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Molecular Basis for the Differences in Lipid and Lipoprotein Binding Properties of Human Apolipoproteins E3 and E4

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Abstract

Human apolipoprotein (apo)E4 binds preferentially to very low density lipoproteins (VLDL) whereas apoE3 binds preferentially to high density lipoprotein (HDL), resulting in different plasma cholesterol levels with the two isoforms. To understand the molecular basis for this effect, the isolated apoE N-terminal domain (residues 1-191) and C-terminal domain (residues 192-299) together with a series of variants containing deletions in the C-terminal domain were engineered and assessed for their lipid and lipoprotein binding properties. Both isoforms can bind to a phospholipid (PL)-stabilized triolein emulsion and residues 261-299 are primarily responsible for this activity. ApoE4 exhibits greater lipid binding ability than apoE3 as a consequence of a rearrangement involving the segment spanning residues 261-272 in the C-terminal domain. The high lipid binding ability of apoE4 coupled with the VLDL particle surface being ~60% PL-covered is the basis for its preference to bind to VLDL rather than HDL. ApoE4 binds much more than apoE3 to VLDL but less than apoE3 to HDL₃, consistent with apoE-lipid interactions being relatively unimportant for binding to HDL. The preference of apoE3 for binding to HDL₃ arises because binding is mediated primarily by interaction of the N-terminal helix bundle domain with the resident apolipoproteins which cover ~80% of the HDL₃ particle surface. Thus, the selectivity in apoE3 and apoE4 binding to HDL₃ and VLDL is dependent upon two factors. 1) The greater lipid binding ability of apoE4 relative to apoE3. 2) The differences in the nature of the surfaces of VLDL and HDL₃ particles, with the former being largely covered with PL and the latter with protein.

Apolipoprotein E (apoE) regulates lipid transport and cholesterol homeostasis in the cardiovascular and central nervous systems (1–2) and is therefore a protein of major biological and clinical importance. In particular, there is great interest in understanding the structure-function relationships of apoE because of its pronounced anti-atherogenic properties (3). A complication in understanding the structure-function relationships of human apoE is the occurrence of three major isoforms (apoE2, apoE3 and apoE4), each differing by a single amino acid substitution (4). ApoE3, the most common isoform, contains cysteine at position 112 and arginine at position 158 whereas apoE2 and apoE4 contain cysteine and arginine, respectively, at both sites. ApoE2 displays defective binding to the low density lipoprotein (LDL) receptor and is associated with Type III hyperlipoproteinemia

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(5). ApoE3 and apoE4 are also associated with different lipoprotein profiles; the presence of the latter isoform leads to higher plasma cholesterol levels and an increased risk of cardiovascular disease relative to individuals with apoE3 (6–7). Unlike the situation with apoE2, the different effects of apoE3 and apoE4 on plasma cholesterol level are not due to variations in interactions with the LDL receptor because both of these isoforms bind similarly to the receptor (8). Rather, the difference is apparently a consequence of altered lipoprotein binding preferences of apoE3 and apoE4 (9–10). The C112R substitution that distinguishes apoE4 from apoE3 occurs in the N-terminal helix bundle domain of the molecule where it destabilizes the helix bundle (11) and loosens its structure (12). As a consequence, interactions between the N- and C-terminal domains of the protein molecule are modified so that, unlike apoE3, apoE4 binds preferentially to very low density lipoprotein (VLDL) as compared to high density lipoprotein (HDL) in plasma (10,13–14). The altered domain-domain interaction in apoE4 is a result of a rearrangement of the R61 side chain in the helix bundle induced by the presence of R112 that allows R61 to interact with E255 in the C-terminal domain (13–14). This interaction causes the C-terminal domain to be organized differently in the two isoforms. For example, the spacing between the N-terminal and C-terminal domains is altered (15–16) and the C-terminal domain in apoE4 around position 264 is less organized and more exposed to the aqueous environment (17). As a result of such alterations in the C-terminal domain, apoE4 binds better than apoE3 to lipid surfaces (14,18). Studies of apoE3 and apoE4 variants containing a progressively truncated C-terminal domain have demonstrated that the region spanning residues 260–299 is important for determining apoE structure, self-association and ability to bind to lipoprotein particles (13,17,19–21).

Despite the insights provided by the above investigations, the molecular basis for why apoE3 and apoE4 partition differently between VLDL and HDL remains to be explained. It is important to understand this effect because it underlies the altered catabolism of triglyceride-rich lipoproteins induced by apoE4 compared to apoE3 (5) and the enhanced cardiovascular disease risk associated with apoE4 (6). Here we address this question by systematically investigating the lipid and lipoprotein binding properties of a series of apoE3 and apoE4 variants with altered C-terminal domains. The results demonstrate that the region spanning residues 261–272 contributes differently to lipid binding in apoE3 and apoE4 such that apoE4 binds better to lipids. The preferential association of apoE4 with VLDL occurs because apoE-lipid interactions control binding to the surface of this lipoprotein particle. ApoE4 does not bind better than apoE3 to HDL because interactions of apoE with other apolipoproteins resident in the HDL particle surface are dominant in this case.

EXPERIMENTAL PROCEDURES

Materials

Human apoE variants were expressed in *E. coli* as thioredoxin fusion proteins and isolated and purified as described (22–23). Full length apoE3 and apoE4 (residues 1–299), their 22kDa N-terminal fragments (residues 1–191) and 12kDa C-terminal fragment (residues 192–299), as well as the C-terminal truncated forms (1–260, 1–272) have been described previously (17,20,23). The variants containing point mutations (apoE3 K146E, apoE3 K146Q, apoE3 P267A, apoE4 E255A) have also been described (18,24). The QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was employed to introduce deletions (Δ 192–260 and Δ 261–272) as well as the point mutation G278P into apoE3 and apoE4. The apoE preparations were at least 95% pure as assessed by SDS-PAGE. The apoE variants were ^{14}C -trace labeled by reductive methylation as described previously (18). In all experiments the apoE sample was freshly dialyzed from 6M GdnHCl and 10mM DTT solution into a buffer solution before use. ApoE concentrations were determined either by a measurement of the absorbance at 280nm or by the Lowry procedure (25). HDL₃ and VLDL

were purified by sequential ultracentrifugation from a pool of normolipidemic human plasma as described (17,26). Dimyristoyl phosphatidylcholine (DMPC) was obtained from Avanti Polar Lipids (Pelham, AL) and egg yolk phosphatidylcholine (PC) and triolein were purchased from Sigma (St. Louis, MO). 8-Anilino-1-naphthalenesulfonic acid (ANS) was purchased from Molecular Probes (Eugene, OR).

