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Influence of Isoforms and Carboxyl-Terminal Truncations on the Capacity of Apolipoprotein E To Associate with and Activate Phospholipid Transfer Protein

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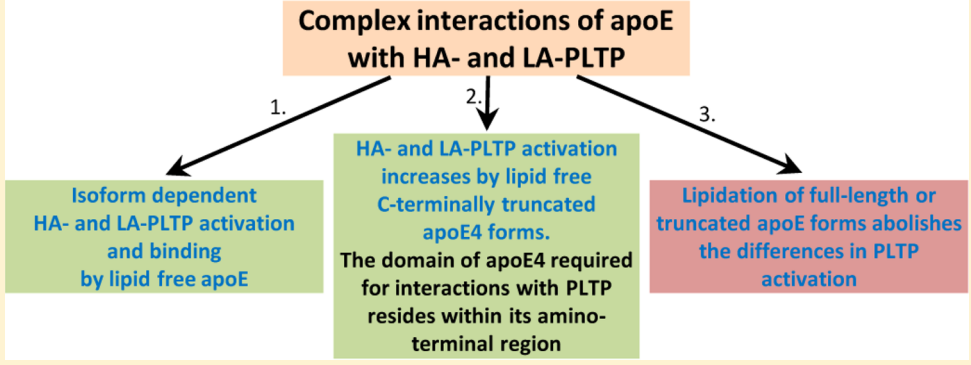
Supporting Information



ABSTRACT: Phospholipid transfer protein (PLTP), a main protein in lipid and lipoprotein metabolism, exists in high-activity (HA-PLTP) and low-activity (LA-PLTP) forms in human plasma. Proper phospholipid transfer activity of PLTP is modulated by interactions with various apolipoproteins (apo) including apoE. The domains of apoE involved in interactions with PLTP are not known. Here we analyzed the capacity of recombinant apoE isoforms and apoE4 mutants with progressive carboxyl-terminal deletions to bind to and activate HA-PLTP and LA-PLTP. Our analyses demonstrated that lipid-free apoE isoforms bind to both HA-PLTP and LA-PLTP, resulting in phospholipid transfer activation, with apoE3 inducing the highest PLTP activation. The isoform-specific differences in apoE/PLTP binding and PLTP activation were abolished following apoE lipidation. Lipid-free apoE4[Δ(260−299)], apoE4[Δ(230−299)], apoE4[Δ(203−299)], and apoE4[Δ(186−299)] activated HA-PLTP by 120−160% compared to full-length apoE4. Lipid-free apoE4[Δ(186−299)] also activated LA-PLTP by 85% compared to full-length apoE4. All lipidated truncated apoE4 forms displayed a similar effect on HA-PLTP and LA-PLTP activity as full-length apoE4. Strikingly, lipid-free or lipidated full-length apoE4 and apoE4[Δ(186−299)] demonstrated similar binding capacity to LA-PLTP and HA-PLTP. Biophysical studies showed that the carboxyl-terminal truncations of apoE4 resulted in small changes of the structural or thermodynamic properties of lipidated apoE4, that were much less pronounced compared to changes observed previously for lipid-free apoE4. Overall, our findings show an isoform-dependent binding to and activation of PLTP by lipid-free apoE. Furthermore, the domain of apoE4 required for PLTP activation resides within its amino-terminal 1−185 region. The apoE/PLTP interactions can be modulated by the conformation and lipidation state of apoE.

Phospholipid transfer protein (PLTP) is an important protein in lipid and lipoprotein metabolism. PLTP transfers phospholipids, diacylglycerol, free cholesterol, α-tocopherol, and lipopolysaccharides among lipoproteins and between lipoproteins and cells.[1,2] Plasma PLTP has been shown to be involved in high-density lipoprotein (HDL) metabolism. More specifically, PLTP remodels HDL particles in a process whereby small HDL3 particles are fused leading to the generation of large fused HDL particles and preβ-HDL.[1,2] In addition, PLTP transfers postlipolytic very low density lipoprotein (VLDL) surface phospholipids to HDL.[1,2] Functions of PLTP which may influence the formation of atherosclerotic lesions include the regulation of plasma HDL levels, generation of acceptors for lipid efflux from cells, modulation of proteins involved in cellular lipid efflux process, protection of lipoproteins from oxidation, and regulation of production of atherogenic lipoproteins.[1,2]

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PLTP expression and activity are differentially regulated in various human diseases, including cardiovascular disease, diabetes, different types of cancer, neuroinflammatory diseases, such as multiple sclerosis and neurodegenerative diseases, such as Alzheimer's disease.[2] Although the role of PLTP in lipoprotein metabolism and atherogenesis in the vascular compartment has been intensively studied using in vitro experiments and gene-targeted mouse models,[1,2] more studies are needed to further delineate the role of PLTP in human metabolism and its functions in specific tissues.

In human plasma, two distinct forms of PLTP are present, one with high activity (HA-PLTP) and the other with low activity (LA-PLTP).[3,4] The two forms exist as different macromolecular complexes in circulation. The mechanism by which LA-PLTP is generated and its functions have not been elucidated. An earlier study suggested that PLTP interactions with apolipoproteins can regulate the activity of PLTP in plasma.[5] HA- and LA-PLTP are surface-active proteins, and the LA-PLTP form was demonstrated to dock more strongly onto a phospholipid monolayer surface as compared to the HA-PLTP form,[6] indicating that the LA-PLTP form could be involved in lipid transfer-independent functions, such as signal transduction.[7,8] Analysis by size-exclusion chromatography demonstrated that the plasma HA-PLTP complex elutes in the position of HDL, while the LA-PLTP complex, that contains approximately 70% of the total PLTP protein, elutes in position corresponding to a higher mass.[3,9] Thus, the two plasma PLTP pools are associated with different types of molecular lipoprotein complexes, suggesting that the PLTP activity in circulation may be modulated by the plasma lipoprotein profile. PLTP is also found in apolipoprotein E (apoE)-containing lipoproteins in the cerebrospinal fluid (CSF).[10,11] ApoE is the main apolipoprotein in human brain, and there is a high correlation between PLTP activity and apoE in CSF, suggesting that the majority of CSF PLTP is in an active form facilitating phospholipid transfer.[11]

Previous studies suggested that the proper phospholipid transfer activity of PLTP is dependent on interactions with apolipoproteins containing amphipathic α-helical domains, such as apoA-I, apoA-II, apoA-IV, and apoE.[12] A thorough characterization of the interactions of PLTP with apoA-I, the main protein component of HDL, showed that the PLTP binding domain of apoA-I resides in its amino-terminal region.[13] The location of PLTP binding domain of apoE and of other apolipoproteins involved in interactions with PLTP is not known.

