Journal of Neurochemistry JNC

JOURNAL OF NEUROCHEMISTRY | 2009 | 110 | 255–263 doi: 10.1111/j.1471-4159.2009.06121.x

Haptoglobin binds apolipoprotein E and influences cholesterol esterification in the cerebrospinal Fluid

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Abstract

Haptoglobin (Hpt) binds the apolipoprotein (Apo) A–I domain, which is involved in stimulating the enzyme lecithin-cholesterol acyltransferase (LCAT) for cholesterol esterification. This binding was shown to protect ApoA–I against hydroxyl radicals, thus preventing loss of ApoA–I function in enzyme stimulation. In this study, we report that Hpt is also able to bind ApoE. The Hpt binding site on the ApoE structure was mapped by using synthetic peptides, and found homologous to the Hpt binding site of ApoA–I. Hydroxyl radicals promoted *in vitro* the formation of ApoE-containing adducts which were detected by immunoblotting. Hpt impaired this oxidative modification whereas albumin did not. CSF from patients with multiple sclerosis or subjects without neurodegeneration contains oxidized forms of ApoE and ApoA–I similar to those observed *in vitro*. CSF was analyzed for its level of ApoA–I, ApoE, Hpt, cholesteryl esters, and unesterified cholesterol. The ratio of esterified with unesterified cholesterol, assumed to reflect the LCAT activity *ex vivo*, did not correlate with either analyzed protein, but conversely correlated with the ratio [Hpt]/([ApoE]+[ApoA–I]). The results suggest that Hpt might save the function of ApoA–I and ApoE for cholesterol esterification, a process contributing to cholesterol elimination from the brain.

Keywords: apolipoprotein A–I, Apolipoprotein E, cholesterol, haptoglobin, lecithin-cholesterol acyltransferase, oxidative stress.

J. Neurochem. (2009) **110**, 255–263.

Liver synthesizes most of circulating Hpt, and a number of other tissues have been recognized as minor sources of this protein (Kalmovarin *et al.* 1991; Smeets *et al.* 2003). Hpt is long far known to bind hemoglobin (Hb) and deliver it to liver for catabolism (Sadrzadeh and Bozorgmehr 2004), and has recently been found able to bind also the apolipoprotein (Apo) A–I (Porta *et al.* 1999). ApoA–I is the major high density lipoprotein (HDL) component and promotes cholesterol (C) efflux from cells, stimulates the enzyme lecithin-cholesterol acyltransferase (LCAT) (EC 2.1.3.45) to esterify cholesterol (CE) for reverse transport to liver, and allows HDL uptake by hepatocytes (Temel *et al.* 1997; Xu *et al.* 1997). Hpt has been proposed to target and protect the ApoA–I effector domain for LCAT against oxidative stress (Salvatore *et al.* 2007). High levels of Hpt, such as those in circulation during inflammation, impair the ApoA–I stimulation on the enzyme activity *in vitro* (Balestrieri *et al.* 2001). In the *in vitro* assay of LCAT, an ApoA–I mimetic peptide with amino acid sequence overlapping the stimulatory site for LCAT (P2a: LSPLGEEMRDRARAHVDALRTHLA) was previously found effective in displacing Hpt from ApoA–I, and able to rescue the stimulatory function of ApoA–I in the presence of high Hpt levels (Spagnuolo *et al.* 2005). Since ApoE contains sequences which are homologous to ApoA–I sequences including that of P2a, and displays activities similar to those of ApoA–I in reverse cholesterol transport (Greenow *et al.* 2005), it seems conceivable that Hpt might affect some roles of ApoE. This apolipoprotein, in free or lipidated form, also plays in the CSF roles which seem important for β-amyloid aggregation or solubilization, two processes involved in neurodegeneration or β-amyloid delivery to circulation respectively (Chan *et al.* 1996; Sadowski *et al.* 2004). HDL-like particles,

Received April 3, 2009; accepted April 20, 2009.

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Abbreviations used: ApoA–I, apolipoprotein A–I; ApoE, apolipoprotein E; BSA, bovine serum albumin; C, unesterified cholesterol; CE, cholesteryl ester; CNBr, cyanogen bromide; GAR-HRP, goat anti-rabbit horseradish-linked peroxidase; Hb, hemoglobin; HDL, high density lipoprotein; Hpt, haptoglobin; HSA, human serum albumin; LCAT, lecithin-cholesterol acyltransferase; MS, multiple sclerosis; PVDF, polyvinylidene fluoride; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis.