Binding of ApoE to Emulsion Particles

Emulsion particles were prepared by sonication of a triolein/egg yolk PC mixture (3.5/1w/w) in pH 7.4 Tris buffer (18,23). The molar ratio of triolein/PC in the emulsion after isolation by ultracentrifugation was $4.6 \pm 0.3/1$ (mean \pm SEM, $n=6$) and the average particle diameter by quasi-elastic light scattering was 86 ± 7 nm. The binding of apoE was monitored by incubating ^{14}C -labeled protein with emulsion for 1h at room temperature and separating free and bound apoE by centrifugation, as described (23).

VLDL/HDL Distribution of ApoE

The partitioning of the apoE variants between human HDL₃ and VLDL was monitored using a previously described, competitive-binding, assay (17) except that the apoE variants were added individually rather than in pairs. In brief, ^{14}C -apoE (5 μg) was incubated at 4°C for 30 min with 0.45mg VLDL protein and 0.9mg HDL₃ protein (these concentrations give approximately equal total VLDL and HDL₃ particle surface areas available for apoE binding) in a total volume of 1ml of Tris buffer (pH 7.4). VLDL, HDL₃ and unbound apoE were then separated by sequential ultracentrifugation.

DMPC Clearance Assay

The kinetics of solubilization of DMPC multilamellar vesicles (MLV) by the apoE variants were measured by monitoring the decrease in absorbance at 325nm, as described before (27).

ANS Fluorescence Measurements

A Hitachi F-4500 fluorescence spectrophotometer was used to measure the fluorescence (400–600nm) from 250 μM ANS in Tris buffer (pH 7.4) in the presence of 50 $\mu\text{g/ml}$ apoE variant at an excitation wavelength of 395nm (18).

Circular Dichroism (CD) Spectroscopy

The average α -helix contents of the apoE variants were determined by measuring CD spectra at room temperature using a Jasco J-810 Spectropolarimeter (28). The α -helix content was derived from the molar ellipticity at 222nm, as described (17).

RESULTS

Influence of C-Terminal Domain Manipulation on ApoE Structure

Since it is known that the C-terminal domain (residues 192-299) is primarily responsible for the lipid affinity of apoE, to understand better the molecular basis for this effect we pursued a mutagenesis strategy aimed at systematically dissecting the contributions of different segments of this domain to lipid binding. The contributions of the N- and C-terminal ends of the domain were evaluated by deleting residues 192-260 (variant apoE (Δ 192-260)) and residues 261-299 (variant apoE (1-260)), respectively. The influence of the latter segment was examined in further detail by deleting either residues 261-272 (variant apoE (Δ 261-272)) or residues 273-299 (variant apoE (1-272)). Contributions of the entire C-terminal domain were examined by employing the isolated N- and C-terminal domains (residues 1-191 and 192-299, respectively). The secondary structures of the above apoE

variants were determined by far-UV CD spectroscopy while hydrophobic surface exposure was monitored by ANS binding using fluorescence spectroscopy. The results of these experiments are summarized in Table 1.

Regarding secondary structure, in agreement with prior reports (11,17,20,29), the human apoE molecule (residues 1-299) is ~ 50% α -helical so that some 150 residues are located in α -helix (Table 1). The exact α -helix content is dependent upon sample history which can affect the degree of self-association of the protein. The isolated N-terminal domain (residues 1-191) contains a bundle of amphipathic α -helices (4,30) comprising about 60% of the residues (Table 1). The separately folded C-terminal domain (residues 192-299) contains a similar level of α -helix. The sum of the numbers of helical residues in the isolated N- and C-terminal domains is greater than that in the intact apoE molecule (Table 1) suggesting that interactions between the two domains when covalently attached prevent some folding into α -helix.

As reported before (17,20–21), C-terminal truncation of apoE3 and apoE4 to either residue 272 or 260 leads to reductions in α -helix content (Table 1); this is consistent with the region spanning residues 261-299 containing some α -helix. At this stage, it is not possible to analyze the helix content data in Table 1 to locate helical residues precisely because the elimination of certain segments can induce changes in secondary structure elsewhere in the protein molecule. For instance, removal of the entire C-terminal domain (residues 192-299) from apoE3 reduces the number of helical residues by 40 while deletions of either residues 192-260 or residues 261-299 cause reductions of 36 and 32 residues, respectively (Table 1). This inconsistency indicates that any decrease in the number of helical residues arising from deletion of a segment of the apoE molecule cannot necessarily be attributed to direct removal of a helix. The CD results summarized in Table 1 demonstrate that the C-terminal domain exerts different structural effects in apoE3 and apoE4. Thus, apart from the case of the Δ 261-272 mutation, the C-terminal domain manipulations induce significantly larger losses of α -helical residues in the latter isoform. The exceptional behavior of the Δ 261-272 variants in this regard indicates that the segment spanning residues 261-272 has a greater helix stabilizing effect in apoE3 than in apoE4. Consistent with this observation, the segment 261-272 (as monitored by the fluorescence of W264) is packed differently in the two isoforms (17).

The ANS binding data in Table 1 confirm that the segments spanning residues 261-272 influence the overall protein structure differently in apoE3 and apoE4. Thus, this deletion in apoE3 reduces ANS binding but increases it in apoE4. This is in contrast to the other variants of apoE3 and apoE4 (Table 1) containing alterations of the C-terminal domain where, relative to the intact protein, ANS binding is reduced similarly for both isoforms. The comparisons in Table 1 of ANS binding to the isolated N- and C-terminal domains confirm prior reports (17–18,20) showing that binding is primarily to the latter domain. It follows that the C-terminal domain has much more exposed hydrophobic surface than the N-terminal helix bundle domain, presumably underlying the greater lipid binding ability of the C-terminal domain (see below).

