Association of apolipoprotein E with α_2-macroglobulin in human plasma

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Abstract **Apolipoprotein (apo) E plays a central role in the transport of lipids among different organs and cell types, whereas α_2-macroglobulin (α_2M) is responsible for the binding and inactivation of plasma proteases, as well as the transport of various cytokines, growth factors, and hormones. In the present study, evidence is presented for direct binding of apoE with α_2M in human plasma, based on the observation that two-dimensional non-denaturing gradient gel electrophoretic separation of plasma resulted in co-migration of apoE with α_2M in a complex intermediate in size (18.5 nm in diameter) between low (LDL) and high density lipoproteins (HDL). ApoE associated with α_2M could be immunoprecipitated from plasma with anti-human α_2M antiserum. Purified apoE, labeled with ^{125}I, bound to native and methylamine-activated α_2M (α_2M-MA) in vitro in a time- and concentration-dependent manner. ApoE bound to α_2M-MA with greater affinity than α_2M. The binding of apoE to both α_2M and α_2M-MA did not depend on the presence of lipid. Ingestion of an oral fat load resulted in a reduction in the amount of apoE associated with α_2M. Sphingomyelin vesicles and very low density lipoproteins (VLDL), but not phosphatidylcholine vesicles or HDL$_3$, inhibited the in vitro binding of ^{125}I-labeled apoE3 to α_2M and α_2M-MA. Binding of ^{125}I-labeled apoE3 was also partially inhibited by an excess of platelet-derived growth factor and β-amyloid protein, but not interferon-γ. Subjects with an apoE 4/4 phenotype had less apoE associated with α_2M in plasma than subjects with an apoE 3/3 or 2/2 phenotype, corresponding to reduced in vitro binding of apoE4 with α_2M or α_2M-MA. Although the functional significance of apoE binding to α_2M remains to be determined, the present results demonstrate that: *1*) apoE is non-covalently bound to α_2M in human plasma, *2*) α_2M-MA has a greater capacity to bind apoE than α_2M, *3*) various proteins or lipoproteins known to bind apoE or α_2M can potentially affect the interaction of apoE with α_2M, and *4*) association of apoE with α_2M or α_2M-MA is dependent on apoE phenotype.—**Krimbou, L., M. Tremblay, J. Davignon, and J. S. Cohn. **Association of apolipoprotein E with α_2-macroglobulin in human plasma.** ***J. Lipid Res.*** **1998.** 39: **2373–2386.**

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Apolipoprotein (apo) E (34.2 kD) plays a central role in the transport of lipids among different organs and cell types (1). It is produced in a variety of cells and is a component of a number of circulating plasma lipoproteins, including very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), and high density lipoproteins (HDL). ApoE interacts with cells through its binding to cell membrane heparan sulfate proteoglycans (HSPG) (2), low density lipoprotein (LDL) receptors (3), and/or the LDL receptor-related protein (LRP) (4). It has been implicated in many physiological processes, including plasma cholesterol and triglyceride homeostasis (1), immune reponse (5), protection against oxidation (6), and the control of neuronal growth (7). ApoE is thus believed to play a significant role in the pathophysiology of Alzheimer's disease (8) and in the onset and development of coronary artery atherosclerosis (9).

Three common apoE isoforms in man (apoE2, apoE3, and apoE4) are the result of either cysteine or arginine residues at positions 112 and/or 158 (10). The most common apoE3 isoform has a cysteine at residue 112 and an arginine at residue 158, whereas apoE2 has cysteine residues and apoE4 has arginine residues at both sites. This structural difference betwen apoE isoforms results in greater affinity of apoE4 for VLDL relative to apoE3 or apoE2 (11). In human plasma, apoE is almost entirely associated with lipoproteins containing apoB or apoA-I (12), though several studies have demonstrated the existence of minor lipoprotein subfractions containing apoE as their only apoprotein component (13, 14). These lipoproteins are found to be intermediate in size between LDL and HDL, and have a diameter between 9 and 18.5 nm, as determined by two-dimensional non-denaturing polyacrylamide gradient gel electrophoresis (14).

Abbreviations: apo, apolipoprotein; α_2M, α_2-macroglobulin; α_2M-MA, methylamine-activated α_2-macroglobulin; Aβ, amyloid protein; d, density; DTNB, 5,5-dithiobis-2-nitrobenzoic acid; ELISA, enzyme-linked immunosorbent assay; EDTA, ethylenediamine-tetraacetate; FPLC, fast protein liquid chromatography; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; LRP, LDL receptor-related protein; PBS, phosphate-buffered saline; PCV, phosphatidylcholine vesicles; SMV, sphingomyelin vesicles; TRL, triglyceride-rich lipoprotein; VLDL, very low density lipoprotein.

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We have observed that apoE associated with particles having a diameter of 18.5 nm consistently migrate to the same position in electrophoretic gels as α_2-macroglobulin (α_2M), raising the possibilty that these two proteins are somehow associated in human plasma. α_2M is a homotetrameric glycoprotein (718 kD) with a plasma concentration of 2–4 mg/ml, which is capable of binding and inactivating a wide range of proteases (15). α_2M (in its native and activated forms) also serves as a carrier of various non-proteolytic proteins including different cytokines, growth factors, and hormones (e.g., transforming growth factors (TGF-β) (16), interleukins (IL-1β) (17), platelet-derived growth factor (PDGF) (18), basic fibroblast growth factor (bFGF) (19), tumor necrosis factor-α (TNF-α) (20), interferon-γ (IFN-γ) (21), insulin (22), and, recently, β-amyloid peptide (Aβ) (23)). Although the exact physiological significance of the association of these peptides with α_2M is uncertain, it has been suggested that α_2M plays a role in controlling cellular growth after vascular injury, and in the formation of Aβ-containing senile plaques characteristic of Alzheimer's disease. The association of non-proteolytic proteins with α_2M is distinct from the reaction of proteases with α_2M, which causes a major conformational change in α_2M and the formation of a nondissociable α_2M/protease complex. α_2M that has undergone this conformational change is said to be "activated," and it can subsequently be recognized by the α_2M/LRP receptor. This receptor is also responsible for the hepatic recognition and uptake of apoE-enriched remnant lipoproteins (24) after interaction with HSPGs (2), and has been shown to mediate the promotion of neurite outgrowth by apoE-containing HDL (25). Although several studies have demonstrated the interaction of apoE and α_2M with different sites on the same receptor (26), the physical association of these two proteins has not been previously reported. It was therefore the aim of the present study to provide both in vivo and in vitro evidence for the association of apoE with α_2M, and to demonstrate how this interaction could be affected by native and activated forms of α_2M, by various proteins or lipoproteins known to bind apoE or α_2M, or by apoE isoform.