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containing ApoE and cholesterol, are present in CSF (Demeester *et al.* 2000) and might vehicle β-amyloid to neurons or across the brain–blood barrier (Donahue and Johanson 2008). Transport of these lipoproteins (and their cholesterol and β-amyloid cargo) in the CSF, and their transcytosis from CSF to blood actually occurs by ApoE-receptors of brain cells and capillary endothelial cells respectively (Rebeck *et al.* 2006). Therefore, brain C, although partly converted into 24S-hydroxyC for free diffusion to blood, can also leave CSF as associated to lipoproteins, and CE are expected to be produced by LCAT and embedded into these lipoproteins for export to circulation and liver-mediated elimination (Vega and Weiner 2007). Both pathway of C elimination are important because C excess in lipid rafts of cell plasma membrane can contribute to β-amyloid formation and its release in CSF (Vance *et al.* 2005). In this frame, LCAT activity and Hpt availability in CSF should be key determinants in C homeostasis in the brain. Hpt in CSF is considered as originated from circulation, and therefore as a marker of brain–blood barrier dysfunction associated to inflammatory conditions (Chamoun *et al.* 2001). However, Hpt has been recently demonstrated to be produced by astrocytes (Lee *et al.* 2002), and its mRNA has been detected in glioblastoma cells (Sanchez *et al.* 2001). Therefore, during oxidative stress triggering neurodegeneration and inflammation, Hpt is expected to work into the CNS. Deregulated lipid metabolism may be of particular importance for CNS injuries and disorders, including multiple sclerosis (MS) (Adibhatla and Hatcher 2008). MS is an inflammatory condition in which the immune system attacks the myelin sheath of nerve cell fibers in the brain and spinal cord. Demyelination is associated with lipid loss and deregulation of C homeostasis in CNS. Therapeutic limitation of C synthesis was actually found reducing the inflammatory components of MS that lead to neurological disability (Vollmer *et al.* 2004). This finding suggests that unbalance between C synthesis and removal from the brain occurs in this neurodegenerative disease. Decreased CSF levels of ApoE and LCAT in MS (Albers *et al.* 1992; Gaillard *et al.* 1998) might contribute to chronic progressive demyelination. On the other hand, Hpt might prevent oxidative injuries to ApoE and ApoA–I, thus saving the stimulatory function of these apolipoproteins on C esterification and removal from the CNS. This work aimed to investigate whether Hpt is able to bind ApoE, and might influence the stimulation of LCAT by ApoA–I and ApoE in CSF of patients with MS.

Experimental procedures

Materials

Chemicals of the highest purity, bovine serum albumin (BSA), cholesterol, egg yolk lecithin, human Hpt (mixed phenotypes: Hpt 1-1, Hpt 1-2, Hpt 2-2), rabbit anti-human Hpt IgG, goat anti-rabbit horseradish peroxidase-conjugated (GAR-HRP) IgG, *o*-phenylenediamine, 4-chloro-1-naphtol, avidin–HRP, cyanogen bromide (CNBr), thrombin, and molecular weight markers were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human ApoA–I and rabbit anti-human ApoA–I IgG were from Calbiochem (La Jolla, CA, USA). Human ApoE (phenotype 3) and goat anti-human ApoE IgG were from Vinci Biochem (Vinci, Italy). [1α,2α-^{3}H] cholesterol (45 Ci/mmol) was obtained from Perkin-Elmer (Boston, MA, USA). Polyvinylidene fluoride (PVDF) transfer membrane (Millipore, Bedford, MA, USA) were used. Organic solvents from Romil (Cambridge, UK), and polystyrene 96-wells plates from Nunc (Roskilde, Denmark) were used.

Peptides, designed to partially overlap the ApoE sequence from D65 to R191 (see Results),were synthesized by INBIOS (Naples, Italy), using standard Fmoc chemistry, with biotinylated N-termini and amidated C-termini. Purity of the peptides was over 98%, as evaluated by HPLC and mass spectrometry analysis by the provider.

CSF from patients with multiple sclerosis (MS), matched for disease duration and disability status (sensory disturbances, reduced coordination, visual impairment), and diagnosed to be in relapsing phase, was provided by P. Bongioanni (Pisa, Italy). Disease diagnosis was based on detection of oligoclonal immunoglobulin in CSF. CSF of subjects without demyelinating disease or any sign of neurodegeneration was used as control. All the patients and controls gave informed consent for use of their CSF samples.

Methods

Fragmentation of ApoE, and immunoblotting of the resulting peptides

A thrombin solution, containing 0.1% (w/v) albumin, was prepared according to the provider instructions. Cleavage of ApoE with CNBr or thrombin was performed, as previously described (Innerarity *et al.* 1983; Gunzburg *et al.* 2007).

The electrophoresis of peptides from ApoE cleavage by CNBr or thrombin was performed in denaturing and reducing conditions essentially as previously described (Schagger and Von Jagow 1987), on gels with 18% or 15% polyacrylamide respectively. Hpt (β chain = 40 kDa, α_2 chain = 21 kDa) ApoE (34 kDa), and Hb (16 kDa) were used as molecular weight markers. After electrophoresis, 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 (Porta *et al.* 1999). 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 T-TBS, that is a solution of TBS (20 mM Tris–HCl, 150 mM NaCl, pH 7.4) containing 0.05% Tween-20. 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 by incubation with 0.02% H_2O_2 and 4 mM 4-chloro-1-naphtol.

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ELISA

ELISA was performed essentially as previously reported (Spagnuolo *et al.* 2003). In particular, in experiments of peptide binding to Hpt, microtiter plate wells were coated by overnight incubation with 1 μg of Hpt in 50 μL of coating buffer (7 mM Na_2CO_3, 17 mM $NaHCO_3$, 1.5 mM NaN_3, pH 9.6) at 10°C. Aliquots of 55 μL of biotinylated peptide (1, 3, 10, 30 μM) were incubated into the wells (1 h at 37°C). Bound peptides were detected following incubation with 60 μL of avidin–HRP (1 : 10 000 dilution in T-TBS supplemented with 0.25% BSA). Color development was produced and measured at 492 nm as previously described (Porta *et al.* 1999).