ApoE, that contains 299 residues, is an important protein of the lipoprotein transport system that has an indisputable role in atherosclerosis, dyslipidemia, and Alzheimer's disease (AD).[14−16] ApoE, that is both an HDL and VLDL component and the main protein of CSF lipoproteins, has three common isoforms (apoE2, apoE3, apoE4) in the general population, each differing in the amino acid positions 112 and 158.[17,18] Structural differences among the apoE isoforms have been proposed to affect their preferences for lipoproteins, their interaction with other proteins and lipids, and their roles in disease.[15] ApoE2 has been associated with type III hyperlipoproteinemia,[19,20] while apoE4 is a major genetic risk factor for AD.[21] Furthermore, apoE4 is associated with carotid atherosclerosis and is a significant risk factor for coronary heart disease.[22,23] ApoE is subject to proteolytic cleavage, and apoE4 is much more susceptible to proteolysis than apoE3.[24−26] Several studies have shown the presence of apoE4 carboxyl-terminal truncated proteolytic fragments in the brains of AD patients.[24,25,27,28] In addition, another study proposed that human plasma HDL3-associated apoE is cleaved in vivo, generating apoE fragments with molecular masses similar to those of the apoE fragments found in human AD brains.[29] The carboxyl-terminal truncations of apoE4 have been shown to induce alterations on the structure of apoE4[30,31] and may be responsible for the abnormal apoE4 functionality in pathogenic processes of various diseases and conditions including AD and hypertriglyceridemia.[24,25,32−34]

On the basis of the combined knowledge that (i) apoE affects PLTP activity,[12] (ii) both apoE and PLTP play important physiological and pathophysiological roles in circulation and brain,[1,2,14,15] and (iii) different truncated apoE4 forms affect distinct processes in AD pathogenesis and plasma lipid homeostasis,[24,25,32−34] our aim here was to gain insight into the interactions of apoE with PLTP. More specifically, the current study was designed to examine the capacity of recombinant apoE isoforms and apoE4 mutants with progressive carboxyl-terminal deletions between residues 186 and 299, in both lipid-free and lipidated forms, to bind and activate HA-PLTP and LA-PLTP. Our analyses showed that of the three apoE isoforms, apoE3 confers the highest PLTP activation, while the isoform-specific differences on apoE/PLTP association and PLTP activation are abolished following lipidation of apoE. Analysis of the effect of carboxyl-terminal truncated apoE4 forms on PLTP activation and binding indicated that these two processes may require different domains of apoE and that the activation domain lies within the amino-terminal domain of apoE, but its ability to function is dependent on the protein conformation. The interactions of PLTP with the apoE isoforms or truncated forms may affect PLTP functions and have an impact on the (patho)physiological roles of PLTP.

MATERIALS AND METHODS

Materials. Unesterified cholesterol and egg-yolk phosphatidylcholine were obtained from Sigma-Aldrich (St. Louis, MO, USA). [^{14}C]-DPPC (L-α-dipalmitoyl-phosphatidylcholine [dipalmitoyl-1-^{14}C]; 0.05 mCi/mL, specific activity 114 mCi/mmol) was from PerkinElmer (Boston, MA, USA) and heparin 5000 IU/mL was from Leo Pharma (Ballerup, Danmark). Cell culture media and other reagents were purchased from Sigma-Aldrich, Bio-Rad (Hercules, CA, USA), Lonza (Verviers, Belgium), Fisher Scientific (Schwerte, Germany), GE Healthcare (Uppsala, Sweden), or other standard commercial sources.

Production and Purification of ApoE Using the Adenovirus System. All plasmids and recombinant adenoviruses containing the wild-type and mutated human apoE genes were constructed as described.[35] ApoE was secreted in the culture medium of human HTB13 cells (SW 1783, human astrocytoma) grown in roller bottles following infection with adenoviruses expressing wild-type apoE2, apoE3, apoE4 or truncated apoE4 forms and was purified from the culture medium as described previously.[31]

Preparation of Discoidal Reconstituted ApoE-Phosphatidylcholine-Cholesterol Particles. Reconstituted discoidal lipoprotein particles containing apoE isoforms or truncated apoE4 forms were prepared, using a molar ratio of 100:10:1:100 of egg phosphatidylcholine (PC): cholesterol (C): apoE: sodium cholate in 50 mM sodium phosphate buffer, pH 7.4, as described previously.[36] Samples were stored at 4 °C under nitrogen to prevent the oxidation of lipids.

PLTP Purification and Activity Assay. HA-PLTP and LA-PLTP were isolated as described.[37] The isolated HA- and LA-PLTP preparations did not contain apoE based on Western blot analysis. For PLTP activation experiments, 50 ng of HA-PLTP or

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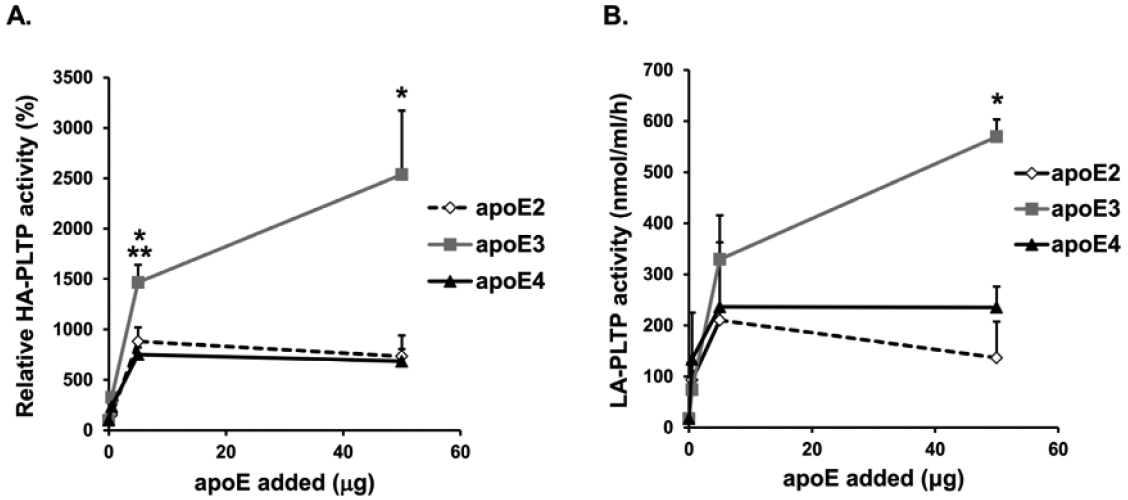

Figure 1. Effect of lipid free apoE isoforms on HA-PLTP and LA-PLTP activity. 50 ng of HA-PLTP (A) or LA-PLTP (B) was incubated in the absence or presence of increasing amounts of lipid-free apoE2, apoE3, or apoE4 for 24 h at room temperature (A) or 37 °C (B) and then assayed for phospholipid transfer activity as described in Materials and Methods. Values represent the mean ± SEM of three independent experiments performed in duplicate. (A) HA-PLTP activity after incubation with apoE isoforms is expressed as percent relative to the HA-PLTP activity in the absence of apoE set to 100%. *$p < 0.05$ for apoE3 50 μg vs apoE2 50 μg or apoE4 50 μg; *$p < 0.05$ for apoE3 5 μg vs apoE2 5 μg; **$p < 0.005$ for apoE3 5 μg vs apoE4 5 μg (B) LA-PLTP activity incubated in the absence or presence of apoE isoforms is expressed as nmol/mL/h *$p < 0.05$ for apoE3 50 μg vs apoE2 50 μg or apoE4 50 μg.

LA-PLTP, in duplicate, was incubated in the absence or presence of indicated amounts of lipid-free or lipoprotein particle-associated apoE isoforms or apoE4 carboxyl-terminal truncated forms, in a total volume of 150 μL of TNE buffer (10 mM Tris-HCl, 150 mM NaCl and 1 mM EDTA, pH 7.4), for 24 h at room temperature (HA-PLTP) or 37 °C (LA-PLTP). The incubations were performed at 37 °C for LA-PLTP due to its much lower starting activity compared to HA-PLTP. Then, the PLTP activity was measured by a radiometric method,[38] using [^{14}C]-DPPC liposomes. The assay tubes contained 80 μL of HA-PLTP/apoE or 150 μL of LA-PLTP/apoE incubation mixtures, HDL_3 (density range 1.125−1.21 g/mL) free of PLTP activity (at 250 μg as HDL_3 protein), 150 nmol of [^{14}C]-PC liposomes and TNE buffer in a final volume of 400 μL. Tubes without PLTP were included in each run. Following 45 min incubation at 37 °C, liposomes were precipitated by the addition of a stop-mix solution yielding final concentrations of 230 mM NaCl, 155 mM $MnCl_2$, 74 IU/mL heparin, and the radioactivity in the supernatant was measured. Phospholipid transfer activity was expressed as nmoles of PC transferred to HDL_3 per milliliter of sample per hour.