DMPC Clearance Assay

We have shown previously (17,27) that the ability of apoE to solubilize DMPC MLV resides largely in the C-terminal domain and the results depicted in Fig. 1 for the apoE variants (1-299), (1-191) and (192-299) are consistent with this concept. Removal of the N-terminal end of the C-terminal domain (residues 192-260) has no effect on the activity of apoE3 and decreases the activity of apoE4 somewhat (Fig. 1). In contrast, removal of the C-terminal end of the domain (residues 261-299) reduces the ability of both apoE3 and apoE4 to solubilize DMPC (see the results for the variants apoE (1-260) in Fig. 1). This finding shows

that residues 261-299 play a key role in the solubilization process. Further dissection of the contribution of this segment of the apoE molecule is complicated by alterations in the degree in self-association when residues in the region 261-299 are deleted. Thus, removal of residues 261-272 (variant (Δ 261-272)) has no effect on DMPC clearance whereas removal of residues 273-299 enhances the rate of solubilization because oligomerization is reduced with this variant (17).

The results in Fig. 1 demonstrate that the DMPC clearance abilities of the 1-299, Δ 192-260, Δ 261-272 and 1-272 variants of apoE4 are greater than those of their apoE3 counterparts and that this effect is not apparent for the 1-191 and 1-260 variants. It follows that the presence of residues in the region 261-299 is required for the isoform effect on DMPC solubilization rate to be evident, and that the presence of either residues 261-272 or residues 273-299 is sufficient.

Binding to Lipid Emulsion

The DMPC clearance results presented in the preceding section give a measure of how alteration of the C-terminal domain of apoE affects the rate of lipid interaction and breakdown of the DMPC MLV. To obtain a complementary measure of how the apoE variants affect steady state binding to a stable lipid-water interface, we employed triolein/egg PC emulsion particles as the lipid substrate in an apoE binding assay. The isotherms in Fig. 2 describing the binding of apoE3 and apoE4 to such emulsion particles are consistent with prior reports (18,20,23) in showing that the isolated N-terminal domain (residues 1-191) binds much less well than intact apoE (residues 1-299). This observation indicates that the C-terminal domain (residues 192-299) plays a key role in the emulsion-binding ability of apoE and the binding isotherm for the isolated C-terminal domain confirms that it binds very well. Interestingly, the isolated C-terminal domain binds better than intact apoE3 (Fig. 2A) but the same as intact apoE4 (Fig. 2B). This result implies that the presence of the N-terminal helix bundle in apoE4 does not interfere with the ability of the C-terminal domain to interact with the emulsion whereas in apoE3 it does.

Comparison of the data for intact apoE3 and apoE4 in Fig. 2A,B confirms that the latter isoform binds better to lipid emulsion, as has been reported previously (14, 18). It is also apparent from Fig. 3 that deletion of residues 192-260 from either isoform has no significant effect on emulsion binding. In marked contrast, removal of residues 261-299 (as in the apoE (1-260) variants) drastically reduces the ability to bind to the lipid emulsion (with the reduction being greater for apoE4), indicating that residues 261-299 are critical for the process (cf. (20)). Furthermore, the poor binding exhibited by the Δ 261-272 and 1-272 variants (Fig. 3A,B) indicates that the entire segment spanning residues 261-299 is required for full binding activity. Interestingly, removal of residues 261-272 has a more damaging effect on the lipid binding activity relative to the effect of removing residues 273-299. No isoform difference in emulsion binding is evident with apoE3 (1-260) and apoE4 (1-260) which lack residues 261-299. Importantly, an isoform effect is seen when either residues 261-272 or 273-299 within the segment 261-299 are present but, opposite to the situation for the full length proteins, apoE4 (1-272) and apoE4 (Δ 261-272) bind less well to lipid emulsion than their apoE3 counterparts (cf. Fig. 3A and B). It follows that these residues contribute more to the emulsion binding of apoE4 than to the emulsion binding of apoE3.

As a basis for understanding the effects of point mutations in different domains of the apoE molecule on the ability to interact with lipoprotein particles (see below), we investigated the ways in which such mutations influence binding to a lipid emulsion (Table 2). Consistent with the results showing that residues 261-299 are critical for lipid binding, mutations in this region (P267A and G278P) reduce binding. The particular importance of the segment spanning residues 261-272 is exemplified by the fact the perturbation via the P267A

mutation in apoE3 drastically reduces binding (cf. (18)). In comparison, the mutation E255A that modifies the interaction between the N- and C-terminal domains in apoE4 (14) has a smaller effect on the level of binding to emulsion. Consistent with the minor contribution of the N-terminal helix bundle domain in lipid binding, the mutations K146E and K146Q in this domain have little or no effect on the ability of apoE3 to bind to emulsion (Table 2).

Distributions of ApoE Variants between VLDL and HDL

The results in Fig. 4 compare the distributions of full length (residues 1-299) apoE3 and apoE4 between human VLDL and HDL₃; experimental conditions were such that approximately equal total VLDL and HDL₃ particle surface areas were available for apoE binding and little or no particle remodeling occurred during the binding (17). The ratio of apoE3 to apoE4 bound to VLDL is ~ 0.45 and the same ratio for HDL₃ is ~ 1.1. The higher apoE3/apoE4 ratio for HDL₃ is consistent with the lipoprotein distribution seen when the isoforms are added to human plasma (10,13,19). The relatively high apoE3/apoE4 ratio in HDL₃ compared to VLDL is also consistent with our prior findings using the same assay but under competitive binding conditions (i.e. the apoE3 and apoE4 were added together rather than individually to the lipoprotein mixture) (17). However, in the competitive binding situation the ability of apoE4 to bind better than apoE3 to VLDL was not apparent; in this case, the isoforms partitioned approximately equally to VLDL. This difference is apparently due to the complication of mixed oligomer formation when the two isoforms are present together; the distribution of a given apoE3 variant between VLDL and HDL₃ is dependent upon the particular competitor that it is paired with. To avoid the confounding issue of the existence of mixed apoE3 and apoE4 oligomers, the single component VLDL/HDL₃ distribution assay was employed in this study. Under these conditions, apoE4 binds more to VLDL than to HDL₃ and apoE3 binds similarly to both lipoproteins (a trend to bind more to HDL₃ did not reach statistical significance) (Fig. 4).