In experiments of competition of peptide with ApoE for Hpt binding, the wells were coated with ApoE. Mixtures of Hpt (0.4 μM) with peptide (0, 1, 5, 10, or 20 μM) in lipidated form were prepared in CB-TBS buffer (5 mM $CaCl_2$, 0.2% BSA, 130 mM NaCl, 20 mM Tris–HCl, pH 7.3), then kept for 2 h at 37°C and, finally, incubated in the wells (2 h, 37°C). Lipidated peptides were prepared by the same procedure above described for ApoE-containing liposomes but labeled cholesterol was omitted. Rabbit anti-Hpt IgG (60 μL of 1 : 1500 dilution in T-TBS supplemented with 0.25% BSA) was used as primary antibody for detecting ApoE-bound Hpt. After this treatment, the immunocomplexes were incubated (1 h at 37°C) with 65 μL of GAR-HRP IgG (diluted, as the primary antibody, 1 : 3000) for color development.

In experiments of titration of Hpt, ApoA–I, and ApoE, 50 μL aliquots of CSF (1 : 50, 1 : 150, 1 : 450, and 1 : 900 dilutions in coating buffer) were incubated into the wells overnight at 10°C. Rabbit IgG (anti-Hpt, anti- ApoA–I, or anti-ApoE) was used as primary antibody, and GAR-HRP as secondary antibody for color development. Calibration curves with different amounts of purified antigens were used for determining the concentration of each antigen.

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) (Langlois and Delanghe 1996). The protein mixture was analyzed, and MW = 53.5 kDa was assigned to the unit αβ. In particular, the Hpt subunits were fractionated by electrophoresis on 15% polyacrylamide gel in denaturing and reducing conditions, and the intensities of the Coomassie-stained bands were measured by densitometry (Cigliano *et al.* 2003). Therefore, the ratio of α_1 (present in phenotypes Hpt 1-1 and Hpt 1-2) with 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.

Oxidation of ApoA–I and ApoE

ApoE and ApoA–I were oxidized essentially according to a published procedure (Salvatore *et al.* 2007). Briefly, each apolipoprotein was incubated with a system of production of hydroxyl radicals, based on Fenton chemistry. Aliquots of standard lapidated ApoA–I or ApoE (4 μM in the reaction mixture) were incubated, in the absence or presence of 8 μM Hpt, with 4 μM Hb, 100 μM ascorbate, and 100 μM hydrogen peroxide at 37°C for 10 min. Apolipoproteins oxidized in the presence of human serum albumin (HSA) were used as controls. After treatment, samples of 40 μL were analyzed by electrophoresis and western blotting. Electrophoresis of ApoA–I or ApoE was performed on 15 or 12.5% polyacrylamide gel, respectively, in denaturing and reducing conditions. The protein bands were blotted, under electric field, onto PVDF membrane, which was then incubated with rabbit anti-ApoA–I or anti-ApoE IgG respectively. The immunocomplexes were detected by GAR-HRP IgG, using hydrogen peroxide and 4-chloro-1-naphtol for color development. Aliquots of CSF samples were processed by electrophoresis and Western blot as above for detecting apolipoprotein oxidation products.

LCAT assay *ex vivo*

The LCAT activity in CSF is assumed to be represented by the molar ratio of CE with C, as only this enzyme is known to promote cholesterol esterification in extracellular fluids. The activity was measured in HDL-like lipoproteins from CSF. In brief, 200 μL aliquots were used for measuring the molar amounts of C and CE in HDL or CSF respectively. An aliquot was incubated (1 h, 75°C) with 0.25 mL of ethanol and water (1 : 1 v/v), while the other one with 2 mL of ethanol and water (1 : 1 v/v) containing 5 M KOH. After incubation, both mixtures were supplemented with NaCl up to 0.35% final concentration and, after addition of 2 mL of ice-cold hexane, vigorously shaken for 2 min. The hexane extract was taken on and the lower phase was likewise extracted two more times. The three extracts were pooled and dried under nitrogen stream. The residue was dissolved in 0.2 mL of acetonitrile : isopropanol (57 : 43, v : v), and 20 μL were processed by reverse-phase HPLC. The chromatography was performed by a RP-C18 column at 40°C, with 1 mL/min flow rate, according to a published procedure (Cigliano *et al.* 2001). The molar amounts of C and CE were measured in samples processed without and with KOH, respectively, and used to calculate the amount of CE as 'total cholesterol minus C'. Calibration curves, obtained by injecting different amounts (n = 12) of standard cholesterol, were used for quantitative analysis.

Procedure used to convert ApoE level into ApoA–I equivalents

In correlation of CE/C with [Hpt]/([ApoA–I] + [ApoE]), ApoE was expressed in terms of 'ApoA–I molar equivalents', which were determined as follows. The *in vitro* assay of LCAT activity was performed to determine the amount of ApoE required to obtain the same stimulation of a given amount of ApoA–I. The enzyme activity was measured, using CSF as LCAT source, and a proteoliposome containing ApoA–I or ApoE (apolipoprotein : lecithin : ^{3}H-cholesterol = 1.5 : 200 : 18, molar contribution) as substrate, according to a published procedure (Spagnuolo *et al.* 2005). This activity was expressed as enzyme units (1 unit = nmoles of cholesterol esterified per h per mL of CSF). Enzyme stimulation was assayed by using apolipoprotein concentrations from 0.02 to 0.4 μM (n = 5, each in triplicate). Linear functions were obtained ($r^2 > 0.998$ for ApoA–I, ApoE2, ApoE3, and ApoE4), and the enzyme activity did not essentially change with the ApoE phenotype (e.g. 1.09, 1.15 or 1.05 units for 0.4 μM ApoE2, ApoE3 or ApoE4 respectively). The data indicated that lipidated ApoA–I stimulates LCAT 1.923 more than lipidated ApoE3, in the concentration range used. Therefore, the molar amounts of ApoE in the analyzed CSFs were divided per 1.923, and the resulting values were expressed as 'ApoA–I molar equivalents', and assumed to be independent on the ApoE phenotype.