Solid Phase Binding Studies. *PLTP Binding to Immobilized ApoE Forms.* The interaction of HA-PLTP or LA-PLTP with apoE isoforms or apoE4−185 was evaluated using an enzyme-linked immunosorbent solid phase assay described before.[13] MaxiSorp 96-well immuno plates were coated with 5 μg/mL of lipid free or lipoprotein particle-associated apoE2, apoE3, apoE4, or carboxyl-terminal truncated apoE4 form apoE4−185, in 50 mM carbonate buffer (pH 9.6) for 4 h at room temperature and then blocked overnight with 1% BSA in PBS at room temperature. Following wash with PBS, HA-PLTP or LA-PLTP was added at amounts of 0−5800 ng or 0−500 ng, respectively, and incubated for 2.5 h at room temperature. The different amounts of HA-PLTP and LA-PLTP used reflect the different binding capacity of apoE for these two forms of PLTP. Subsequently, the plate was washed with PBS containing 0.05% Tween 20 and incubated with rabbit polyclonal anti-PLTP antibody R180[39] for 1 h at room temperature, followed by wash and incubation with horseradish peroxidase-conjugated goat antirabbit IgG (Bio-Rad) for 1 h at room temperature. Bound PLTP forms were detected spectrophotometrically at 490 nm after addition of *o*-phenylenediamine dihydrochloride as substrate for horseradish peroxidase. Noncoated wells with apoE and blocked with 1% BSA in PBS were used as control for nonspecific binding.

ApoE Isoforms Binding to Immobilized PLTP. MaxiSorp 96-well immune plates were coated with 115 ng of HA-PLTP displaying phospholipid transfer activity of 8700 nmol/h/mL, in 50 mM carbonate buffer (pH 9.6) for 4 h and then blocked overnight with 1% BSA in PBS at room temperature. Following wash with PBS, 0−1000 ng of lipid free or lipoprotein particle-associated apoE2, apoE3, or apoE4 was added, and incubation was continued for 2.5 h at room temperature. The plate was washed with PBS containing 0.05% Tween 20, and finally, horseradish peroxidase-conjugated rabbit polyclonal anti-apoE antibody R107[40] was added for 1 h at room temperature. Bound apoE was detected at 490 nm after addition of *o*-phenylenediamine dihydrochloride as substrate for horseradish peroxidase. Noncoated wells with PLTP and blocked with 1% BSA in PBS were used as control for nonspecific binding.

Circular Dichroism (CD) Measurements. Far-UV CD spectra were recorded using a Jasco 715 (USA) spectropolarimeter at 20 °C in a 1 mm path-length quartz cuvette. The concentration of the protein component (apoE4 forms) of lipoprotein particles was 0.1 mg/mL in sodium phosphate buffer (pH 7.4). Spectra were recorded from 195 to 260 nm by using the following measurement parameters: bandwidth 1 nm, response 8 s, step size 0.2 nm, and scan speed 50 nm/min. During the measurements a Jasco PTC-348 WI Peltier temperature controller was connected to the instrument for thermostatting the cuvette chamber. Each spectrum was the average of three accumulations. All spectra were obtained by subtracting the buffer baseline. Helical content was calculated using the molecular ellipticity at 222 nm as described[41] using the equation:

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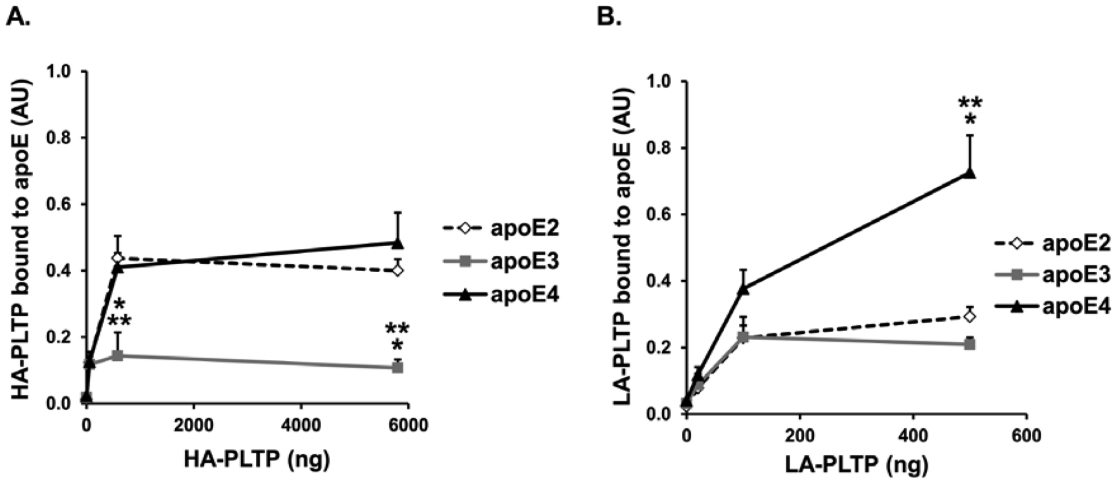

Figure 2. Binding of HA-PLTP or LA-PLTP to lipid free apoE isoforms. MaxiSorp immuno plates were coated with 5 μg/mL of apoE2, apoE3, or apoE4 and incubated with increasing amounts of HA-PLTP (A) or LA-PLTP (B). The binding (total) of PLTP was monitored with the polyclonal anti-PLTP antibody R180 as described in Materials and Methods. Nonspecific binding of PLTP to noncoated cells was subtracted from total binding. (A) Values represent the means ± SEM of three independent experiments performed in triplicate. *p < 0.05 for HA-PLTP 580 ng/apoE3 vs HA-PLTP 580 ng/apoE2 and HA-PLTP 5800 ng/apoE3 vs HA-PLTP 5800 ng/apoE4; **p < 0.005 for HA-PLTP 580 ng/apoE3 vs HA-PLTP 580 ng/apoE4 and HA-PLTP 5800 ng/apoE3 vs HA-PLTP 5800 ng/apoE2 (B) Values represent the means ± SEM of two independent experiments performed in duplicate. *p < 0.05 for LA-PLTP 500 ng/apoE4 vs LA-PLTP 500 ng/apoE3; **p < 0.01 for LA-PLTP 500 ng/apoE4 vs LA-PLTP 500 ng/apoE2. AU: arbitrary units.

$$\%\alpha\text{-helix} = ([\Theta]_{222} + 3000)/(36000 + 3000) \times 100$$

For thermal denaturation measurements, the change in molar ellipticity at 222 nm was monitored while the temperature in the range of 20−100 °C was varied at a rate of 1 °C/min. The thermal denaturation curve was fitted to a Boltzman simple sigmoidal model curve using the GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). $T_{1/2}$ is the midpoint of the transition of the thermal denaturation curve, at which unfolding of apoE was half complete.