MATERIALS AND METHODS

Materials

Human α_2M and anti-human α_2M monoclonal antibody were purchased from Biodesign International (Kennebunk, ME). Immunopurified polyclonal anti-human apoE antibody was a generous gift from Genzyme Corp. (Cambridge, MA). Methylamine hydrochloride, bovine brain sphingomyelin, dimyristoyl l-α-phosphatidylcholine, sphingomyelinase, phosphatidylcholine-specific phospholipase C, phosphatidylinositol-specific phospholipase C, and phospholipase A_2 were purchased from Sigma (St. Louis, MO). Protein A bound to agarose was from Bio-Rad Labs. (Hercules, CA). Partially purified anti-human α_2M polyclonal antibody and anti-human IgG polyclonal antibody were purchased from ICN Pharmaceuticals Inc. (Aurora, OH). Recombinant human platelet derived growth factor-BB and recombinant human interferon-γ were purchased from Life Technologies (Gibco BRL Products, Gaithersburg, MD). Lipase from *Rhizopus arrhizus* was purchased from Boehringer Mannheim, FRG. Human β-amyloid peptide 1-40 (Aβ) was obtained from Calbiochem (La Jolla, CA).

Blood sampling

Blood samples analyzed during the course of the present studies were obtained after an overnight fast from 9 male subjects. Four normolipidemic subjects with an apoE 3/3 phenotype acted as controls (plasma cholesterol: 4.3 ± 0.2 mmol/l, plasma triglyceride: 1.0 ± 0.1 mmol/l, HDL cholesterol: 1.2 ± 0.2 mmol/l; mean ± SD). Blood samples were also obtained from 3 individuals with an apoE 2/2 phenotype and 2 individuals with an apoE 4/4 phenotype. Blood was drawn from an arm vein into evacuated tubes containing ethylenediamine-tetraacetate (EDTA, final concentration: 1.5 mg/ml). Blood collection tubes were immediately placed in ice. Plasma was obtained by centrifugation (3000 rpm, 15 min) and was kept in ice until separation of lipoproteins, or was frozen at −70°C until analysis of lipids and apolipoproteins.

Gel electrophoresis

Separation of plasma samples by two-dimensional non-denaturing gradient gel electrophoresis was carried out as described previously (14). Briefly, plasma samples (200 μl) were separated in the first dimension (according to charge) by 0.75% agarose gel electrophoresis (100 V, 8 h, 4°C), and in a second dimension (according to size) by 3–24% polyacrylamide concave gradient gel electrophoresis (125 V, 24 h, 4°C). In some experiments, each gel (15 cm × 15 cm) was used to separate a single sample. In other experiments, up to 6 samples were analyzed together on the same gradient gel. Only the α_2M-containing pre-β_2-migrating segments (~2 cm) of agarose gels were separated in the second dimension, as described previously for the separation of γ-migrating lipoproteins (27). Samples from in vitro binding experiments were separated on 2–36% non-denaturing gradient gels, as described by Asztalos et al. (28). The electrophoretic migration of plasma proteins and lipoproteins was compared to high molecular weight protein standards (Pharmacia, Piscataway, NJ), which were radiolabeled with ^{125}I (29), and incorporated into 0.5-cm slices of agarose (approximately 100,000 cpm per slice) for separation on gradient gels. Alternatively, ^{125}I-labeled standards were added to the outside wells of gradient gels used to separate samples that were not subjected to an initial separation on agarose. The molecular size of apoE-containing particles was determined by comparison with the size of the protein standards using Image Quat software (Molecular Dynamics, Sunnyvale, CA).

Detection of proteins after electrophoresis

Gradient gels were stained for 1 h with 0.1% Coomassie Brilliant Blue in methanol–acetic acid–water 3:1:6 and were destained by several changes of the same solvent, in order to detect proteins after electrophoresis. Immunodetection of specific proteins was achieved by electrotransferring (20 h, 30 V, 4°C) separated proteins with a Trans-Blot System (Bio-Rad Laboratories, Hercules, CA) onto nitrocellulose membranes (Hybond ECL, Amersham Life Science, Buckinghamshire, England). Coomassie Blue staining of gels after electrotransfer confirmed that transfer of proteins was essentially 100%. Non-specific binding sites on membranes were blocked for 30 min with phosphate-buffered saline (PBS) containing 5% non-fat milk powder. Membranes were incubated (3 h) with immunopurified polyclonal anti-apoE or anti-α_2M antibody (Genzyme Corp, Cambridge, MA), which had been labeled with ^{125}I (29). After incubation with antibodies, membranes were washed three times (30 min) with PBS containing 0.05% (v/v) Tween-20 and the presence of

labeled antibodies was detected by autoradiography using XAR-2 Kodak film. In some experiments, films exposed to labeled antibodies were scanned with an IS-1000 Digital Imaging System (Alpha Innotech Corp., San Leandro, CA) and proteins were quantitated by densitometry.

Lipoprotein separations

Plasma samples (1 ml) were separated by automated gel filtration chromatography on an FPLC system (Pharmacia, Uppsala, Sweden). Samples were automatically loaded onto a 50-cm column packed with cross-linked agarose gel (Superose 6 prep grade, Pharmacia) and were eluted with 0.15 M NaCl (pH 7.4) at a rate of 1 ml/min, as described previously (30). VLDL ($d < 1.006$ g/ml) and HDL_3 ($1.12 < d < 1.21$ g/ml) were isolated from normolipidemic plasma by sequential ultracentrifugation using a Beckman ultracentrifuge (Fullerton, CA).

Immunoprecipitation and immunoaffinity procedures

ApoE associated with α_2M was isolated from human plasma by immunoprecipitation. Plasma (500 μl) was incubated (overnight at 4°C) with 200 μl anti-human α_2M antibody (or with control anti-human IgG antibody). In some experiments, the formation of an immunoprecipitate was augmented by the addition of Protein A bound to agarose (30 μl). Immunoprecipitates were centrifuged at 10,000 rpm for 6 min, and washed three times with buffer (20 mM HEPES, pH 7.5, 0.15 M NaCl, 0.1% Triton, and 10% glycerol). They were analyzed by 4–22.5% SDS-polyacrylamide gel electrophoresis, together with molecular weight standards (Pharmacia). Presence of apoE and α_2M in immunoprecipitates was detected by immunoblotting, as described for non-denaturing gradient gels. The efficiency of α_2M immunoprecipitation was assessed as being >95%, based on the absence of α_2M detected in the supernates. Plasma without apoA-I-containing lipoproteins was prepared by immunoaffinity chromatography using anti-apoA-I-antibody bound to latex (Genzyme Corp., Cambridge, MA) (31). Plasma (50 μl) was added to 250 μl of latex suspension, gently mixed for 15 min at room temperature, and then centrifuged at 12,000 rpm for 10 min. The infranate, containing plasma devoid of apoA-I, was concentrated using centricon-10 concentrators (Amicon, Beverly, MA), before being separated by electrophoresis. Less than 1% of total plasma apoA-I was detected by ELISA assay or by electrophoresis in the unbound fraction; furthermore, this residual apoA-I did not co-migrate with apoE-containing particles.