As is seen with apoE binding to lipid emulsion (Fig. 2), deletion of residues 192-299 in apoE3 and apoE4 to give the isolated N-terminal domains (residues 1-191) reduces binding to VLDL (Fig. 4) (cf. (19,26)). The decrease in binding is greater for apoE4 than for apoE3 so that the isoform preference is reversed with apoE4 (1-191) binding less well than its apoE3 counterpart (Fig. 4). Consistent with the C-terminal domain (residues 192-299) being primarily responsible for apoE binding to VLDL, the isolated C-terminal domain binds extremely well to VLDL (Fig. 4) as is seen with emulsion binding (Fig. 2). The strong binding of apoE (192-299) to VLDL has also been observed using surface plasmon resonance (26). Significantly, removal of the C-terminal domain has a relatively minor effect on binding of apoE to HDL₃ (Fig. 4); apoE3 and apoE4 binding is reduced by about one-third and two-thirds, respectively. It is also apparent from Fig. 4 that the isolated N-terminal helix bundle domains (residues 1-191) of both isoforms bind better to HDL₃ than to VLDL. In contrast, the isolated C-terminal domain binds much less well to HDL₃ than to VLDL (Fig. 4).

Previous work has shown that residues 261-299 play a key role in mediating the binding of apoE to lipoprotein particles (13,17,19,26). In order to be able to systematically compare the effects of C-terminal truncation of the apoE3 and apoE4 molecules on lipid binding ability (Fig. 1-3) and lipoprotein binding (VLDL/HDL₃ distribution) we obtained the results summarized in Fig. 5. It is apparent that removal of the N-terminal part of the C-terminal domain (i.e. residues 192-260) does not affect binding of either of apoE3 or apoE4 to VLDL; furthermore, the enhanced binding of the apoE4 variant is maintained. In marked contrast, removal of residues 261-299 (to give the 1-260 variant) greatly reduced binding to VLDL, with the effect being greater for apoE4. This observation confirms that the presence of residues 261-299 is critical for effective VLDL binding. As was seen with emulsion binding (Fig. 3), removal of either residues 261-272 or 273-299 is sufficient to disrupt

binding to VLDL (Fig. 5). No isoform specificity in VLDL binding is evident for either of these apoE variants. Broadly similar effects of altering the C-terminal domain are evident for the binding of the apoE variants to HDL₃, except that the alterations in binding are smaller than seen with binding to VLDL (Fig. 5); this effect has been observed previously using surface plasmon resonance to monitor binding of apoE to these lipoproteins (26). As was seen with binding to VLDL, removal of residues 192-260 from apoE does not impair binding and actually enhances binding of apoE3 to HDL₃. Another significant difference in the effects of C-terminal truncation on binding of apoE to VLDL and HDL is that deletion of residues 273-299 in apoE3 does not impair binding to HDL₃. Inspection of the data in Fig. 5 also reveals that the apoE3 variants bind as well or better to HDL₃ than to VLDL. In contrast, the apoE4 molecules containing residues 261-299 (i.e. variants (1-299) and (Δ 192-260)) bind much better to VLDL than to HDL₃, in marked contrast to the variants lacking the complete segment 261-299 (i.e. variants (1-260), (Δ 261-272) and (1-272)) which bind better to HDL₃ than to VLDL. Overall, the data in Fig. 5 demonstrate that residues 261-299 play a key role in mediating apoE binding to HDL₃ and VLDL particles; their contribution to VLDL binding is larger and they are required for the ability of apoE4 to bind to VLDL better than apoE3 does.

The similarity in the effects of altering the C-terminal domain on binding of apoE to a lipid emulsion (Fig. 2 and 3) and to VLDL (Fig. 4, 5) is consistent with apoE-lipid interactions being dominant in both cases. The fact that modifying the C-terminal domain has different effects on apoE binding to VLDL and HDL is consistent with different interactions mediating the binding to HDL. As we have suggested before, protein-protein interactions may also contribute to the binding of apoE to HDL (26). To further explore this concept, we employed in the VLDL/HDL₃ distribution assay the apoE variants containing point mutations that were investigated for their abilities to bind to lipid emulsions (Table 2). Most of the point mutations have similar consequences for apoE binding to lipid emulsion and VLDL. Notable exceptions are the K146E and K146Q mutations in the helix bundle domain which greatly enhance apoE3 binding to VLDL but not to lipid emulsion (Table 3). A possible explanation for this effect is that, in addition to apoE-lipid interaction, some apoE-apoB interaction contributes to apoE binding to VLDL. Interestingly, the same mutations do not affect apoE3 binding to HDL₃ (Table 3) suggesting that electrostatic interactions between this region of the apoE molecule and apolipoproteins resident in the HDL₃ particle surface are not important for apoE binding.

DISCUSSION

The goal of this study is to understand the mechanisms responsible for the preferences in VLDL and HDL binding exhibited by apoE3 and apoE4. To this end, we have pursued a mutagenesis strategy designed to identify the particular region of the apoE molecule that confers lipid binding ability and compare the properties of this region in the two isoforms. Also, by comparing apoE3 and apoE4 binding to a lipid emulsion on the one hand and VLDL/HDL on the other, we addressed the question of how the lipid-binding capabilities of the two isoforms influence binding to the two types of lipoprotein particles.

Lipid Interactions of ApoE3 and ApoE4

The results in Fig. 1 and Fig. 2 showing that the C-terminal domain (residues 192-299) binds lipids much better than the N-terminal domain (residues 1-191) confirm prior literature (for reviews, see (4,31)). Furthermore, the region spanning residues 261-299 is primarily responsible for this lipid binding ability because deletion of this segment from either apoE3 or apoE4 leads to the same reduction in ability to bind to a lipid emulsion as seen when the entire C-terminal domain is removed (cf. Fig. 2 and 3). The idea that residues 261-299 control apoE binding to lipid is confirmed by the observation that removal of residues

192-260 from the C-terminal domain does not decrease emulsion binding in either apoE3 or apoE4 (Fig. 3). Thus, the entire C-terminal domain is not required for efficient lipid binding. Importantly, relative to apoE3, the emulsion-binding capability of apoE4 is higher and more sensitive to removal of residues 261-299; the latter point is reflected by the fact that the reduction in binding relative to intact apoE4 is larger than the equivalent reduction for apoE3 (Fig. 3) (cf. (17,26)). While both ends of the C-terminal segment spanning residues 261-299 contribute to lipid binding (cf. the results for the Δ 261-272 and 1-272 variants in Fig. 3), the fact that deletion of residues 261-272 induces the greater reduction in emulsion binding (Fig. 3) is consistent with this segment exerting the dominant effect on lipid binding. The contribution of this segment to lipid binding is greater in apoE4 than in apoE3 and the reason for this apparently is that residues 261-272 exert different structural effects in apoE3 and apoE4. The α -helix contents and ANS binding results listed in Table 1 show that, while elimination of residues 273-299 (to give the apoE (1-272) variant) has similar effects in apoE3 and apoE4, elimination of residues 261-272 (to give the apoE (Δ 261-272) variant) exerts different effects in the two isoforms. Namely, the reduction in α -helix content is much larger in apoE3 and the exposure of hydrophobic surface is decreased in apoE3 but increased in apoE4 (Table 1). At this point, it is apparent that residues 261-272 are most involved in the variations in C-terminal domain properties seen between apoE3 and apoE4, as well as in the enhanced lipid binding capability of the latter isoform. However, understanding of the exact structural basis for the effects of C-terminal domain modification on apoE lipid binding properties must await elucidation of the secondary and tertiary structures of the C-terminal domain, and more knowledge of how it interacts with the N-terminal helix bundle domain.