RESULTS

Effect of Lipid-Free ApoE Isoforms on Activation of and Binding to HA-PLTP or LA-PLTP. To examine and compare the effect of lipid-free apoE isoforms on HA-PLTP and LA-PLTP, increasing amounts of apoE isoforms were incubated with HA-PLTP or LA-PLTP (Figure 1). The molar ratios of apoE:PLTP were 23.5:1, 235:1, and 2350:1 mol/mol and within the range that had been demonstrated previously to promote the activation of LA-PLTP by apoE3.[5] As shown in Figure 1A all three lipid-free apoE isoforms promoted the activation of HA-PLTP (with activity 790 ± 20 nmol/h/mL in the absence of apoE) at all the apoE amounts used, while apoE3 showed the highest activating capacity of HA-PLTP (330−2540% of HA-PLTP activity in the absence of apoE). Lipid-free apoE2 and apoE4 exhibited similar capacity to activate HA-PLTP (150−880 and 230−750%, respectively, of HA-PLTP activity in the absence of apoE). Lipid-free apoE isoforms promoted also the activation of LA-PLTP, which as expected has very low activity (18 ± 7 nmol/h/mL) in the absence of apoE (Figure 1B). ApoE3 (at the highest amount used) promoted the highest activation of LA-PLTP, while apoE2 and apoE4, used at the same amount, induced lower activation of LA-PLTP. These results suggest that all apoE isoforms in the lipid-free form can activate both LA-PLTP and HA-PLTP *in vitro*. The apoE3, the most common apoE isoform in the general population, was shown to be the stronger activator of PLTP, as compared to apoE2 and apoE4.

To explore further the interactions of PLTP/apoE we studied the binding of lipid-free apoE isoforms to HA-PLTP or LA-PLTP using an ELISA platform method. Microplates coated with lipid-free apoE2, apoE3, or apoE4 were incubated with increasing amounts of HA-PLTP or LA-PLTP. PLTP/apoE binding was monitored with the rabbit polyclonal anti-PLTP antibody R180 after incubation with horseradish peroxidase-conjugated anti-rabbit IgG, as described in Materials and Methods. HA-PLTP or LA-PLTP was added at amounts of 0−5800 ng and 0−500 ng, respectively, since preliminary tests showed a stronger binding capacity of apoE for LA-PLTP. The analysis showed that all three lipid-free apoE isoforms bind both HA-PLTP (Figure 2A) and LA-PLTP (Figure 2B). However, apoE3, which displayed the stronger HA- and LA-PLTP activating capacity (Figure 1), binds less HA-PLTP compared to apoE2 and apoE4 (Figure 2A). In addition, apoE3 binds to the same extent to LA-PLTP as apoE2 and to a lesser extent to LA-PLTP compared to apoE4 (Figure 2B). The binding of apoE to HA-PLTP was also studied with another ELISA method, in which HA-PLTP (instead of apoE) was immobilized on microplates and then incubated with increasing amounts of lipid-free apoE2, apoE3, or apoE4 (Supplemental Figure 1A). PLTP/apoE binding was monitored with the horseradish peroxidase-labeled rabbit polyclonal anti-apoE antibody R107, as described in Materials and Methods. This analysis also showed that, consistent with the results of Figure 2A, HA-PLTP binds less apoE3 than apoE2 or apoE4 (Supplemental Figure 1A). Therefore, it seems that the binding capacity pattern of lipid-free apoE isoforms to PLTP (Figure 2, Supplemental Figure 1A) does not follow the pattern of PLTP activation by lipid-free apoE isoforms (Figure 1), suggesting that the activation of PLTP by lipid-free apoE is not a direct result of apoE/PLTP binding. The two effects may be independent and mediated by separate functional domains of PLTP and lipid-free apoE isoforms.

Effect of Lipidated apoE Isoforms on Activation of and Binding to HA-PLTP or LA-PLTP. To examine whether the lipidation of apoE isoforms affects their capacity to interact with HA-PLTP or LA-PLTP, we performed similar activating and binding studies as those described above using reconstituted discoidal lipoprotein particles (designated PC/C/apoE) con-

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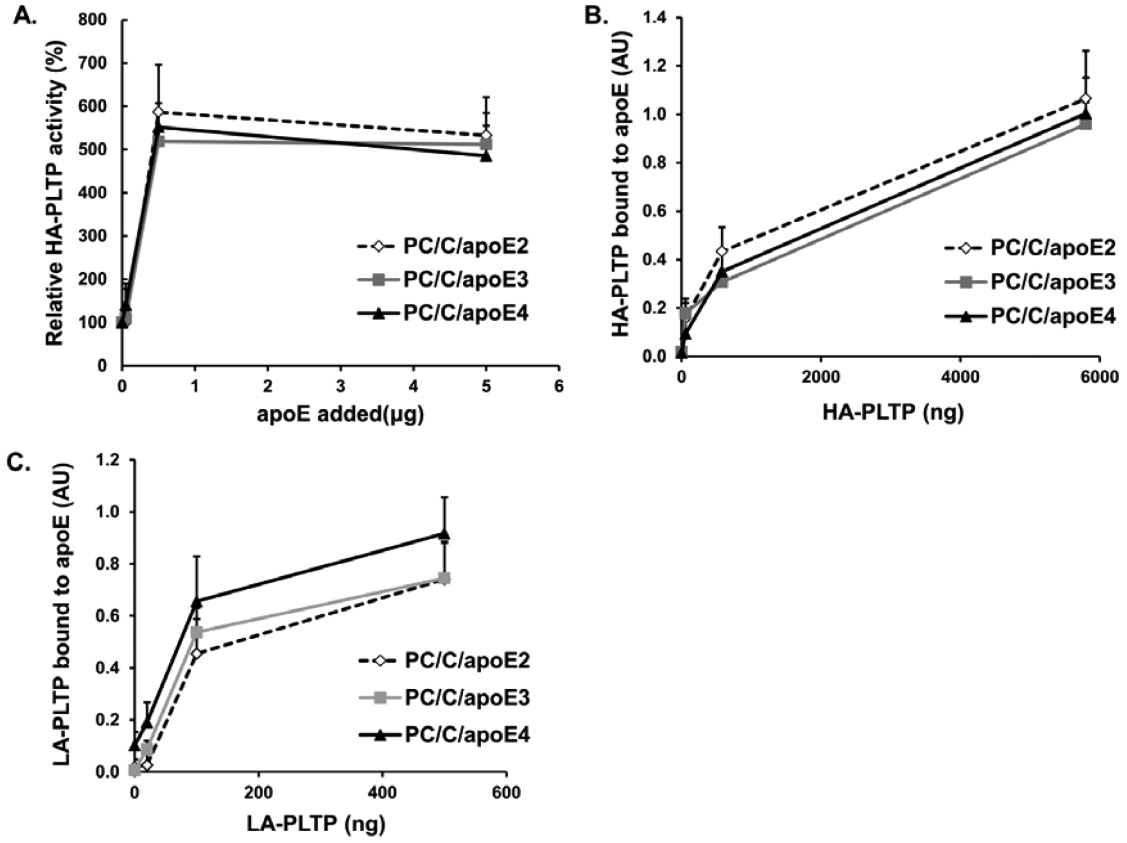

Figure 3. Effect of lipoprotein particle-associated apoE isoforms on activation of and binding to HA-PLTP or LA-PLTP. (A) 50 ng of HA-PLTP were incubated in the absence or presence of increasing amounts (of the protein component) of PC/C/apoE particles containing apoE2, apoE3, or apoE4 for 24 h at room temperature and then assayed for phospholipid transfer activity as described in Materials and Methods. Values represent the mean ± SEM of four independent experiments performed in duplicate. HA-PLTP activity after incubation with PC/C/apoE particles is expressed as percent relative to the HA-PLTP activity in the absence of apoE set to 100%. (B, C) MaxiSorp immuno plates were coated with 5 μg/mL (of the protein component) of PC/C/apoE particles containing apoE2, apoE3, or apoE4 and incubated with increasing amounts of HA-PLTP (B) or LA-PLTP (C). The binding (total) of PLTP was monitored with the polyclonal anti-PLTP antibody R180 as described in Materials and Methods. Nonspecific binding of PLTP to noncoated cells was subtracted from total binding. Values represent the means ± SEM of two independent experiments performed in duplicate. AU: arbitrary units.