Preparation of apoE isoforms, α_2M-MA, and phospholipid vesicles

Human apoE isoforms (E2, E3, E4) were prepared from VLDL ($d < 1.006$ g/ml) freshly separated from plasma by ultracentrifugation. After delipidation, apoE was separated from other VLDL apolipoproteins by preparative SDS-polyacrylamide gel electrophoresis (32). In certain experiments, α_2M was also isolated from preparative two-dimensional gradient gels by electroelution (where the α_2M was localized visually as a refractive protein mass in gels, as verified by immunoblotting). The purity of apoE isoforms was confirmed by SDS-polyacrylamide gel electrophoresis. Activation of commercially available human α_2M was achieved by incubation at room temperature (3 h) with 200 mM methylamine in 50 mM Tris buffer, pH 8.2, for 3 h (33). After extensive dialysis against 10 mM sodium phosphate, 0.15 M NaCl, pH 7.4, at 4°C (phosphate-buffered saline, PBS), reaction of α_2M with methylamine was verified by observing that the α_2M-MA preparations had "faster" migration in non-denaturing gradient gels (33). For the preparation of phospholipid multilamellar vesicles, phosphatidylcholine or sphingomyelin (20 mg) was dissolved in methanol and the solvent was evaporated with N_2 at room temperature (remaining solvent was removed by vacuum at room temperature). Buffer (5 ml, 0.01 M Tris, 0.15 M NaCl, pH 8.0) was added and samples were warmed to 50°C. Lipids were then vortexed and placed in a low-power sonication bath for 30 min to obtain turbid multilamellar vesicle dispersions.

In vitro binding studies

Iodination of human apoE3, α_2M-MA, and Aβ was performed using IODO-GEN® Iodination Reagent (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril, Pierce Chem. Co., Rockford, IL) (29). Free iodine was removed by PD10 column chromatography and iodinated proteins were dialyzed extensively at 4°C against PBS, pH 7.4. Binding experiments were carried out in plastic Eppendorf tubes, which were blocked with 0.4 mM BSA in PBS containing 3 mM sodium azide (27°C, 24 h) and then for an additional 24 h with 0.1% (v/v) Tween 20 in the same buffer. Tubes were rinsed twice with H_2O immediately before use. Less than 5% of ^{125}I-labeled apoE3 bound non-specifically to tubes prepared in this way. Purified apoE3 (5 μg), purified apoE4 (5 μg) or ^{125}I-labeled apoE3 (0.15–1.3 μg), quantified by ELISA assay, were incubated with native α_2M or α_2M-MA (20–50 μg) in PBS for 3 h at 37°C. The effect of various proteins, lipoproteins, and phospholipid vesicles on binding of apoE to α_2M was determined by adding these substances (as specified for each experiment) to reaction tubes prior to the addition of ^{125}I-labeled apoE3. In certain experiments, samples were delipidated or treated with phospholipases before addition of ^{125}I-labeled apoE3, as described in the caption to Fig. 5. Bound and unbound apoE were separated by non-denaturing gradient gel electrophoresis (28). For determination of dissociation constants, samples were separated on 2.5–18% gradient gels (27) at 60 V (16 h, 15°C), after equilibration of gels at 125 V (30 min, 15°C). No dissociation of ^{125}I-labeled apoE3 bound to α_2M was detected with this electrophoretic system, as assessed by reseparating electroeluted ^{125}I-labeled apoE3/α_2M or ^{125}I-labeled apoE3/α_2M-MA complexes. In experiments using unlabeled apoE, gradient gels were transferred, blocked, and apoE bound to α_2M was immunolocalized and quantitated as described previously. In experiments using ^{125}I-labeled apoE, gels were dried and exposed at −70°C to Kodak X-Omat S film for 1–3 days. Exposed films were used as a template to identify the position of appropriate bands for excision and counting.

Lipid and lipoprotein analyses

Cholesterol and triglyceride concentrations were determined enzymatically on an autoanalyzer (Cobas Mira, Roche). HDL-cholesterol concentration was determined by measuring cholesterol in the supernate after heparin-manganese precipitation of apoB-containing lipoproteins in the $d > 1.006$ g/ml fraction of plasma prepared by ultracentrifugation. Plasma apoB and apoA-I concentrations were measured by nephelometry (Behring Nephelometer 100 Analyzer). ApoE was determined by enzyme-linked immunosorbent assay (ELISA) (30). ApoA-I was determined in immunoaffinity prepared fractions with an in-house ELISA. Plasma HDL-apoE concentration was determined by measuring apoE in the supernate after precipitation of plasma apoB-containing lipoproteins with an equal volume of 13% (w/v) polyethylene glycol 6000 (34). ApoE phenotypes were determined by immunoblotting of plasma separated by minigel electrophoresis (35).

RESULTS

Separation of human plasma by two-dimensional non-denaturing gradient gel electrophoresis has previously been used in our laboratory to investigate apoE-containing lipoproteins in the HDL size range (14, 27). As shown in

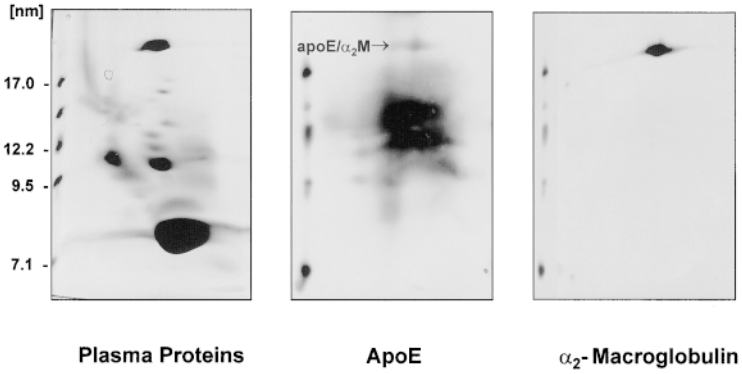

Fig. 1. Separation of human plasma by two-dimensional gradient gel electrophoresis, demonstrating the presence of apoE co-migrating with plasma α_2-macroglobulin (α_2M). Plasma (200 μl) from a normolipidemic subject (with an apoE 3/3 phenotype) was separated according to charge in the first dimension (from left to right) by agarose gel electrophoresis, and then according to size in the second dimension (top to bottom) by polyacrylamide gradient (3–24%) gel electrophoresis. Plasma proteins stained with Coomassie Blue are shown in the left-hand panel, together with molecular weight standards (left-hand lane). ApoE was detected with ^{125}I-labeled anti-apoE antibody after transfer of proteins to a nitrocellulose membrane (middle panel). ^{125}I-labeled molecular weight standards are shown in the left-hand lane. Presence of α_2M was detected by immunoblotting with an ^{125}I-labeled anti-α_2M antibody (right-hand panel). ApoE comigrating with α_2M is indicated by an arrow in the middle panel.

the middle panel of **Fig. 1**, apoE is characteristically found in association with particles having pre-β-mobility and diameters ranging from 9 to 18.5 nm. (Plasma lipoproteins with diameters greater than 30 nm, corresponding to VLDL and IDL, were not detected with this gel system as they were too large to enter the gel.) The majority of apoE evident in the middle panel was associated with lipoproteins representing large HDL; however, it was apparent that several plasma proteins (as depicted in the left-hand panel of Fig. 1) migrated to the same region of the gradient gel as apoE. α_2M was consistently found to co-migrate with a fraction of apoE, having a molecular diameter of 18.5 nm. The position of α_2M is shown in the right-hand panel, detected with ^{125}I-labeled anti-α_2M monoclonal antibody, and apoE migrating in the same position as α_2M is indicated in the middle panel.

