culture and cholesterol internalization assay

The human hepatoblastoma-derived cells (HepG2), kindly provided by M. Russo (CNR Institute of Food Science and Technology, Avellino, Italy), were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 $U \cdot mL^{-1}$ penicillin, and 100 $\mu g \cdot mL^{-1}$ streptomycin, and grown at 37 °C in a humidified atmosphere of 5% CO_2 in air. The internalization of labelled proteoliposomes into HepG2 cells was performed as previously described [42]. Exponentially growing cells were seeded at a density of 0.15×10^6 mL^{-1} into 12-well plates. After 48 h of culture, the cells were washed twice with DMEM, and then incubated in DMEM containing 2 mM L-glutamine. After 2 h, this medium was supplemented with the proteoliposome suspension with or without 3 μM Hpt. After further incubation (3 h), the medium was removed, and the cells, after six washes with NaCl/P_i, were lysed in 300 μL of 0.1 M NaOH. The lysates were analysed for their radioactivity and protein concentration by scintillation and Bradford [43] analysis, respectively. The values of cholesterol internalized by the cells were expressed as d.p.m. per mg cell protein. In control experiments, Hb (6 μM) was added to the culture medium to displace Hpt from proteoliposomes. The effect of albumin on proteoliposome internalization was evaluated by performing the assay in the presence of 5 μM HSA. Unlabelled liposomes, prepared as above but without apolipoproteins, and antibodies against either apolipoprotein were used to evaluate specific internalization of ApoE-containing or ApoA-I-containing proteoliposomes. In detail, non-apolipoprotein-mediated uptake of cholesterol was evaluated by incubating the cells with a 100-fold excess of unlabelled liposome, in the presence of saturating concentrations of anti-ApoA-I IgG or anti-ApoE IgG. The obtained values of labelled cholesterol internalization were considered to represent nonspecific uptake by the cells (background).

Liposomes containing ApoA-I (or ApoE) and lipids were prepared by the cholate dialysis procedure described above, but with an ApoA-I/lecithin/cholesterol molar ratio of 1 : 100 : 5, or an ApoE/lecithin/cholesterol molar ratio of 1 : 100 : 2 [44]. Tritium-labelled cholesterol (specific activity of 3.2×10^6 or 16.5×10^6 $d.p.m \cdot nmol^{-1}$ in proteoliposomes containing ApoA-I or ApoE, respectively) was used. After preparation, proteoliposomes with ApoA-I were depleted of possible ApoA-I-free micelles by affinity chromatography. In detail, the liposomes were loaded on a small column (0.3 mL) of anti-ApoA-I IgG coupled with Sepharose. Liposomes lacking ApoA-I were washed off with NaCl/P_i. When radioactivity was no longer detected in the effluent buffer, the proteoliposomes with ApoA-I were recovered by elution with 0.1 M glycine-HCl at pH 3. The eluted material was extensively dialysed against diluted NaCl/P_i (1 : 10 in H_2O). The suspension was then concentrated 10-fold by centrifugation (6 h at 500 ***g***) under vacuum. The same procedure, but with goat anti-ApoE IgG linked to Sepharose, was used to remove ApoE-free micelles from ApoE-containing liposomes.

Statistical analysis

Each sample in all of the experiments was processed at least in triplicate, and the datum was expressed as mean value ± standard error of the mean (SEM). The program GRAPH PAD PRISM 3 (Graph Pad Software, San Diego, CA, USA) was used to obtain trend curves and to perform regression analysis or *t*-tests.

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