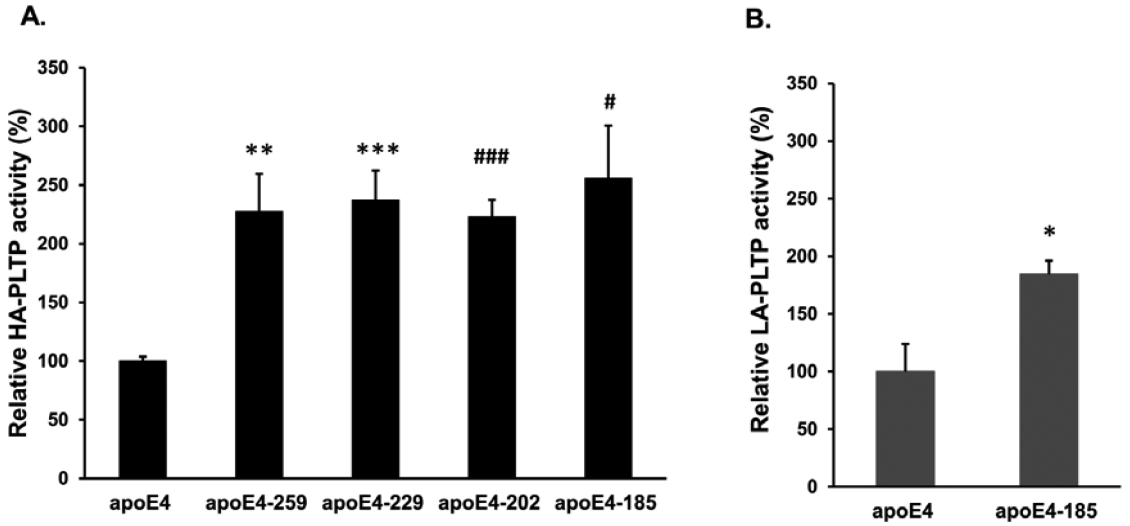

Figure 4. Effect of lipid free full-length and carboxyl-terminal truncated apoE4 forms on HA-PLTP and LA-PLTP activity. 50 ng of HA-PLTP (A) or LA-PLTP (B) was incubated in the presence of 50 μg of lipid-free full-length or carboxyl-terminal truncated apoE4 forms for 24 h at room temperature (A) or 37 °C (B) and then assayed for phospholipid transfer activity as described in Materials and Methods. Values represent the mean ± SEM of three independent experiments performed in duplicate. PLTP activity after incubation with apoE4 carboxyl-terminal truncated forms is expressed as percent relative to PLTP activity after incubation with full-length apoE4 set to 100%. $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$, $^{\#}p < 0.01$, $^{\#\#\#}p < 0.0001$ vs apoE4.

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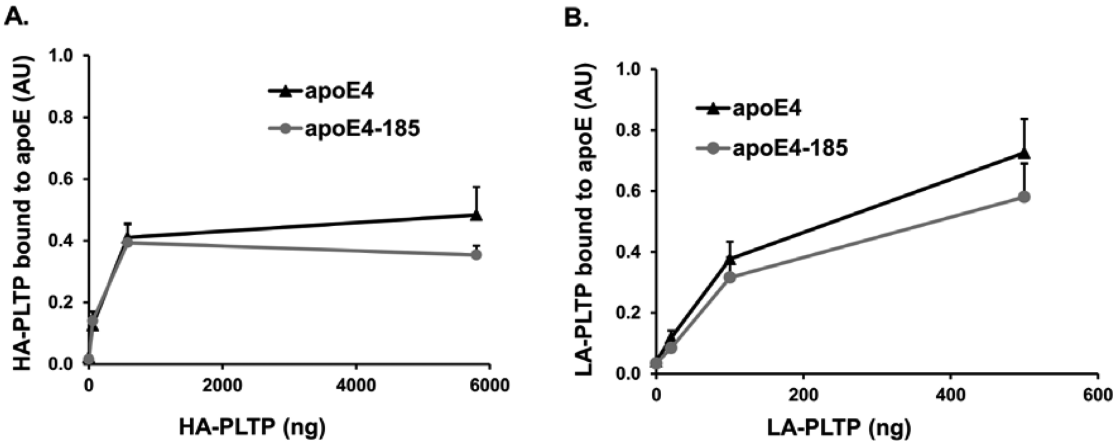

Figure 5. Binding of HA-PLTP or LA-PLTP to lipid free full-length apoE4 and carboxyl-terminal truncated apoE4 form apoE4−185. MaxiSorp immuno plates were coated with 5 μg/mL of full-length apoE4 or apoE4−185 and incubated with increasing amounts of HA-PLTP (A) or LA-PLTP (B). The binding (total) of PLTP was monitored with the polyclonal anti-PLTP antibody R180 as described in Materials and Methods. Nonspecific binding of PLTP to noncoated cells was subtracted from total binding. Values represent the means ± SEM of three independent experiments performed in triplicate. AU: arbitrary units.