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Statistical analysis

Each sample was processed at least in triplicate, and the datum was expressed as mean value ± SD. The program 'Graph Pad Prism 3' (Graph Pad Software, San Diego, CA, USA) was used to perform t-test analysis.

Results

Binding of Hpt to specific amino acid sequences of ApoE

ApoE fragmentation by CNBr results in a number of peptides. These peptides were separated by electrophoresis, in denaturing and reducing conditions, according to their sizes. After blotting onto PVDF membrane, and incubation with Hpt, complexes of peptides with Hpt were detected by anti-Hpt antibodies (Fig. 1). These peptides were analyzed by mass spectrometry, and sequences of residues 65–272 and 68–272 were identified. Thrombolytic cleavage of ApoE produced two primary fragments (residues 1–191 and 216–299), which were separated and analyzed by Hpt binding as the CNBr-derived fragments. The larger thrombolytic fragment was mostly detected by anti-Hpt antibodies (Fig. 2), accompanied by traces of undigested ApoE. Albumin was detected by both Coomassie-staining and immunoblotting as expected. This protein was actually present in the thrombin solution used, and might slightly bind Hpt (Eckersall and Conner 1990). On the basis of these data, it seemed conceivable that the Hpt binding region on ApoE sequence was harbored in the sequence from D65 to R191. Nine distinct peptides with overlapping sequences, spanning this ApoE sequence, were synthesized (Fig. 3) and conjugated to biotin. The biotinylated peptides (named PE1 to PE9) were scored for their activity of binding to Hpt linked on stationary phase, in microtiter plates for ELISA, using avidin-linked

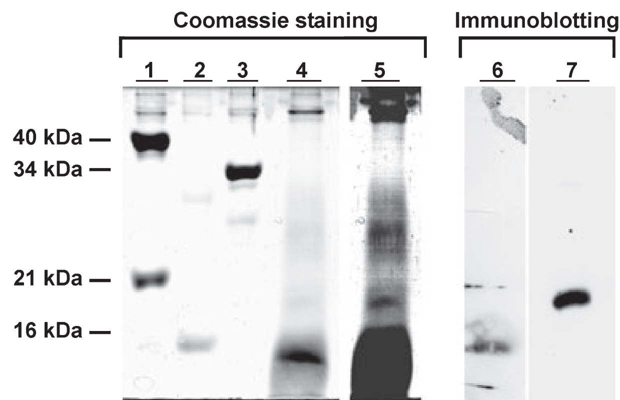

Fig. 1 SDS-18% PAGE and Immunoblotting of ApoE CNBr fragments. Samples of 100 μg in 40 μL were loaded on the gel. The proteins were fractionated by SDS-PAGE and stained by Coomassie R250 (lanes 1–5), or blotted onto PVDF membrane for immunostaining with rabbit anti-Hpt IgG and HRP-GAR (lanes 6 and 7). Lane 1: Hpt; lane 2: Hb; lane 3: ApoE; lane 4: ApoE digested by CNBr; lane 5: ApoE digested by CNBr (higher contrast); lane 6: Hb, following membrane incubation with Hpt and immunoblotting; lane 7: Hpt bound to ApoE fragments, following membrane incubation with Hpt and immunoblotting.

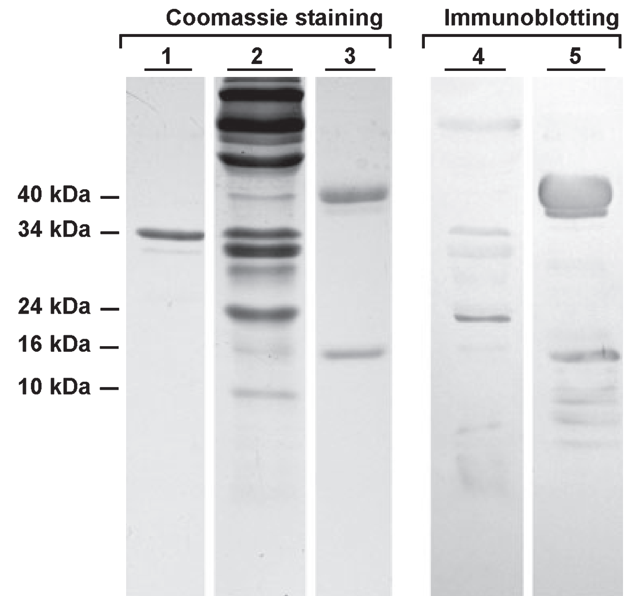

Fig. 2 SDS-15% PAGE and immunoblotting of ApoE thrombolytic fragments. Samples of 100 μg in 40 μL were loaded on the gel. The proteins were fractionated by SDS-PAGE and stained by Coomassie R250 (lanes 1–3), or blotted onto PVDF membrane for incubation with Hpt followed by immunostaining with rabbit anti-Hpt IgG and HRP-GAR (lanes 4 and 5). Lane 1: ApoE; lane 2: ApoE digested by thrombin; lane 3: Hpt; lane 4: ApoE digestion fragments, following membrane incubation with Hpt and immunoblotting; lane 5: Hpt, immunoblotting.