ApoE in association with a particle having a diameter of 18.5 nm has been consistently detected in the plasma of more than 50 human individuals. Quantitatively, this apoE represented ~5% of apoE associated with particles in the HDL size range (as determined by densitometric scanning). It was estimated that 0.1 mg/dl (0.029 μmol/l) of apoE (mol. mass: 34.2 kD) was on average associated with 3 mg/ml (4.18 μmol/l) of α_2M (mol. mass: 718 kD) in human plasma (a molar ratio of 1:144), indicating that apoE was associated with about one in every 150 molecules of α_2M in human plasma. ApoE associated with α_2M was also detected in human cerebrospinal fluid and in the medium of cultured human monocyte-macrophages (data not shown). It was assumed that apoE/α_2M complexes in plasma contained only one molecule of α_2M, as the presence of 2 or more molecules of α_2M would have increased their size and caused them to migrate a shorter distance during electrophoresis. Although it could not be ruled out that the association of apoE with α_2M occurred in vitro after blood sampling (i.e., during preparation of plasma from isolated blood, or during separation of plasma by electrophoresis), the amount of apoE co-migrating with α_2M was not different in serum versus plasma of the same individual nor in blood collected in the presence of DTNB or iodoacetate (agents that inhibited disulfide bond formation and the dimerization of apoE). When the plasma of three normolipidemic subjects was separated by automated gel filtration chromatography (a method that does not cause dissociation of apoE from lipoprotein particles (36)), the majority of α_2M and the majority of apoE migrating in the same position as α_2M (top panels, **Fig. 2**) eluted in association with LDL-sized lipoproteins (fraction I), with a lesser amount eluting with HDL (fraction II), pointing out that these complexes are in fact intermediate in size between LDL and HDL. It was also significant that apoE remained in apparent association with α_2M during chromatographic separation by FPLC. This is in contrast to the small amount of apoE that was found to co-migrate with α_2M after electrophoresis of the $d > 1.21$ g/ml fraction of plasma prepared by ultracentrifugation (data not shown), suggesting that the interaction of apoE with α_2M could be affected by ultracentrifugation.

In order to provide evidence for a direct association of apoE with α_2M, a purified anti-α_2M IgG fraction was used to immunoprecipitate α_2M from human plasma. The amount of apoE in this immunoprecipitate was assessed by SDS polyacrylamide gel electrophoresis followed by immunodetection of apoE (**Fig. 3**, panel A). Non-specific association of apoE with the immunoprecipitate was assessed by immunoprecipitating a second aliquot of plasma with non-specific anti-human IgG antibodies. The absence and presence of α_2M, detected by immunoblotting is evident in the control (lane a) and test samples (lane b) in the right-hand panel of Fig. 3A. Considerably more apoE (having an appropriate molecular mass of ~34 kD) was detected in the test versus control samples (left-hand panel, Fig. 3A), demonstrating that apoE was directly associated with α_2M. (The lighter concave band with an apparent molecular mass of 45 kD in the left-hand panel of Fig. 3A represents non-specific binding of ^{125}I-labeled anti-apoE antibody to Protein A, which was added in this experiment to aid the precipitation of α_2M bound to antibody.)

Support for the aforementioned result was provided by the finding that apoE was present in commercial prepara-

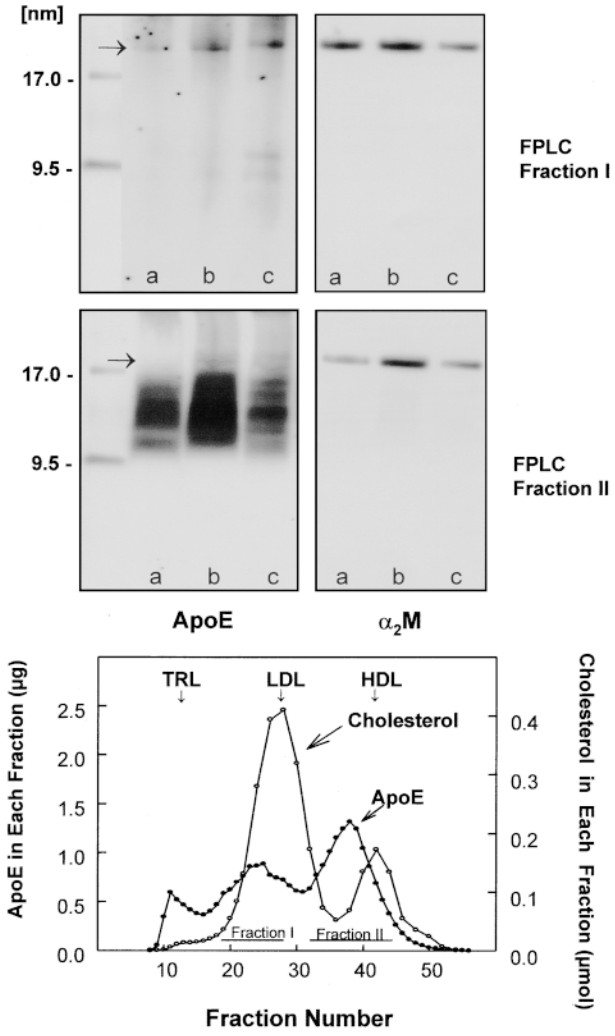

Fig. 2. Presence of apoE and α_2M in plasma separated by automated gel filtration chromatography on an FPLC system. Plasma (1 ml) from three normolipidemic subjects (a–c) with an apoE 3/3 phenotype was separated on a 6% Superose column that was eluted with 0.15 M NaCl (1.0 ml/min). Cholesterol and apoE were measured in each elution fraction (shown for plasma b in bottom panel). Peaks of cholesterol identified the elution of different-sized lipoproteins (i.e., TRL, LDL, and HDL as indicated). Elution fractions were combined to give two pooled Fractions I and II containing apoE associated with LDL- and HDL-sized lipoproteins. Fractions I and II were then concentrated and separated by one-dimensional non-denaturing gel electrophoresis and the presence of apoE and α_2M was detected by immunoblotting (left- and right-hand panels, as indicated). ApoE associated with α_2M is indicated by horizontal arrows in the two top panels.