Lipoprotein Interactions of ApoE3 and ApoE4

The fact that apoE4 binds better than apoE3 to VLDL (Fig. 4) raises the question as to whether this effect is a consequence of the enhanced lipid binding ability of apoE4. This seems to be the case because mutations that alter the emulsion binding properties of apoE3 and apoE4 (Fig. 2 and 3) induce parallel effects on VLDL binding (Fig. 4 and 5). The idea that apoE-lipid interactions dominate binding to a VLDL particle is reasonable given that most of the particle surface is covered by PL. Knowing the amounts of PL and protein present in VLDL (diameter 40 nm) and HDL₃ (diameter 8 nm) particles (32) and assuming that the PL occupies 0.65 nm²/molecule (33) and the proteins occupy an average molecular area of 0.15nm²/amino acid residue (34), it can be estimated that ~ 60% of the VLDL particle surface is covered by PL. The greater lipid binding ability of apoE4 compared to apoE3 promotes more binding to this PL surface, explaining the preferential VLDL binding of apoE4 (see the model in Fig. 6). In addition, in apoE4 the interaction of R61 with E255 may stabilize an extended helical structure in the C-terminal domain that is better accommodated on the less curved VLDL surface (14). The apoE-PL surface interaction occurs by a two-state mechanism in which the C-terminal domain binds first followed by opening of the N-terminal helix bundle domain (4,26,28,31); the degree of helix bundle opening probably depends upon the available surface area (23,35).

As far as apoE binding to HDL₃ is concerned, lipid interactions apparently play some role (more obvious with apoE4) because deletion of either the entire C-terminal domain (Fig. 2 and 4) or residues 261-299 (Fig. 3 and 5) has parallel effects on lipid emulsion and VLDL binding on the one hand and HDL binding on the other hand. However, the effects of the deletions are smaller with binding to HDL (Fig. 5) indicating that apoE-lipid interactions play a lesser role in this case. This conclusion is perhaps unsurprising given that the HDL₃ surface is predominantly covered with protein; surface area calculations of the type described above for VLDL indicate that ~80% of the HDL₃ particle surface is protein-covered. Interactions with this protein surface are important for apoE binding and, because

apoE-lipid interactions play only a minor role, the enhanced lipid binding capability of apoE4 does not promote more binding to HDL (Fig. 6). The fact that apoE3 tends to bind better than apoE4 suggests that apoE3 participates in stronger protein-protein interactions in the HDL particle surface. By analogy to the situation with structurally related apoA-I whose interaction with the HDL particle surface has been monitored by fluorescence spectroscopy (36), the N-terminal helix bundle domain of apoE is likely to be involved in interactions with proteins resident on the HDL particle surface.

How are the apoE N- and C-terminal domains involved in the interactions with the apolipoproteins resident on the HDL surface? The apoE C-terminal domain is less important for binding to HDL than for binding to VLDL because the isolated N-terminal domain binds relatively well to HDL₃ but poorly to VLDL (Fig. 4) (cf. (26)). Also, unlike the case with lipid emulsion (Fig. 2) and VLDL binding (Fig. 4) where the isolated C-terminal domain binds more than the isolated apoE3 N-terminal domain, the C-terminal and apoE3 N-terminal domains bind similarly to HDL₃ (Fig. 4). The isolated apoE3 N-terminal domain binds better than its apoE4 counterpart to HDL₃ (Fig. 4) implying that the more stable apoE3 helix bundle participates in stronger protein-protein interactions at the HDL surface. These interactions apparently do not involve electrostatic interactions with basic residues in the LDL receptor binding region of apoE3 (4) because the K146E and K146Q mutations do not inhibit binding to HDL₃ (Table 3).

Additional evidence for the different nature of apoE interactions with the surfaces of VLDL and HDL₃ particles comes from the observation that alterations in the C-terminal domain can have different effects on binding to the two types of lipoprotein particle. For example, deletion of residues 192-260 promotes binding of apoE3 to HDL₃ but not to VLDL (Fig. 5). Along the same lines, deletion of residues 273-299 does not alter apoE3 binding to HDL₃ but decreases binding to VLDL (Fig. 5); the fact that the same deletion in apoE4 does reduce binding to HDL₃ implies that lipid interactions play a larger role with this isoform.

As summarized in the model depicted in Fig. 6, there are two factors that contribute to the selectivity in apoE3 and apoE4 binding to VLDL and HDL₃. 1) The greater lipid binding ability of apoE4 which arises from the reorganized C-terminal domain (especially around the segment spanning residues 261-272) in this isoform. 2) The differences in the nature of the surfaces of VLDL and HDL₃ particles; the former is largely PL-covered whereas the latter is mostly covered by the resident apolipoproteins. In combination, these factors cause apoE4 to bind better than apoE3 to VLDL predominantly via apoE-lipid interactions and apoE3 to bind better to HDL₃ predominantly via apoE-protein interactions. These differential apoE binding effects underlie the variations in plasma VLDL-cholesterol and HDL-cholesterol levels seen in carriers of the apoE3 and apoE4 isoforms, and are responsible for the different cardiovascular disease risk associated with this apoE polymorphism.