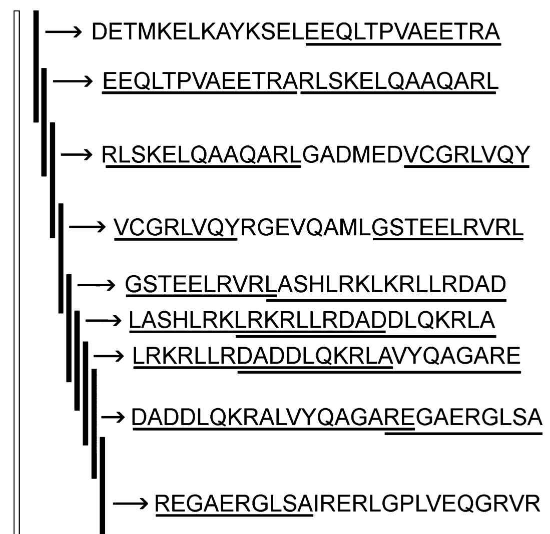

Fig. 3 Peptides synthesized from the ApoE amino acid sequence. The amino acid sequence of ApoE from residue D65–R191 is represented by a vertical bar on the left. Nine distinct peptides with overlapping sequences (underlined), spanning this ApoE sequence, were synthesized.

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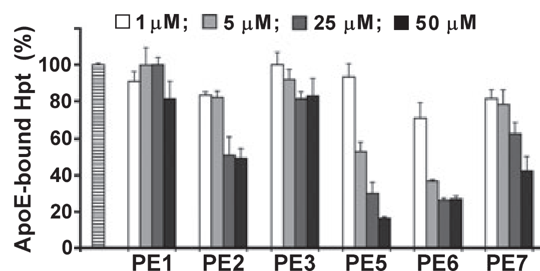

Fig. 4 Competition of ApoE mimetic peptides with ApoE for binding Hpt. ApoE mimetic peptides (PE1, PE2, PE3, PE4, PE5, PE6, PE7, PE8, PE9) were lipidated and analyzed for their ability to interfere with Hpt binding to native ApoE. Aliquots of 0.4 μM Hpt (50 μL), previously incubated with different amounts of peptidosome (0, 1, 5, 10, or 20 μM peptide), were incubated into ApoE-coated wells. Rabbit anti-Hpt IgG, and then GAR-HRP IgG with H_2O_2 and o-phenylendiamine were used to detect ApoE-bound Hpt. The amount of immunocomplexes was determined by measuring the absorbance at 492 nm. Hpt binding was not detected in the presence of 1 μM ApoA–I. The data are reported as percent of the value obtained by incubation of Hpt alone (bar with horizontal lines, on the left). Each sample was processed in triplicate, and bars indicate SD values. Mean and SD values of competitions with PE4, PE8, and PE9 were very similar to those obtained with PE1, and are not reported in the figure.

HRP for detection. The results obtained (data not shown) suggested that the sequences ^{88}ETRARLSKELQAAQARL^{104}, present in part of PE2 and PE3, and ^{131}EELRVRLASHLRK LRKLRLL^{150}, present in part of PE5 and PE6, might harbor distinct binding sites for Hpt. Both sequences, known as repetitive regions in ApoE helices 3 and 4 respectively, display significant structure homology with the ApoA–I mimetic peptide P2a (LSPLGEEMRDRARAHVDALRTH-LA), which overlaps the apolipoprotein stimulatory site for LCAT and binds Hpt (Spagnuolo *et al.* 2005). A second approach for identifying Hpt-binding peptides was carried out by ELISA. Each peptide was lipidated, and the peptidosomes were separately analyzed for their ability to compete with native ApoE for Hpt binding. Lipidated PE5 and PE6 (and, at minor extent, lipidated PE2 and PE7) bound and displaced Hpt with dose-dependent effect (Fig. 4). These results indicate that Hpt can interact with ApoE, and suggest that the apolipoprotein helix 4 is mostly involved in the binding.

Effect of Hpt binding to ApoE exposed to hydroxyl radicals

It was recently demonstrated that Hpt protects ApoA–I structure against hydroxyl radicals, thus saving the apolipoprotein function in LCAT stimulation (Salvatore *et al.* 2007). To investigate whether Hpt might also protect the structure of ApoE and save the stimulatory function of this apolipoprotein during oxidative conditions, proteoliposomes containing 4 μM ApoE were incubated with a source of hydroxyl radicals, in the presence or absence of 8 μM Hpt, and then analyzed for protein structure alteration. ApoE structure was altered by oxidation, as demonstrated by formation of antigens with electrophoretic mobility higher than that of native protein, but Hpt was able to limit or prevent such oxidative modifications as shown in Fig. 5