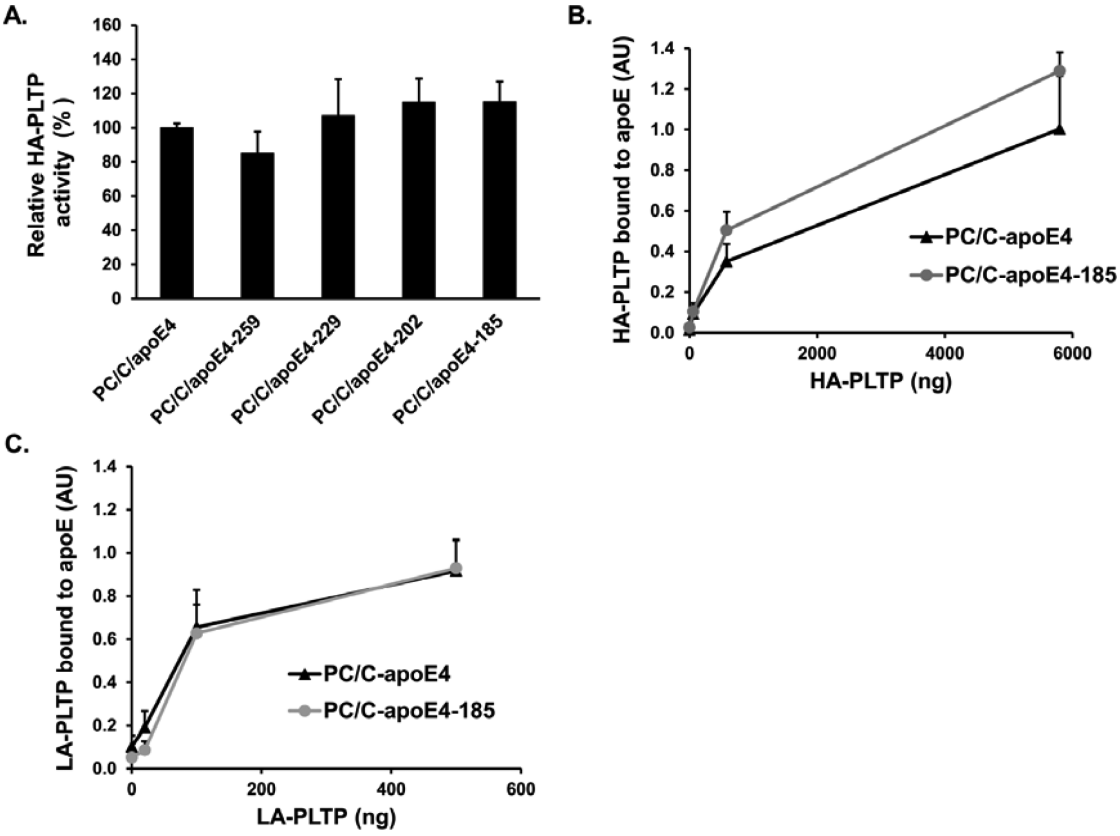

Figure 6. Effect of reconstituted lipoprotein particle-associated full-length and carboxyl-terminal truncated apoE4 forms on activation of and binding to HA-PLTP or LA-PLTP. (A) 50 ng of HA-PLTP was incubated in the absence or presence of 5 μg (of the protein component) of PC/C/apoE particles containing full-length or carboxyl-terminal truncated apoE4 forms for 24 h at room temperature and then assayed for phospholipid transfer activity as described in Materials and Methods. Values represent the mean ± SEM of four independent experiments performed in duplicate. HA-PLTP activity after incubation with PC/C/apoE particles containing apoE4 carboxyl-terminal truncated forms is expressed as percent relative to the HA-PLTP activity after incubation with lipoprotein particles containing full-length apoE4 set to 100%. (B, C) MaxiSorp immuno plates were coated with 5 μg/mL (of the protein component) of PC/C/apoE particles containing full-length apoE4 or apoE4−185 and incubated with increasing amounts of HA-PLTP (B) or LA-PLTP (C). The binding (total) of PLTP was monitored with the polyclonal anti-PLTP antibody R180 as described in Materials and Methods. Nonspecific binding of PLTP to noncoated cells was subtracted from total binding. Values represent the means ± SEM of two independent experiments performed in duplicate. AU: arbitrary units.

taining apoE isoforms along with phosphatidylcholine and cholesterol at a defined PC:C:apoE molar ratio of 100:10:1. Similar apoE-containing lipoprotein particles have been used previously to study the interactions of apoE with proteins of the

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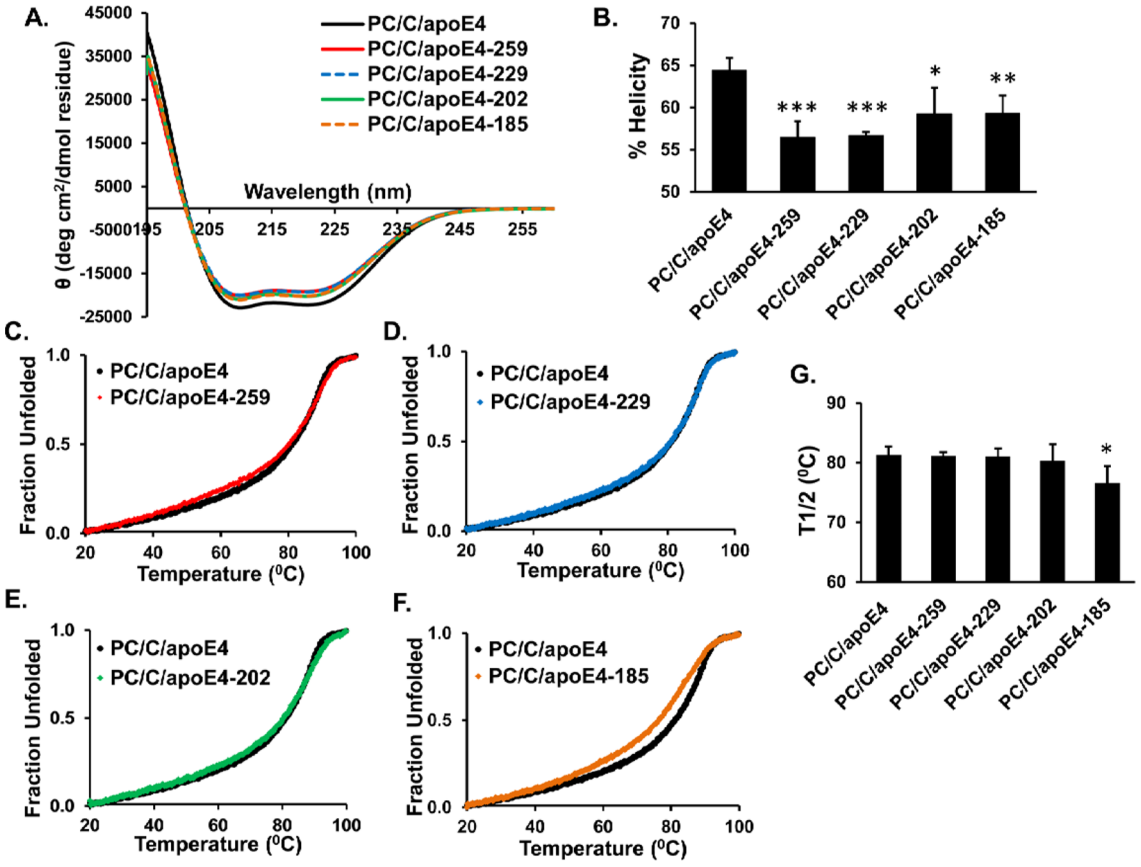

Figure 7. Biophysical characterization of full-length apoE4 or carboxyl-terminal truncated apoE4 forms in reconstituted lipoprotein particles. Reconstituted discoidal lipoprotein particles consisting of full-length or truncated apoE4 forms, phosphatidylcholine, and cholesterol (PC/C/apoE4) were prepared as described in Materials and Methods. (A) Far UV CD spectra of the protein component (full-length or truncated apoE4) of PC/C/apoE4 particles. Spectra are averages of three separate experiments. (B) Percentage of helical content calculated on the basis of the molar ellipticity at 222 nm, as described in Materials and Methods. Values represent the means ± SD of five independent experiments. *$p < 0.5$, **$p < 0.005$, ***$p < 0.0001$ vs full-length apoE4. (C−F) Thermal denaturation profiles of full-length and truncated apoE4 forms in lipoprotein particles. The y axis has been normalized to correspond to the fraction of the protein in the unfolded state. (G) Temperature ($T_{1/2}$) at the midpoint of the transition of the thermal denaturation curve. Values represent the means ± SD of five independent experiments. *$p < 0.5$ vs full-length apoE4.

HDL metabolism pathway.[42−44] Analysis of the effect of lipidated apoE on the activation of HA-PLTP showed that the activation could be achieved using 10 times less apoE compared to lipid free apoE and that all three lipidated apoE isoforms were equally efficient in increasing HA-PLTP activity (approximately 580% of HA-PLTP activity in the absence of apoE) (Figure 3A). In contrast, the lipidated apoE isoforms did not promote the activation of LA-PLTP at any amount used (data not shown). ApoE/PLTP binding studies showed that either when immobilized PC/C/apoE particles were incubated with HA-PLTP (Figure 3B) or immobilized HA-PLTP was incubated with PC/C/apoE particles (Supplemental Figure 1B) all apoE particles bound HA-PLTP at similar levels. Furthermore, our studies showed that lipidated apoE isoforms also bound LA-PLTP, despite the fact that they did not activate LA-PLTP, and that all lipidated apoE isoforms display a comparable LA-PLTP binding pattern (Figure 3C) as it is observed for HA-PLTP. Overall, these data suggest that the apoE lipidation abolishes the isoform-specific differences observed for the effect of lipid-free apoE on PLTP activation and binding.