tions of α_2M, purified by metal chelate chromatography (37). As shown in lane b of the left-hand panel of Fig. 3B, a significant amount of apoE was immunodetectable in commercial α_2M, separated (in this case) by non-denaturing gradient gel electrophoresis. This apoE co-migrated with α_2M present in the preparation (lane b, right-hand panel) and was found to have an apparent diameter of 18.5 nm, as was the case for apoE/α_2M in plasma. The control sample in this experiment (lane a) was α_2M isolated by electroelution from a non-denaturing gel, which contained bound apoE, even after electroelution and extensive dialysis. Methylamine activation of commercial α_2M did not cause dissociation of apoE from α_2M, as shown by the similar amount of apoE co-migrating with α_2M and α_2M-MA (Fig. 3C, left-hand panel, lanes a and b, respectively) after non-denaturing gel electrophoresis. Under non-reducing conditions, SDS gel electrophoresis caused the majority of apoE to dissociate from commercial α_2M (data not shown), providing evidence for the non-covalent association of apoE with α_2M. This did not, however, rule out the possibility that a small proportion of apoE was covalently linked to α_2M. Indeed, SDS polyacrylamide gel electrophoresis of immunoprecipitated or commercial α_2M, in the presence of a reducing agent (2-mercaptoethanol), resulted in more than 90% but not complete dissociation of apoE from α_2M (data not shown).

The binding of apoE to activated (α_2M-MA) and non-activated α_2M was investigated in vitro by incubating ^{125}I-labeled apoE3 at 37°C with different concentrations of α_2M for varying periods of time. Bound and unbound ^{125}I-labeled apoE3 was separated by non-denaturing gel electrophoresis, and ^{125}I-labeled apoE3 associated with α_2M was quantitated by densitometric scanning or direct scintillation counting. ApoE binding was found to occur in a time- and concentration-dependent manner. Maximum binding was reached after 6 h for both α_2M and α_2M-MA, and remained constant for the remaining 18 h of the experiment (data not shown). α_2M-MA had a 2-fold greater capacity to bind apoE3 relative to α_2M (**Fig. 4**). ^{125}I-labeled apoA-II was used as a negative control in these experiments and did not bind to α_2M. ^{125}I-labeled apoA-I, on the other hand, did associate with α_2M (data not shown). The affinity of apoE binding to both forms of α_2M was assessed from a double-reciprocal plot of the concentration-dependent binding data (right-hand panel). Dissociation constants (K_D) were calculated from the slope of the regression lines, and were 2.23 and 1.18 μm for the non-activated and activated forms of α_2M, respectively, indicating that apoE had a greater affinity to bind to α_2M-MA than to α_2M.

In order to verify that the association of apoE with different forms of α_2M was not dependent on the presence of lipid, we determined whether the binding of apoE to α_2M or to α_2M-MA could be affected by phospholipase treatment or delipidation. Phospholipase treatment was chosen as a method for breaking apart complexes dependent on protein–lipid interactions, based on the fact that lipoproteins in the HDL-size range are rich in phospholipids and depend upon these phospholipids for their structural integrity (38). Commercial α_2M (having detectable quantities of bound apoE), as well as MA-activated commercial α_2M (containing a similar amount of apoE, Fig. 3C) were incubated for 12 h at 37°C with phosphatidylcholine-specific phospholipase C (PC-PLC), sphingomyelinase (SM-ase), or were delipidated (3 times) with ethanol–ether 3:1. The presence of apoE bound to different forms of α_2M was then determined after gel electrophoresis (**Fig. 5A**, bottom panel). Neither the specific hydrolysis of phospholipids nor removal of total lipid caused a detectable change in the amount of apoE associated

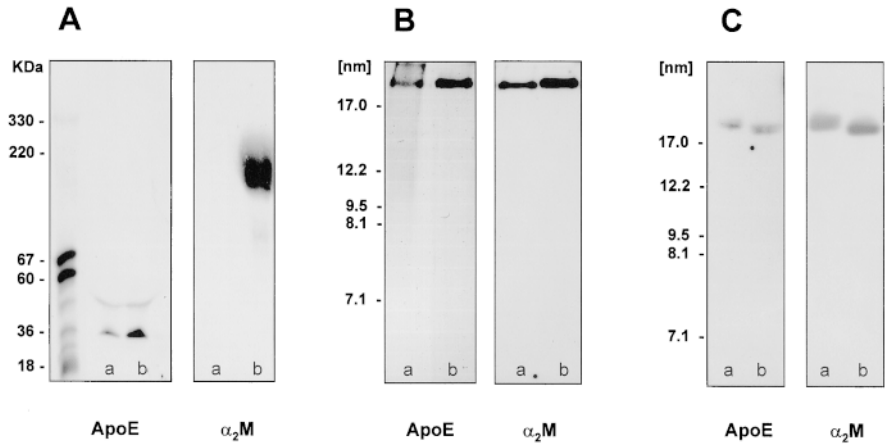

Fig. 3. Association of apoE with α_2M isolated by immunoprecipitation or electroelution, and with commercially available α_2M before and after methylamine treatment. Panel A: normolipidemic plasma (500 μl) was immunoprecipitated with non-specific anti-human-IgG antibodies or with anti-human-α_2M antibodies. Immunoprecipitates were separated by SDS polyacrylamide gradient-gel electrophoresis and apoE and α_2M were detected by immunoblotting; lanes a and b: control anti-IgG and anti-α_2M immunoprecipitates, respectively (migration of ^{125}I-labeled molecular weight markers is indicated). Panel B: α_2M was isolated by electroelution after two-dimensional non-denaturing gel electrophoresis of plasma from a normolipidemic subject; electroeluted α_2M (50 μg) and commercially available α_2M (100 μg) (lanes a and b, respectively) were reseparated by one-dimensional non-denaturing gel electrophoresis, and apoE and α_2M were detected by immunoblotting. Panel C: immunodetection of apoE and α_2M in native (lane A) and methylamine-treated commercial α_2M (lane B) separated by non-denaturing gel electrophoresis.

with either α_2M or α_2M-MA. Interestingly, PC-PLC (but not SM-ase) caused α_2M to become "activated" (as evidenced by the smaller size and increased mobility of α_2M, Fig. 5A); however, this did not cause a reduction in the amount of apoE already bound to α_2M. A second experiment was carried out in order to determine whether binding of apoE could be prevented by prior treatment of α_2M or α_2M-MA with phospholipases or delipidating solvents (Fig. 5B). In fact, no decrease was observed in binding of ^{125}I-labeled apoE3 to treated compared to non-treated α_2M and α_2M-MA preparations. The amount of ^{125}I-labeled apoE3 associated with PC-PLC-treated α_2M actually increased compared to that of untreated or SM-ase-treated α_2M, consistent with the concept that apoE binds more avidly to activated than to non-activated α_2M (Fig. 4). These experiments indicated that the association of α_2M and α_2M-MA with apoE was due to a direct molecule-to-molecule interaction between these molecules, which was not dependent on the presence of lipid.