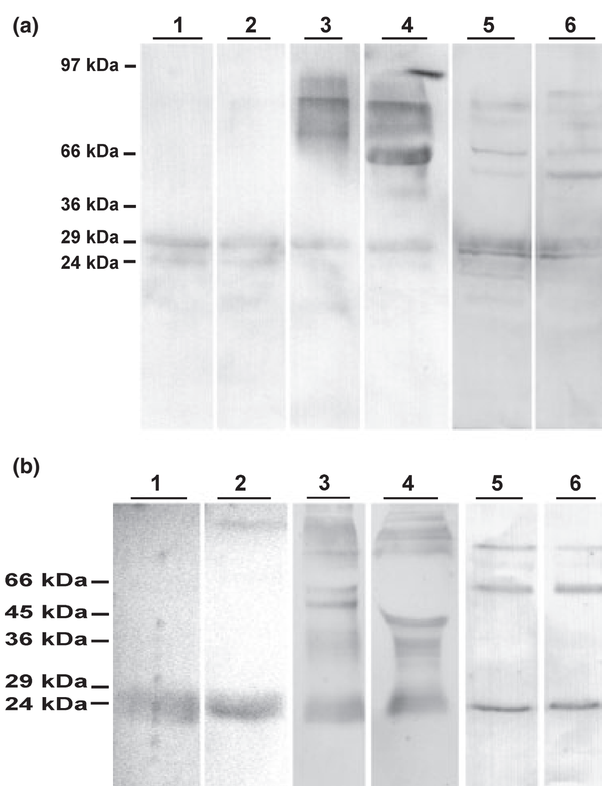

Fig. 5 Immunoblotting of lipidated ApoE and ApoA–I treated by oxidative stress. Panel (a) ApoE on liposomes or CSF lipoproteins. The liposomes were oxidized in the presence or absence of Hpt. Aliquots of 40 μl of liposome or CSF samples, containing 4 μM ApoE, were loaded on 12.5% polyacrylamide gel. After electrophoresis in reducing and denaturing conditions, the proteins were blotted onto PVDF membrane for immunostaining with rabbit anti-ApoE IgG and GAR-HRP. Lane 1: mixture of liposomes incubated only with 4 μM Hb; lane 2: liposomes oxidized by the Hb/H_2O_2/ascorbate system (4/100/100 μM respectively) in the presence of 8 μM Hpt; lane 3: liposomes oxidized by the Hb/H_2O_2/ascorbate system in the absence of Hpt; lane 4: liposomes oxidized by the Hb/H_2O_2/ascorbate system in the presence of 8 μM HSA; lane 5: CSF from controls; lane 6: CSF from MS patients. Panel (b) ApoA–I on plasma HDL or CSF lipoproteins. The HDL were oxidized in the presence or absence of Hpt. Aliquots of 40 μl of HDL or CSF samples, containing 4 μM ApoA–I, were loaded on 15% polyacrylamide gel. After electrophoresis in reducing and denaturing conditions, the proteins were blotted onto PVDF membrane for immunostaining with rabbit anti-Apo A-I IgG and GAR-HRP. Lane 1: Mixture of HDL with only 4 μM Hb; lane 2: HDL oxidized by the Hb/H_2O_2/ascorbate system (4/100/100 μM respectively) in the presence of 8 μM Hpt; lane 3: HDL oxidized by the Hb/H_2O_2/ ascorbate system in the absence of Hpt; lane 4: HDL oxidized by the Hb/H_2O_2/ascorbate system in the presence of 8 μM HSA; lane 5: CSF from controls; lane 6: CSF from MS patients.

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(panel a, lanes 1–4). In the same figure, the protective effect of Hpt on proteoliposomes with ApoA–I, exposed to hydroxyl radicals, is shown (panel b, lanes 1–4) as it has previously been reported (Salvatore *et al.* 2007). Adducts of ApoE or ApoA–I with other protein sequences were also detected in samples of CSF, following electrophoresis and immunoblotting, from MS patients or controls (lanes 5 and 6 in both panels of Fig. 5). The finding of CSF antigens containing ApoE or ApoA–I epitopes, displaying similar electrophoretic mobility of the molecules originated by hydroxyl attack to ApoE and ApoA–I, suggests that such an attack might occur also in CSF. Significant differences in molecular mass, or amount (relevant to that of apparently unmodified native apolipoproteins) of these antigens, among CSF samples analyzed, were not found. Furthermore, the data suggest that the structure of either apolipoprotein, in CSF, is not altered in MS patients more than in controls.

These data, taken together, provide further evidence on the binding of Hpt to ApoE, and suggest that the Hpt protection of ApoE and ApoA–I from oxidative injuries depends on the concentrations of the interacting proteins, according to the mass action law.

LCAT activity in CSF of patients with multiple sclerosis

LCAT activity in CSF might be altered during neurodegeneration. In fact, neurodegeneration is associated to oxidative stress, which might affect the function of the effectors stimulating the enzyme. Structure and function of ApoA–I were previously demonstrated to be protected by Hpt against oxidative stress (Salvatore *et al.* 2007). ApoE is here reported to be protected by Hpt as well. Therefore, Hpt was expected to importantly contribute to regulate LCAT in CSF, under oxidative conditions. Thus, we investigated whether and how the LCAT activity might be correlated with the levels of the enzyme effectors (i.e. Hpt, ApoA–I, and ApoE). These levels and the CE/C ratio, which was assumed to reflect the enzyme activity, were determined in CSF samples from MS patients (n = 9, in the remitting-relapsing phase) or controls (n = 5) (Table 1). The two groups were analyzed both separately and as a single population for possible correlations between a given parameter and each other. No correlation was found between the level of ApoE and that of ApoA–I (as it does occur in the plasma during acute inflammation) or Hpt or CE/C. No correlation was found between the level of ApoA–I and that of Hpt or CE/C. No correlation was found between CE/C and the level of Hpt, or the molar ratios Hpt/ApoE, Hpt/ApoA–I, ApoA–I/ApoE, or the sum of the level of ApoE with that of ApoA–I. These data demonstrate that the enzyme is not regulated just by the levels of its stimulators. Since Hpt might influence ApoA–I and ApoE stimulatory functions, the enzyme regulation should result from the ratio of the Hpt level with the sum of the levels of ApoA–I and ApoE (i.e. [Hpt]/([ApoA–I]+[ApoE]). This ratio was calculated for the all samples, and the values obtained were matched with those of the ratio CE/C. As shown in Fig. 6, a positive correlation was found (r = 0.70114, p < 0.01). The data indicate that, in all the CSFs analyzed, the Hpt level was under the value associated to LCAT inhibition *in vitro* (Salvatore *et al.* 2007). In these conditions, Hpt might play the role of protecting both ApoA–I and ApoE against oxidative stress, and higher protection seems to be associated to higher ratio of Hpt with the positive effectors.