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ABBREVIATIONS

ANS	8-anilino-1-naphthalenesulfonic acid
apo	apolipoprotein
DMPC	dimyristoyl phosphatidylcholine

HDL	high density lipoprotein
LDL	low density lipoprotein
MLV	multilamellar vesicles
PC	phosphatidylcholine
PL	phospholipid
VLDL	very low density lipoprotein

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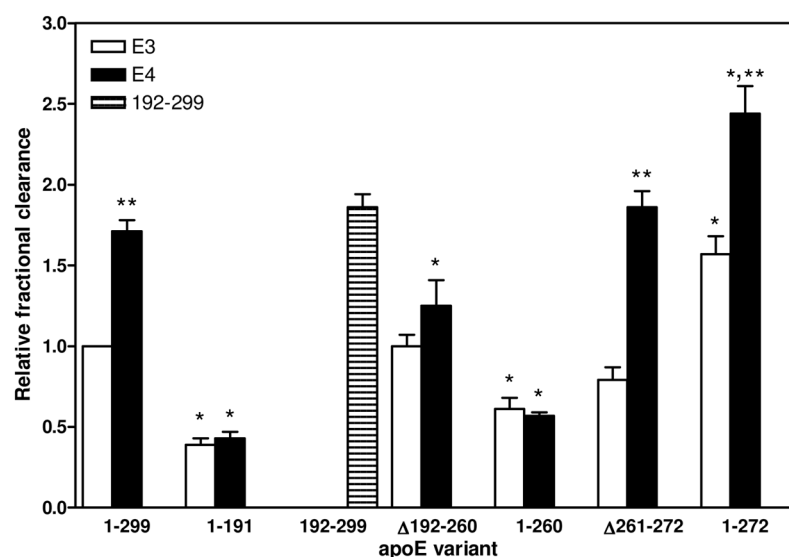


Fig. 1. Influence of truncation of the C-terminal domain of apoE3 and apoE4 on the ability to solubilize DMPC multilamellar vesicles
 Mixtures of DMPC (0.25mg/ml) and apoE variants (0.1mg/ml) were incubated at 24°C and the fraction of turbidity cleared in 10 min measured. The fractional decreases in turbidity are normalized to the value observed with WT apoE3 (1-299). The results are from three to five independent experiments. The open and filled bars are for apoE3 and apoE4 variants, respectively. *Significantly different ($p < 0.001$) from value for respective WT apoE (1-299) (one-way analysis of variance followed by the Tukey multiple comparison test using Graphpad Prism 4.0). **ApoE4 variant significantly different ($p < 0.001$) to equivalent apoE3 variant.

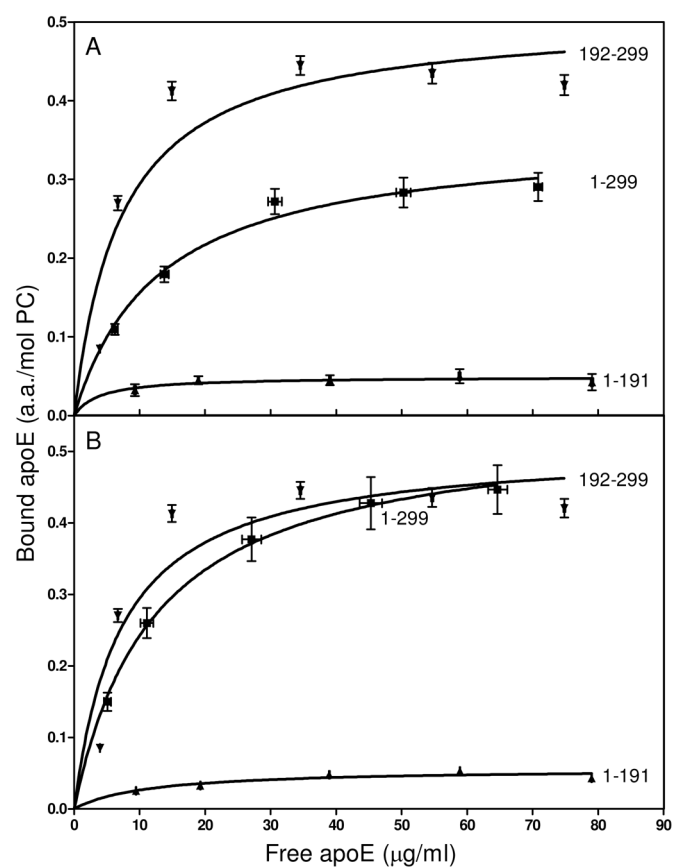


Fig. 2. Binding of apoE N- and C-terminal domains to triolein/PC emulsion particles
 A. Binding isotherms for apoE3 (residues 1-299) (■), 22-kDa apoE3 (residues 1-191) (▲) and 12-kDa apoE3 (residues 192-299) (▼). B. The equivalent binding isotherms for the apoE4 counterparts are depicted.

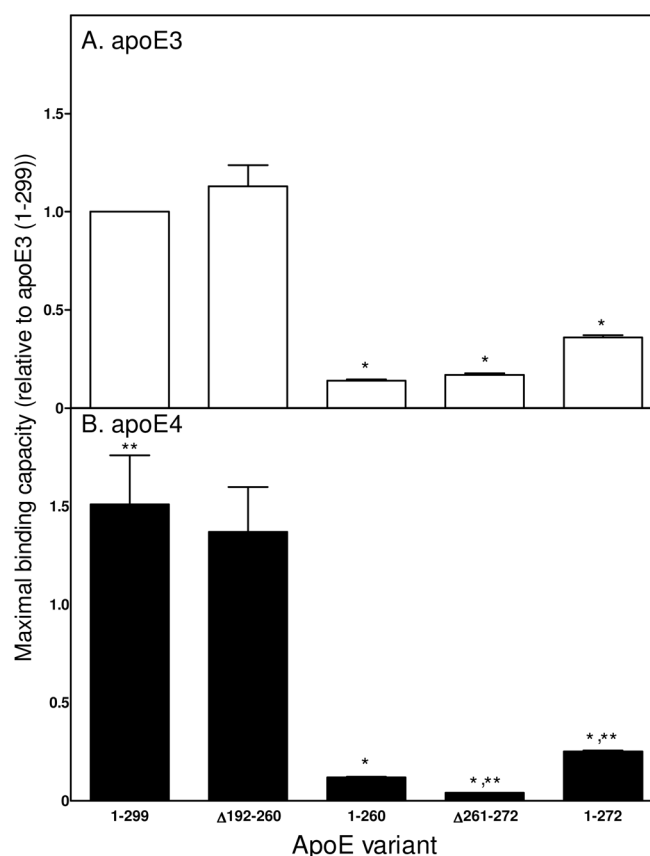


Fig. 3. Binding of apoE3 and apoE4 C-terminal variants to triolein/PC emulsion particles
The binding parameters were derived from isotherms of the type shown in Fig. 2 using a one binding site model. A. Relative maximal binding capacities of apoE3 (1-299), apoE3 (Δ 192-260), apoE3 (1-260), apoE3 (Δ 261-272) and apoE3 (1-272). B. Relative maximal binding capacities of the equivalent apoE4 variants. * Significantly different ($p < 0.001$) from value for WT apoE (1-299) by one-way analysis of variance. ** Significant difference between apoE3 and apoE4 variants.