Similar experiments were carried out to determine the effect of phospholipases on the association of apoE with α_2M in plasma. Incubation of plasma alone for 12 h at 37°C did not result in a significant increase or decrease in apoE associated with α_2M and had no apparent effect on the amount of immunodetectable α_2M (**Fig. 6**). (A second slower-migrating α_2M-reactive band was observed in the experiment shown in the top panels of Fig. 6, as in Fig. 5A, perhaps representing the presence of mannosylated α_2M (39). This band was not affected by incubation alone or by the action of phospholipases.) Phospholipase A2 caused a reduction in the amount of apoE associated with pre-β-migrating particles and a relative increase in apoE associated with smaller-sized particles. The amount of apoE associated with α_2M did not change significantly. In contrast, sphingomyelinase resulted in an almost complete disappearance of apoE from HDL-sized complexes with pre-β-mobility, consistent with previous data showing that sphingomyelinase causes the break-down and disappearance of apoE- as well as apoA-I-containing HDL (40). The amount of apoE associated with α_2M was also significantly reduced. Phosphatidylinositol-specific phospholipase C had little effect, while phosphatidylcholine-specific phospholipase C caused a significant decrease in apoE associated with smaller pre-β-migrating particles and an increase in apoE associated with particles larger than 18.5 nm (Fig. 6). PC-PLC treatment resulted in an increase in the amount of apoE associated with α_2M, consistent with the concept that PC-PLC caused α_2M to become activated (Fig. 5) and consequently bind more apoE (Fig. 4).

As it is well known that the distribution of apoE between plasma lipoproteins changes after the ingestion of a fat-rich meal (resulting in an increase in TRL-apoE and a decrease in HDL-apoE) (41), it was of interest to determine whether this physiological perturbation would affect the amount of apoE associated with α_2M. Thus, 2 normolipidemic subjects with an apoE 3/3 phenotype were given liquid cream to drink (1 gram of fat per kg body weight). Blood samples were obtained in the fasting state (T_{0H}) and then at 2-h intervals for 8 h. ApoE and α_2M were detected by immunoblotting of electrophoretically separated plasma samples, depleted of apoB- and apoA-I-containing lipoproteins (allowing for visualization of apoE not associated with the principle plasma lipoproteins). Results for one subject are shown in **Fig. 7**. Con-

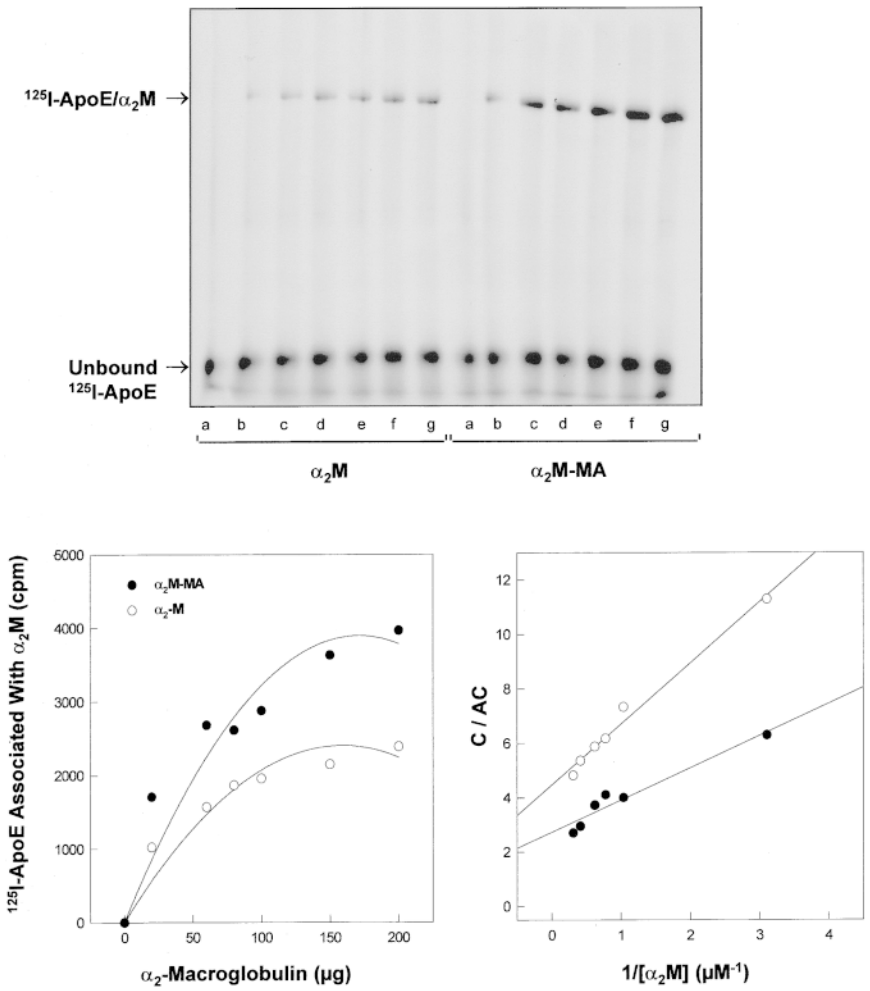

Fig. 4. Concentration-dependent association of ^{125}I-labeled apoE3 with native and methylamine-treated α_2M (α_2M-MA). ^{125}I-labeled apoE3 (150 ng) was incubated for 3 h at 37°C with increasing amounts of α_2M or α_2M-MA (0, 20, 60, 80, 100, 150, 200 μg) and was separated (lanes a to g, top panel) by electrophoresis. ^{125}I-labeled apoE3 associated with α_2M or α_2M-MA was quantitated by scintillation counting of excised bands (left-hand, bottom panel). The affinity of apoE binding to both forms of α_2M was assessed from a double-reciprocal plot of the binding data (right-hand panel). ApoE had a greater affinity to bind to α_2M-MA than to α_2M (K_D = 1.18 and 2.23 μm, respectively). C represents the number of radioactive counts in unbound apoE; AC represents the number of counts in apoE bound to α_2M or α_2M-MA (ref. 43).

centration of α_2M was essentially unchanged during the course of the experiment (upper right-hand panel). Total plasma HDL-apoE concentration, measured by ELISA after precipitation of apoB-containing lipoproteins, decreased postprandially (bottom right-hand panel) and was accompanied by a decrease in all fractions of non-lipoprotein-associated apoE, including apoE associated with α_2M (arrowed in the upper left-hand panel and quantified densitometrically in the lower left-hand panel). This was particularly apparent 2 and 4 h after the fat load. We have determined that about one-third of apoE associated with HDL-sized particles in human plasma is not associated with apoB or apoA-I, and under normal circumstances this apoE is readily transferable to postprandial TRL, similar to apoE associated with apoA-I-containing lipoproteins (data not shown).