Table 1 Titration of Hpt, ApoA–I, ApoE and CE/C in CSF. Samples of CSF from MS patients (1 to 9) and controls (10 to 14) were analyzed. ELISA was used to titrate Hpt, ApoA–I and ApoE. The molar level of C was measured, in hexane extracts from samples, before and after saponification. CE was determined as difference between these values, and the CE/C ratio was calculated. Each sample was analyzed in triplicate, and SD was calculated

Sample	Hpt (μM)	ApoA–I (μM)	ApoE (μM)	CE/C
1	0.055 ± 0.002	0.100 ± 0.017	0.057 ± 0.009	1.16 ± 0.07
2	0.031 ± 0.004	0.192 ± 0.008	0.065 ± 0.000	0.91 ± 0.07
3	0.052 ± 0.004	0.228 ± 0.014	0.054 ± 0.001	0.96 ± 0.08
4	0.118 ± 0.000	0.339 ± 0.000	0.041 ± 0.007	0.72 ± 0.09
5	0.033 ± 0.001	0.181 ± 0.010	0.054 ± 0.004	0.66 ± 0.08
6	0.029 ± 0.002	0.097 ± 0.003	0.060 ± 0.005	0.41 ± 0.07
7	0.019 ± 0.002	0.049 ± 0.009	0.041 ± 0.000	0.45 ± 0.08
8	0.038 ± 0.004	0.182 ± 0.000	0.032 ± 0.004	1.00 ± 0.08
9	0.196 ± 0.011	0.307 ± 0.012	0.032 ± 0.005	1.50 ± 0.11
10	0.046 ± 0.006	0.034 ± 0.004	0.031 ± 0.000	1.50 ± 0.09
11	0.062 ± 0.013	0.093 ± 0.015	0.036 ± 0.006	0.82 ± 0.07
12	0.034 ± 0.001	0.050 ± 0.001	0.024 ± 0.000	1.56 ± 0.10
13	0.012 ± 0.000	0.033 ± 0.002	0.028 ± 0.007	1.00 ± 0.09
14	0.036 ± 0.002	0.059 ± 0.005	0.020 ± 0.003	1.27 ± 0.05

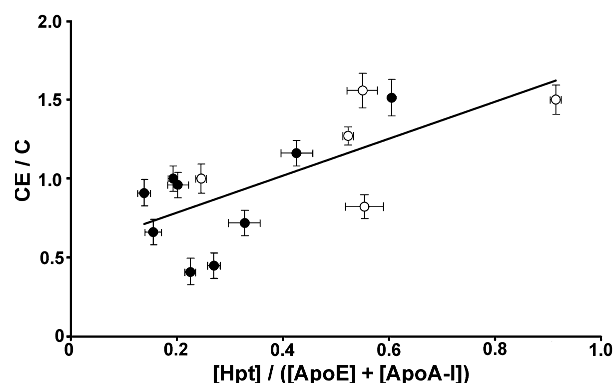

Fig. 6 Correlation between [Hpt] /([ApoA–I] +[ApoE]) and CE/C. CSF samples from MS (n = 9, full circles) or control (n = 5, open circles) were used to measure the molar concentration of Hpt, ApoE, ApoA–I, C and CE. The ratios of [Hpt] with ([ApoA–I]+[ApoE]), and [CE] with [C] were calculated from triplicates. [ApoE] was expressed as "[ApoA–I] equivalents" as described in Methods. Bars indicate SD values.

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Discussion

Hpt is a pleiotropic protein, and a body of information on its roles is available (Langlois and Delanghe 1996; Wassel 2000; Yerbury *et al.* 2005). A novel role was recently proposed for this protein. In particular, ApoA–I function for stimulating LCAT is altered by oxidative stress, but Hpt can save such a function as binding the apolipoprotein and protecting its structure from oxidative injuries (Salvatore *et al.* 2007). In this study, we demonstrate that Hpt binds and protects from oxidative damage also ApoE. The assay of ApoE protection against hydroxyl radicals supports the data on the ApoE binding by Hpt. We chose to use these radicals, instead of other reactive oxygen or nitrogen species, because they are very aggressive and attack more types of targets on protein structure (Halliwell and Gutteridge 1999). Furthermore, hydroxyl radicals are expected to greatly and chiefly originate from Hb which, infiltrated into CSF when the blood–brain barrier is damaged, binds just to Hpt and, therefore, might be kept close to Hpt-bound apolipoproteins. Hpt is actually able to bind both Hb and ApoA–I or ApoE, owing to its oligomeric structure with two or more binding sites depending on its structure polymorphism. Masking of apolipoprotein, rather than non-specific scavenger activity, should be the cause of protection by Hpt, as demonstrated by failure of HSA to protect the apolipoprotein structures from oxidative alteration. Oxidized forms of both apolipoproteins, very similar to those obtained *in vitro*, were observed by electrophoresis and immunoblotting of CSF proteins. This finding suggests that these oxidized forms might be produced by hydroxyl radicals *in vivo*. However, no significant difference between normal and MS CSF was found in relative amounts of either oxidized apolipoprotein. This suggests that most ApoE and ApoA–I might be sufficiently protected by Hpt against radicals in CSF.