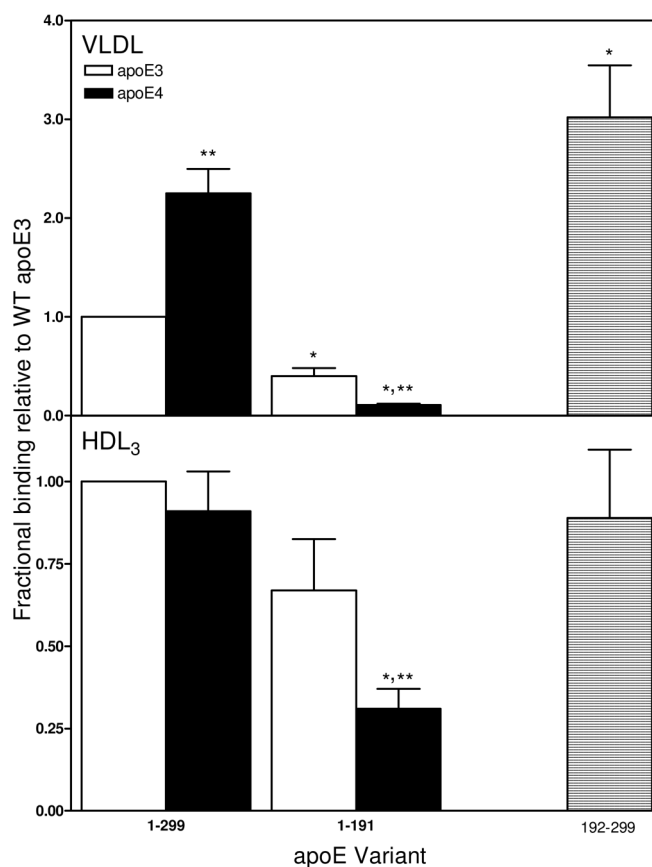


Fig. 4. Binding of apoE N- and C-terminal domains to VLDL and HDL₃

The fractional binding of ¹⁴C-labeled apoE3 and apoE4 domains was determined using the VLDL/HDL₃ distribution assay (see Experimental Procedures). A. Fractional binding to VLDL normalized to the value for apoE3. B. Fractional binding to HDL₃ normalized to the value for apoE3. The open and filled bars are for apoE3 and apoE4 domains, respectively. *p<0.01 compared to full-length (1-299) apoE. ** apoE4 variant significantly different (p<0.01) to equivalent apoE3 variant.

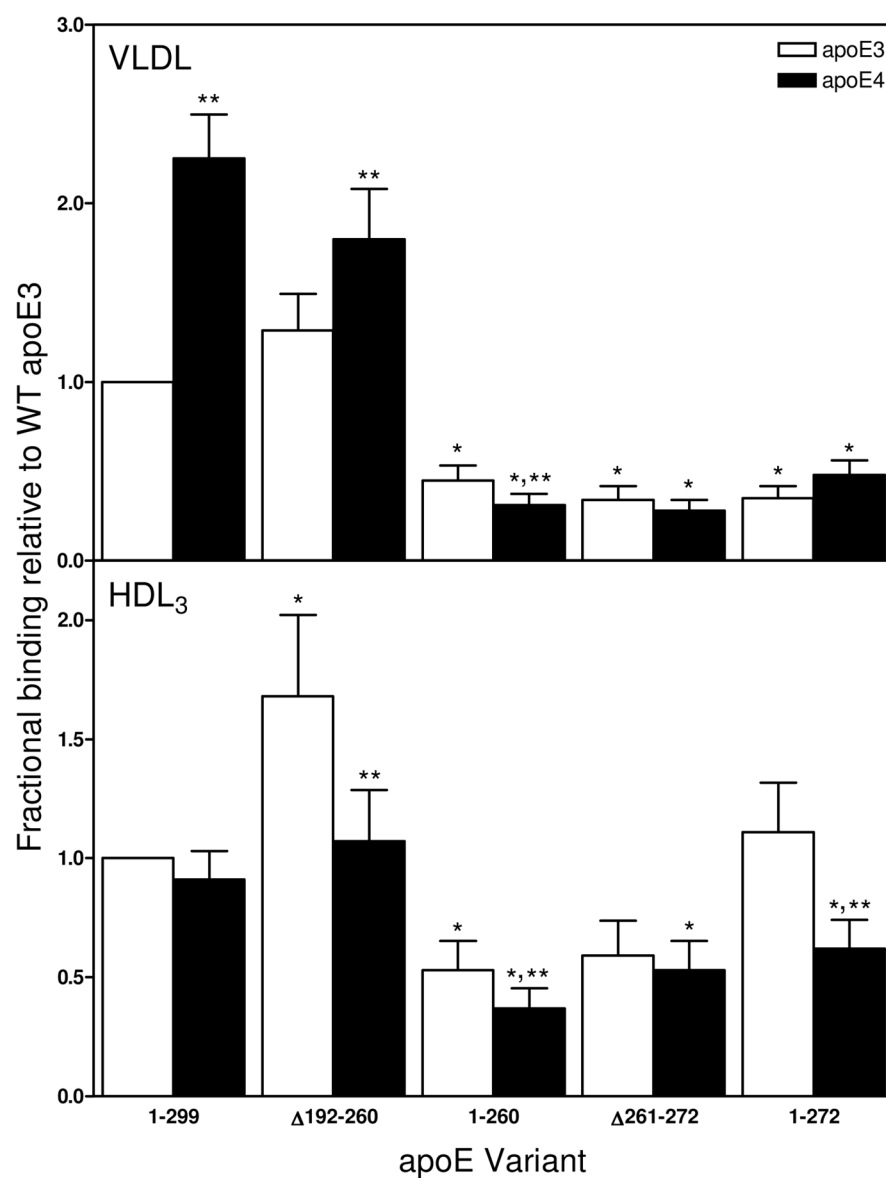


Fig. 5. Binding of apoE3 and apoE4 C-terminal variants to VLDL and HDL₃

The relative fractional binding data to VLDL (A) and HDL₃ (B) were derived as described in the legend to Fig. 4. The open and filled bars are for apoE3 and apoE4 variants, respectively. * $p < 0.05$ compared to full-length (1-299) apoE. ** apoE4 variant significantly different ($p < 0.01$) to equivalent apoE3 variant.