The ability of other lipoproteins to affect the binding of apoE to α_2M was investigated in vitro by determining the effect of sphingomyelin vesicles (SMV), as well as phosphatidylcholine vesicles (PCV) on the binding (3 h at 37°C) of ^{125}I-labeled apoE3 to native and activated forms of α_2M (**Fig. 8**, left-hand panel). Incubation of ^{125}I-labeled apoE3 with phospholipid vesicles alone resulted in the appearance of apoE-containing particles (20–23 nm) smaller in size than normal LDL (24–27 nm). More apoE was associated with SMV than PCV. No particle with the same size as apoE/α_2M or apoE/α_2M-MA was evident in these samples. SMV reduced (although not completely) the association of ^{125}I-labeled apoE3 with α_2M and α_2M-MA, while PCV had little effect. Similar experiments were carried out with human VLDL and HDL_3 (Fig. 8, right-hand panel). VLDL but not HDL_3 was found to inhibit the association of ^{125}I-labeled apoE3 with both α_2M and α_2M-MA. Addition of lipase to samples containing VLDL re-

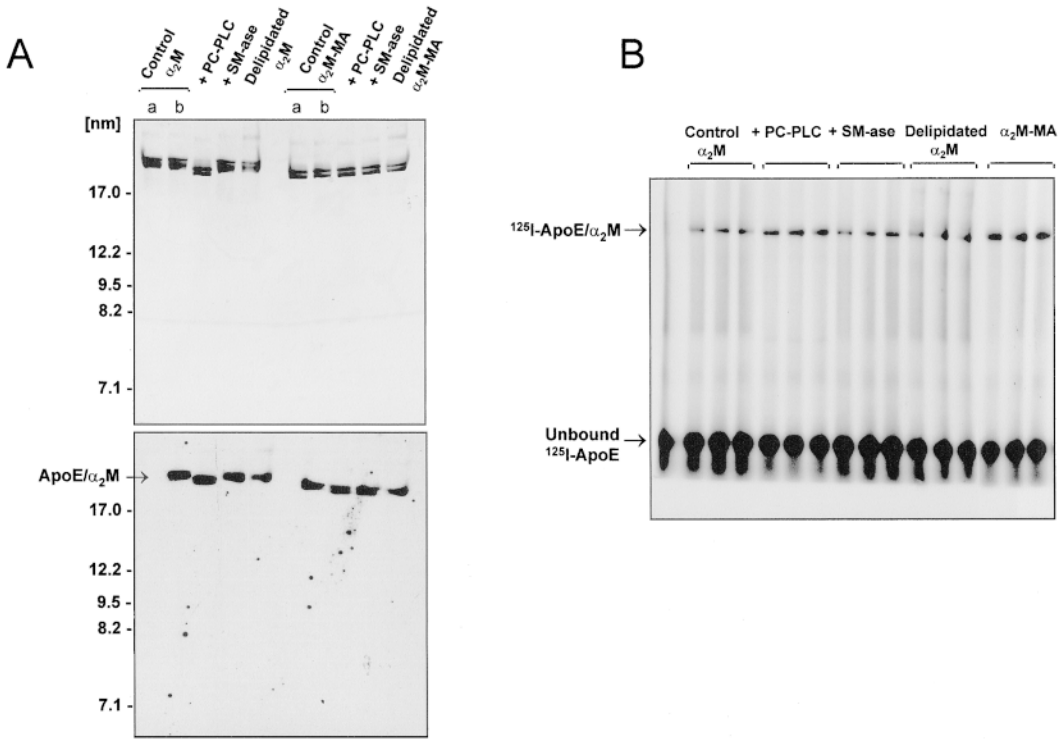

Fig. 5. Effect of phospholipase treatment or delipidation on the in vitro binding of apoE to α_2M and α_2M-MA. A: Commercial α_2M (having detectable quantities of bound apoE) as well as MA-activated commercial α_2M (containing a similar amount of apoE) were incubated for 12 h at 37°C with phosphatidylcholine-specific phospholipase C (PC-PLC), sphingomyelinase (SM-ase), or were delipidated (3 times) with ethanol–ether 3:1. Preparations of α_2M (80 μg) were incubated with 5 U phospholipase/100 μg protein, and excess phospholipase was separated from α_2M with 500 kD exclusion filters. Samples were separated by gradient gel electrophoresis and the presence of α_2M or α_2M-MA was detected with Coomassie Blue staining (A, top panel). Lanes "a" contained non-incubated α_2M or α_2M-MA; lanes "b" contained α_2M or α_2M-MA incubated for 12 h at 37°C in the presence of 30 μl of TBS alone. The presence of apoE was detected by immunoblotting with ^{125}I-labeled anti-apoE antibody (A, bottom panel). No sample was added in lanes "a" (bottom panel). B: Binding of ^{125}I-labeled apoE3 to α_2M or α_2M-MA after treatment of α_2M preparations with PC-PLC, SM-ase, or after delipidation (3 times) with ethanol–ether 3:1. ^{125}I-labeled apoE3 bound to α_2M or α_2M-MA is indicated, and was clearly separated from unbound ^{125}I-labeled apoE3.

sulted in the appearance of apoE associated with particles somewhat larger than α_2M and α_2M-MA, but did not result in consistent evidence of ^{125}I-labeled apoE3 bound to α_2M or α_2M-MA. (^{125}I-labeled apoE3 in bands situated in the middle of the gels shown in Fig. 8, below α_2M or α_2M-MA, represent dimerized and aggregated forms of apoE, and in the case of samples containing HDL_3, represent apoE bound to these added lipoproteins.)

Having determined that certain phospholipid and lipoprotein particles with a high affinity to bind apoE had the capacity to inhibit the association of apoE with different forms of α_2M, the question was posed whether known ligands of α_2M could have a similar effect. The results of in vitro binding experiments with platelet-derived growth factor (PDGF-BB), interferon-γ (INF-γ), and β-amyloid protein (Aβ) are shown in **Fig. 9**. INF-γ had no effect and PDGF only a partial effect on the association of ^{125}I-labeled apoE3 with α_2M or α_2M-MA. Despite clear evidence of binding of ^{125}I-labeled Aβ to both α_2M or α_2M-MA (Fig. 9, right-hand panel), unlabeled Aβ was able to only partially inhibit the association of ^{125}I-labeled apoE3 with α_2M-MA. Increasing the amount of Aβ 5-fold, in order to have a 50-fold excess of Aβ compared to apoE, resulted in greater inhibition of apoE/α_2M-MA formation (data not shown).