Ability of Carboxyl-Terminal Truncated apoE4 Forms To Activate and Bind HA-PLTP or LA-PLTP. To gain further insight on the interactions of apoE with PLTP, we examined the ability of apoE4 carboxyl-terminal deletion mutants to activate and bind HA-PLTP or LA-PLTP. The apoE4 forms, with progressive truncations between residues 186−299 of the carboxyl-terminal domain, used were apoE4[Δ(260−299)] (designated as apoE4−259), apoE4[Δ(230−299)] (designated as apoE4−229), apoE4[Δ(203−299)] (designated as apoE4−202), and apoE4[Δ(186−299)] (designated as apoE4−185).

Analysis of the effect of lipid-free carboxyl-terminal truncated apoE4 forms on HA-PLTP activation showed that all four truncated forms increased the activation of HA-PLTP compared to full-length apoE4. All carboxyl-terminal truncated apoE4 forms displayed similar HA-PLTP activation, which was 220−260% of the HA-PLTP activation capacity of full-length apoE4 set to 100% (Figure 4A). In addition, the shortest carboxyl-terminal truncated apoE4 form apoE4−185 also increased the activation of LA-PLTP compared to full-length apoE4 (185% of the LA-PLTP activation capacity of full-length apoE4) (Figure 4B). Study of binding of lipid-free full-length apoE4 and apoE4−185 deletion mutant to HA-PLTP or LA-PLTP showed that apoE4−185 binds both HA-PLTP (Figure 5A) and LA-PLTP (Figure 5B), displaying a similar binding profile to that of full-length apoE4. These data indicate that the amino terminal domain 1−185 of lipid-free apoE4 is sufficient for the binding of apoE4 to HA- and LA-PLTP and that deletion of the carboxyl-

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terminal domain from apoE4 enhances the activation of HA- and LA-PLTP by lipid-free apoE4.

Studies using reconstituted lipoprotein particles containing full-length apoE4 or apoE4 carboxyl-terminal deletion mutants showed that PC/C/apoE4−185 binds both HA-PLTP (Figure 6B) and LA-PLTP (Figure 6C), displaying a similar binding profile to that of PC/C/apoE4, as it was observed for lipid free full-length apoE4 and apoE4−185 (Figure 5). However, the lipidation of apoE4−185, as well as of the other truncated apoE4 forms activated HA-PLTP to the same extent as the full-length apoE4 (Figure 6A). Furthermore, the lipidated apoE4 carboxyl-terminal deletion mutants did not promote the activation of LA-PLTP (data not shown), as it was observed for lipidated full-length apoE4 and other apoE isoforms.

Overall, the above data suggest that the amino-terminal region 1−185 of apoE4 is sufficient for the binding of lipid-free or lipidated apoE4 to HA-PLTP or LA-PLTP. Deletion of the carboxyl-terminus of lipid-free apoE4 enhances its capacity to activate HA-PLTP or LA-PLTP, but this effect is abolished as a result of lipidation of the full-length and carboxyl-terminal truncated apoE4 forms.

Biophysical Characterization of Lipoprotein Particles Containing apoE4 Carboxyl-Terminal Deletion Mutants. We examined whether apoE4 carboxyl-terminal deletion mutants in reconstituted lipoprotein particles show structural and thermodynamic changes compared to lipoprotein particles containing the full-length apoE4. CD spectroscopic analysis showed that all apoE4 carboxyl-terminal deletion mutants have decreased helical content in lipoprotein-associated form (Figure 7A,B). However, thermal denaturation measurements did not reveal any significant perturbations in the unfolding transitions for apoE4−259, apoE4−229, and apoE4−202 in reconstituted lipoprotein particles compared to lipoprotein particles containing the full-length apoE4. Only a small perturbation was observed for apoE4−185 in reconstituted lipoprotein particles. The midpoint of the transition for this mutant was 4.7 °C lower than that of full-length apoE4 in reconstituted lipoprotein particles (Figure 7C−G). These findings suggest that although the truncation of carboxyl-terminal segments of apoE4 may result in small changes of the structural or thermodynamic properties of apoE4 in reconstituted lipoprotein particles these changes are much less pronounced compared to changes observed previously for lipid-free apoE4.[31] This observation may underlie the different effects on PLTP activation seen for lipid-free and lipidated apoE.

DISCUSSION

The present study has focused on the functional interactions between apoE and PLTP that result in PLTP activation. ApoE and PLTP play important (patho)physiological roles in lipid homeostasis and other processes both in circulation and brain.[1,2,14,15] Two forms of PLTP exist in the circulation, one active (HA-PLTP) and one with very low activity (LA-PLTP),[3,4] while the majority of CSF PLTP has been suggested to be active in phospholipid transfer.[11] The physiological implications of the distribution of PLTP between HA- and LA-PLTP forms, and the mechanisms of PLTP inactivation/activation are poorly understood. Previous studies have suggested that apoE can interact with PLTP,[5,12] but no systematic analysis on the effect of apoE allelic background and lipidation status on activation of and binding to the two PLTP forms has been performed. Here, we examined and compared the capacity of apoE2, apoE3, and apoE4 and carboxyl-terminal truncated apoE4 forms, both in lipid-free and lipoprotein particle associated form, to activate and bind to the two PLTP forms HA- and LA-PLTP.

We showed that lipid-free apoE3 induces the higher activation of both HA- and LA-PLTP, compared to apoE2 and apoE4. However, the binding capacity pattern of lipid-free apoE isoforms to either HA- or LA-PLTP does not follow the pattern of PLTP activation. These findings suggest a complex mechanism for the activation of HA- or LA-PLTP by lipid-free apoE. It is conceivable that different functional domains in apoE and PLTP are responsible for binding and activation, and as a result these two events, that may be sequential steps in the same mechanism, are distinct and independent. Our current data do not allow us to distinguish whether apoE participates in protein−protein interactions resulting in conformational changes in PLTP that may facilitate the phospholipid transfer, the binding of PLTP to phospholipid vesicles, the binding of PLTP to HDL3 or in all three processes.

On the basis of our findings on the effect of lipid-free apoE isoforms on PLTP activity, it could be postulated that apoE molecules that can be exchanged readily from the surface of lipoprotein particles[45] may modulate the ratio of HA- and LA-PLTP activities and therefore the total activity of PLTP. In addition, since lipid-free apoE cannot be found in substantial amount in plasma, the potential physiological relevance of studying the functions of lipid-free apoE is associated with the capacity of most tissues to secrete apoE.[16] ApoE expression by a number of nonhepatic cells, including macrophages in the arterial wall and brain cells, indicates that the local production may be related to specific functional traits.[16] Furthermore, PLTP is also widely distributed among tissues and cells, such as macrophages in the arterial wall and brain cells,[2] and it has been proposed that one function of the peripheral PLTP is the transfer of cellular lipids to acceptor lipoprotein particles.[46] Therefore, it can be postulated that the interactions of PLTP with lipid-free apoE forms may have an impact on lipid homeostasis in cells and tissues.