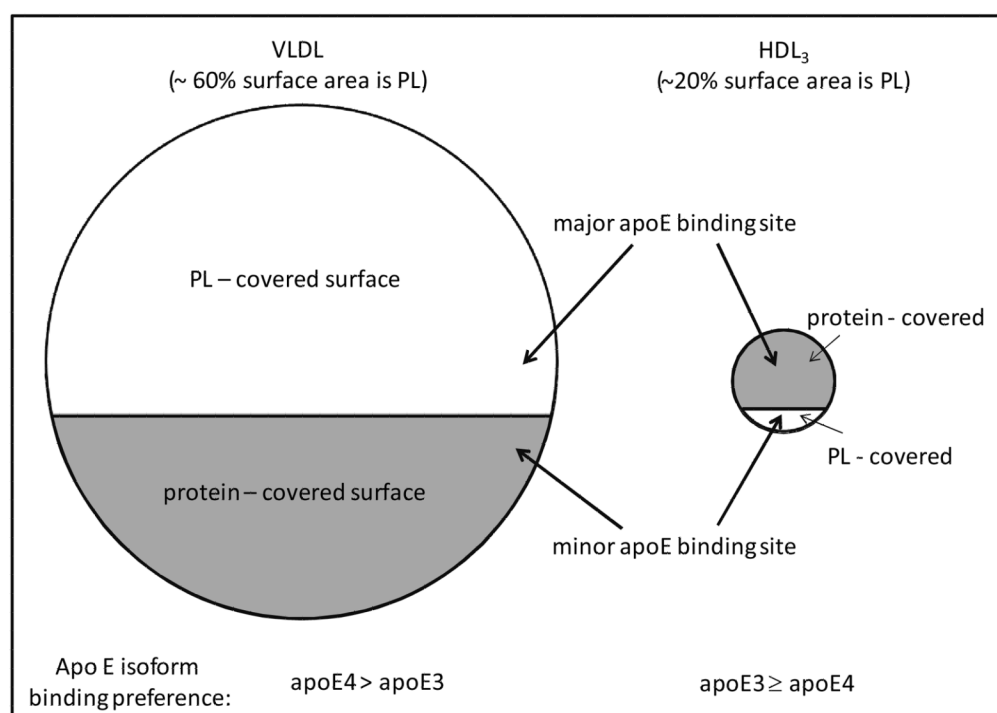


Fig. 6. Model for isoform specificity of human apoE binding to human VLDL and HDL₃
 ApoE4 binds better than apoE3 to lipid surfaces such as that of phospholipid (PL)-stabilized triolein emulsion particles because of C-terminal domain structural differences induced by the C112R substitution that distinguishes apoE4 from apoE3. The lipid binding ability of apoE is particularly mediated by the C-terminal segment spanning residues 261-299, and the better lipid binding ability of apoE4 is a consequence of the region encompassing residues 261-272 being organized differently in apoE4 compared to apoE3. As shown in the diagram, the surface area of a VLDL particle (~40nm diameter) is about 60% PL-covered (see text) so that the higher lipid-binding ability of apoE4 causes it to bind more than apoE3. ApoE-resident apolipoprotein (primarily apoB-100) interactions play a relatively minor role in the binding of apoE to VLDL. In contrast, apoE-resident apolipoprotein (especially apoA-I and apoA-II) interactions are significant for binding of apoE to HDL₃ (8nm diameter) because these proteins cover about 80% of the particle surface. In this case, the protein-protein interaction is mediated primarily by the apoE N-terminal helix bundle domain (residues 1-191) and apoE3 tends to bind better than apoE4 to HDL₃. In the diagram, the relative sizes of the VLDL and HDL₃ particles are shown approximately to scale.

Table 1

α – Helix Content and ANS Binding for ApoE3 and ApoE4 C-Terminal Variants

apoE variant	% α -helix ^a		no. of amino acid residues in α -helix		ANS fluorescence intensity ^b	
	apoE3	apoE4	apoE3	apoE4	apoE3	apoE4
1-299	50 \pm 3	56 \pm 2	150	169	1.0	1.2
1-191	57 \pm 1	61 \pm 1	110	118	0.3	0.4
192-299	59 \pm 1		65		1.1	
Δ 192-260	49 \pm 1	38 \pm 2	114	88	0.8	0.7
1-260	45 \pm 1	38 \pm 1	118	100	0.5	0.5
Δ 261-272	27 \pm 1	46 \pm 1	78	133	0.8	1.3
1-272	42 \pm 1	39 \pm 1	115	107	0.8	0.9

^aMeans \pm SD from at least three measurements.

^bValues are ratios to apoE3 (1-299). Estimated error is within \pm 0.1

Table 2

Influence of Point Mutations in ApoE3 and ApoE4 on Binding to Triolein/PC Emulsion Particles

ApoE variant	relative binding to emulsion ^a
<u>apoE3</u>	
WT	1.0
K146E	0.9 ± 0.1
K146Q	1.2 ± 0.2
P267A	0.2 ± 0.1
G278P	0.7 ± 0.1
<u>apoE4</u>	
WT	1.0
E255A	0.9 ± 0.2
G278P	0.7 ± 0.2

^aMaximal binding of the apoE variants was determined from binding isotherms of the type shown in Fig. 2 and is expressed relative to the value for the respective WT proteins. The values are mean ± SD.

Table 3

Influence of Point Mutations in ApoE3 and ApoE4 on Binding to Lipoprotein Particles

ApoE variant	Relative binding ^a	
	VLDL	HDL ₃
<u>apoE3</u>		
WT	1.0	1.0
K146E	2.1 ± 0.5	1.2 ± 0.3
K146Q	2.6 ± 0.5	1.0 ± 0.2
P267A	0.5 ± 0.1	0.5 ± 0.1
G278P	0.7 ± 0.2	0.6 ± 0.2
<u>apoE4</u>		
WT	1.0	1.0
E255A	0.4 ± 0.1	0.5 ± 0.1
G278P	0.8 ± 0.1	0.6 ± 0.1

^aFractional binding of the apoE variants was determined using a VLDL/HDL₃ distribution assay under defined conditions (see Experimental Procedures) and is expressed relative to the value for the respective WT protein. The values are mean ± SEM.