The Hpt-bound ApoE site was mapped by using synthetic peptides, and found to be homologous to the Hpt-bound ApoA–I site belonging to the enzyme stimulating domain (Spagnuolo *et al.* 2005). Since ApoE stimulates LCAT as ApoA–I, it is conceivable that high Hpt levels might influence the function of both ApoA–I and ApoE in C esterification. Therefore, CE formation in CSF might depend not only on LCAT activity and apolipoprotein level, but also on Hpt level. The ratio CE/C was assumed to express the LCAT activity in CSF. Data are reported here on the correlation between the CE/C ratio and a parameter which highlights the possible role of Hpt in LCAT regulation in CSF, that is the ratio of the Hpt level with the levels of the enzyme stimulators. Previous data indicated that LCAT activity is correlated with the level of ApoA–I or ApoE in CSF, and no evidence on the possible correlation between enzyme activity and the measured ratio ApoE/ ApoA–I was given. These data, based on the enzyme assay *in vitro*, actually referred to the enzyme amount in CSF but did not provide information on the enzyme regulation by the two apolipoproteins *in vivo*. Such a regulation is expected to change as the ratio of ApoE/ ApoA–I changes, because these apolipoproteins differently stimulate the enzyme, and both binds Hpt which might influence their function. Just this consideration prompted us to calculate the ratio [Hpt]/ ([ApoA–I] + [ApoE]) in searching for a parameter which could represent the contribution of all these three effectors to the enzyme regulation, and might thus correlate with the *ex vivo* LCAT activity.

The finding that rats with up-regulated LCAT are resistant to neurodegeneration (Swanberg *et al.* 2006), and data on reduced LCAT activity in Alzheimer disease (AD) (Demeester *et al.* 2000) suggest that C esterification is important for normal CSF function. Interestingly, C accumulation in brain promotes the cleavage of the amyloid precursor protein into amyloidogenic components with the consequence of the acceleration of neuronal degeneration (Simons *et al.* 1998) and C-lowering agents may reduce the risk for the development of dementia (Jick *et al.* 2000; Wolozin *et al.* 2000). However, epidemiological data do not support the hypothesis that LCAT deficiency might affect onset or progression of neurodegenerative diseases. To date, LCAT is not believed to play a major role in C removal from the brain, although CE are formed (Demeester *et al.* 2000) and transported by lipoproteins into the circulation (Pitas *et al.* 1987). Under normal conditions, the mechanism of apolipoprotein-mediated CE formation might be of little quantitative importance for efflux of C from the brain, but it may play a large part in transport within the human brain (Björkhem *et al.* 1997; Björkhem 2002). C is generally believed to be mostly converted to 24S-hydroxyC in the neurons prior to be eliminated from the brain (Lütjohann *et al.* 1996). This oxysterol was shown to be a potent neurotoxin *in vitro* (Kölsch *et al.* 1999). It is therefore reasonable to hypothesize that 24S-hydroxyC might promote neural death when it accumulates *in vivo*. A possible role of LCAT in CSF might be to convert this compound into mono- or di-esters which would be embedded in lipoproteins for transport to blood and elimination. LCAT can actually form esters from oxysterols (Szedlacsek *et al.* 1995). In this frame ApoE, in addition to preventing precipitation or promoting solubilization and cell uptake of β-amyloid, might also be responsible of removal of C from cell membrane, thus preventing membrane structure conditions which promote an accelerated cleavage of amyloid precursor protein into amyloidogenic components (Simons *et al.* 1998; Puglielli *et al.* 2003) leading to the formation of amyloid plaques in susceptible brain regions with consecutive neurodegeneration (Fassbender *et al.* 2001). Thus, LCAT and its effectors might play crucial roles for cell survival and function in brain. In particular, the enzyme effectors are here proposed to be key determinants of the removal and transport efficiency of C or

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24S-hydroxyC from CSF to blood. Such an efficiency is expected to be required to impair the transformation of macrophages to lipid-laden foam cells, an inflammatory process which is a clear sign of defective reverse C transport and atherosclerosis onset in the vascular wall (Curtiss *et al.* 2006). Also in CNS such a process can occur, as inflammation is associated with C accumulation (Koudinov and Koudinova 2005; Rahman *et al.* 2005; Vance *et al.* 2005). In fact, MS is a chronic inflammatory disease of CNS, characterized by neuron demyelination, leukocyte infiltration from blood, and foam cell formation caused by lipid (including C) loading from myelin degradation. In MS, and other neurodegenerative diseases as well, markers for monitoring of disease activity and treatment are required. In conclusion, this study provides information on the regulation of an enzyme with high potential for elimination of excess C, and suggests the ratio CE/C might represent a reliable marker of the enzyme activity in CSF.

Acknowledgments

The authors thank Dr. P. Bongioanni for diagnosis of MS, and CSF samples from patients and controls. The skilful technical assistance of A. Carrizzo is acknowledged. This study was supported partly by a grant from the Università di Napoli Federico II (Ric. Dip. 10112/2008) and partly in the context of the I-TECHPLAT 1 project (Docup 2000-2006, Misura 3.4 'Iniziative multiassiali di supporto all'attività economica', Regione Piemonte).

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