Analysis of the effect of lipid-bound apoE in the form of PC/C/apoE particles on PLTP activation showed that all three apoE isoforms promoted equally the enhancement of HA-PLTP activity and demonstrated similar binding to HA-PLTP. The three lipoprotein particle-associated apoE isoforms also displayed a similar capacity to interact with LA-PLTP. However, while all lipidated apoE isoforms were capable to bind to LA-PLTP, displaying comparable binding capacity, none of them promoted the activation of LA-PLTP. This finding suggests that binding of lipidated apoE to LA-PLTP alone is not sufficient to induce activation, indicating a complex mechanism for PLTP activation, as proposed for lipid free apoE. Our data are consistent with a previous study in a normal Finnish population sample which demonstrated that serum total PLTP activity was similar in three apoE isoform subgroups (group 1, E3/E3; group 2, E4/E4, E4/E3; group 3, E2/E2, E2/E3, E2/E4).[47] However, our findings indicate that the effect of lipidated apoE on HA- and LA-PLTP activation is drastically different, something not previously evaluated.[47]

ApoE has structural and functional similarities to apoA-I.[48−50] Previous studies demonstrated that PLTP binds to the amino-terminal domain of apoA-I.[13] Therefore, we examined whether, in analogy to apoA-I, carboxyl-terminal truncated apoE forms are also capable of binding to and/or activating HA- and LA-PLTP. Of the three apoE isoforms, apoE4 has been proposed to be more prone to proteolysis,[24−26] and several studies have shown the presence of apoE4 carboxyl-terminal truncated proteolytic

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fragments in brains of AD patients.[24,25,27,28] Furthermore, different truncated apoE4 forms have been shown to affect distinct processes in AD pathogenesis and plasma lipid homeostasis.[24,25,32−34] Additionally, PLTP is secreted by brain cells (neurons, microglia, and astrocytes), and PLTP levels are altered in the brain and CSF of AD patients compared to controls.[10,11] Thus, the study of the interactions of carboxyl-terminal truncated apoE4 forms with PLTP is physiologically relevant. Our studies demonstrated that the amino-terminal region 1−185 of apoE4 is sufficient for the binding of lipid-free apoE4 to HA- or LA-PLTP and that deletion of the carboxyl-terminal domain (186−299) of lipid-free apoE4 enhances the capacity of apoE4 to activate both HA- and LA-PLTP. Furthermore, the amino-terminal region 1−185 of apoE4 is also sufficient for the binding of reconstituted lipoprotein particle-associated apoE4 to HA- or LA-PLTP and for HA-PLTP activation. Overall, these findings suggest that the amino-terminal region 1−185 of lipid-free or lipoprotein particle-associated apoE4 participates in the functional interactions with PLTP.

Several studies have suggested that the structural and biophysical properties of apoE can dictate the function of the protein and have provided insight into the mechanisms by which apoE is involved in cardiovascular and neurological diseases.[15,31,51,52] Here we showed that the allelic background of lipid-free apoE can influence the interactions of apoE with PLTP. These functional differences may be related to the previously described differences in structure and structural plasticity between the lipid-free apoE isoforms.[15,51,53−55] More specifically, functional differences between apoE4 and apoE3 that can affect disease predisposition have been previously attributed to altered interdomain interactions brought about by the polymorphism at position 112 of the amino-terminal domain, which results in alterations in the thermodynamic properties of the molecule.[15,51,53−55] Similarly, differences in structure, function, and disease predisposition have been established between apoE3 and apoE2 variants, which also differ by a single amino acid at position 158.[15,51,55]

Upon lipidation apoE undergoes large conformation change, but after lipidation the apoE isoforms can adopt similar conformations depending on the particle lipid composition.[56,57] Thermal denaturation analysis of the protein component of PC/C/apoE particles containing apoE3 or apoE4 showed a similar profile for the two apoE isoforms (data not shown). The adoption of similar conformations for lipoprotein particle-associated apoE isoforms could explain the similar capacity of these proteins, in lipidated form, to interact with PLTP.

Our studies showed that all lipid-free apoE4 carboxyl-terminal deletion mutants have increased capacity to activate HA-PLTP compared to full-length apoE4. One explanation for this effect could be that while full-length apoE4, in micromolar concentrations used in the current study, exists predominantly as tetramers, removal of the carboxyl-terminal domain favors the monomeric state in apoE4.[58,59] Thus, the presence of more monomers could enhance the interactions of apoE4 with PLTP that result in activation. However, continuous size distribution analysis of progressive carboxyl-terminal truncated apoE4, at concentrations similar to those used in our study, showed that the self-association of apoE4 is affected by the length of the carboxyl-terminal domain that is removed. Specifically it was shown that apoE4−191 exists predominantly as monomer together with some tetramer, apoE4−231 exists equally as monomer and tetramer, and apoE4−271 exists mostly as tetramer.[58] Our analyses, however, showed that even the smallest carboxyl-terminal truncation of apoE4 was sufficient to induce the maximum activation of HA-PLTP. These results could be explained by a simple model where the activation domain of apoE4 for HA-PLTP lies within its amino-terminal region. It is possible that this domain is maximally accessible in the truncated apoE4 forms but is hindered in the full-length apoE4. Carboxyl-terminal truncations in lipid-free apoE4 have been previously shown to affect the conformational plasticity of the protein.[31] Consequently, it is possible that the 260−299 region of apoE4 can either directly interact with the amino-terminal domain of apoE4 and obstruct the HA-PLTP activation or can indirectly affect structural transitions necessary to expose the activation domain of apoE4 to PLTP. Therefore, the interdomain interactions in lipid-free apoE4 could be crucial for PLTP activation. Furthermore, our data suggested that the binding domain of apoE4 for PLTP lies also within its amino-terminal region and that the transition from the tetrameric to monomeric state of lipid-free apoE4 does not affect the binding to PLTP since lipid-free full-length and highly truncated apoE4−185 display a similar binding pattern to PLTP. In any case, the lack of correlation between binding of lipid-free apoE4 carboxyl-terminal deletion mutants to PLTP and activation of PLTP indicates a multistep mechanism for PLTP/apoE4 interaction that leads to PLTP activation.

While carboxyl-terminal truncation increased the capacity of lipid-free apoE4 to activate the HA-PLTP, we did not observe a similar result for lipoprotein particle-associated apoE4. All reconstituted lipoprotein particles containing apoE4 carboxyl-terminal deletion mutants displayed similar HA-PLTP activation compared to reconstituted lipoprotein particles containing full-length apoE4. These findings suggest that lipidation may have similar structural effects on full length or carboxy-terminal truncated apoE4 forms in exposing the HA-PLTP activation domain. This is consistent with the amelioration of any thermodynamic differences that exist between the apoE4 truncated mutants when the protein is lipidated.

In summary, we present here a systematic analysis of the interactions of apoE, in lipid-free or lipoprotein particle-associated form, with HA- and LA-PLTP and discuss how the structural properties of apoE forms may affect their functions. Our data show distinct effects of apoE isoforms, carboxyl-terminal truncations, and lipidation status on HA- and LA-PLTP activation. These findings suggest that apoE may play a role in regulating PLTP activity in circulation and may affect various PLTP-dependent metabolic processes related to (patho)-physiological conditions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00681.

Supplemental Figure 1. Binding of lipid free or reconstituted lipoprotein particle associated apoE isoforms to HA-PLTP (PDF)

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Notes

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■ ABBREVIATIONS

AD, Alzheimer's disease; apo, apolipoprotein; C, cholesterol; CD, circular dichroism; CSF, cerebrospinal fluid; [^{14}C]-DPPC, L-α-dipalmitoyl-phosphatidylcholine [dipalmitoyl-1-^{14}C]; HA, high activity; HDL, high density lipoprotein; LA, low activity; PC, phosphatidylcholine; PLTP, phospholipid transfer protein

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