All experiments described up to this point had been carried out with the apoE3 isoform, and we therefore posed the question whether interaction of apoE with α_2M could be affected by apoE isoform. The amount of apoE associated with α_2M was determined in the plasma of patients homozygous for different apoE phenotypes. Results for six individuals are shown in **Fig. 10**. In the general population, subjects with an apoE 2/2 phenotype characteristically have a higher plasma total and HDL apoE concentration than apoE 3/3 individuals, who in turn tend to have higher apoE concentrations than apoE 4/4 subjects (42). Subjects were selected who corresponded with these differences, and subsequently, the plasma HDL apoE concentrations for the pairs of subjects in Fig. 10 with apoE 2/2, apoE 3/3, and apoE 4/4 phenotypes, were: 2.5 and 2.3, 1.9 and 1.3, and 1.0 and 1.0 mg/dl, respectively. These concentrations are reflected by the different amounts of immunodetectable apoE found in the pre-β-migrating HDL for each subject. ApoE associated with α_2M is indicated by an arrow. Although the subjects appeared to have similar levels of plasma α_2M (Fig. 10, top right-hand panel), patients with an apoE 4/4 phenotype tended to have significantly less apoE bound to α_2M. In order to ensure that this was due to less apoE directly asso-

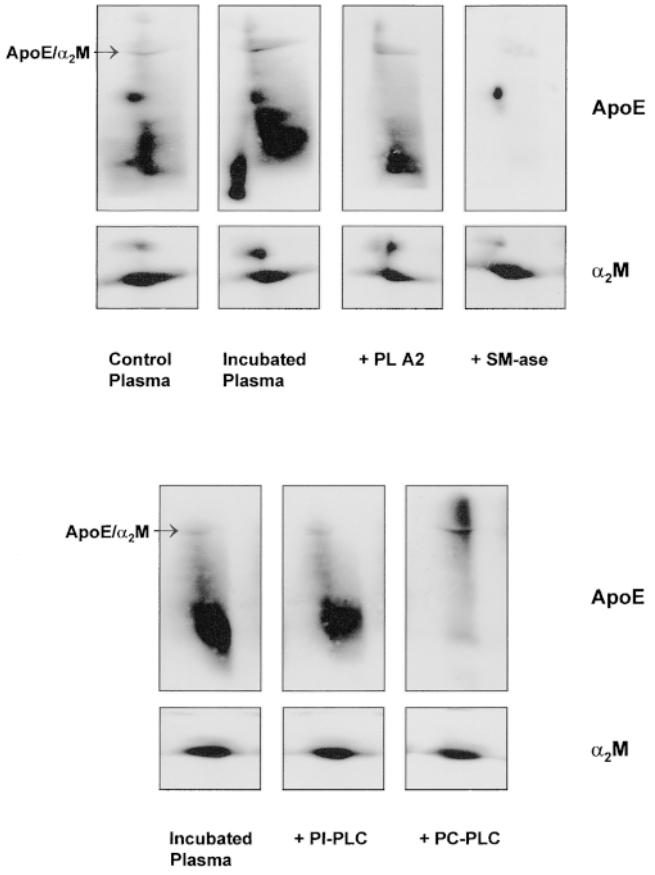

Fig. 6. Effect of phospholipase treatment on the association of apoE with α_2M in human plasma. Upper panels (left to right): plasma (100 μl) was: *a*) kept in ice (12 h) with Tris-buffered saline (TBS, 30 μl, pH 7.4), *b*) incubated for 12 h at 37°C with TBS (30 μl), *c*) incubated for 12 h at 37°C with 10 units phospholipase A2 (PL A2), or *d*) incubated with 10 units of sphingomyelinase (SM-ase) before being separated by two-dimensional gel electrophoresis. ApoE and α_2M in the pre-β-migrating region were detected by immunoblotting, as indicated. ApoE associated with α_2M is indicated. Lower panels (left to right): plasma (100 μl) was: *a*) incubated for 12 h at 37°C with TBS (30 μl), *c*) incubated for 12 h at 37°C with 8 units phosphatidylinositol-specific phospholipase C (PI-PLC), or *d*) incubated with 10 units of phosphatidylcholine-specific phospholipase C (PC-PLC), before being separated by two-dimensional gel electrophoresis.

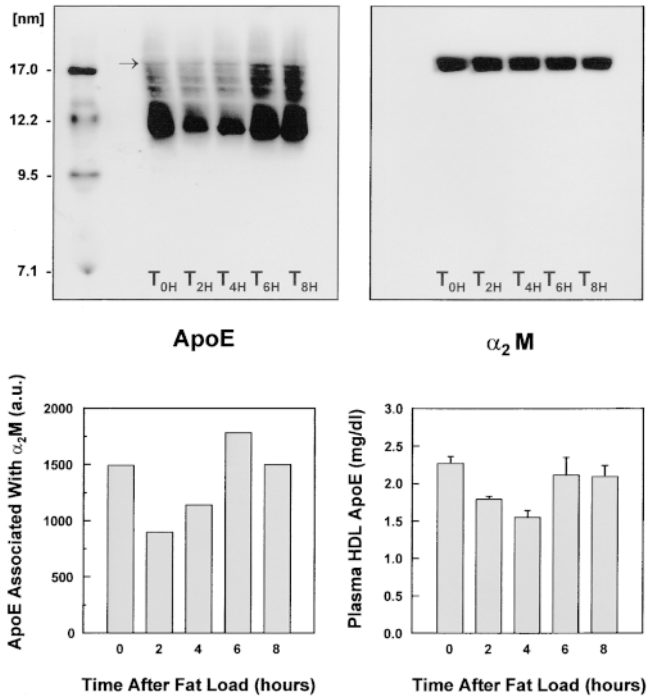

Fig. 7. Effect of an oral fat load on apoE associated with HDL-sized lipoproteins and with α_2M. After an overnight fast, normolipidemic subjects (n = 2) with an apoE 3/3 phenotype were given liquid cream to drink (1 gram of fat per kg body weight). Blood samples were obtained in the fasting state (T_{0H}) and then at 2-h intervals for 8 h. Plasma was depleted of apoB-containing lipoproteins by precipitating them with polyethylene glycol 6000, and then depleted of apoA-I-containing lipoproteins by immunoaffinity precipitation using anti-apoA-I antibody-bound latex. Plasma samples were then separated by one-dimensional non-denaturing gel electrophoresis, and apoE and α_2M were detected by immunoblotting (results for one subject are shown). The hydrated diameters of molecular standards are indicated in the top left-hand panel. ApoE associated with α_2M was quantitated by densitometric scanning. ApoE associated with HDL was quantitated (±SD, n = 3) in plasma samples precipitated with polyethylene glycol.

ciated with α_2M and not simply a reduced amount of co-migrating HDL-apoE, α_2M was immunoprecipitated from plasma with non-specific anti-human IgG antibodies or with anti-human-α_2M antibodies. Co-precipitated apoE was assessed by SDS polyacrylamide gel electrophoresis followed by immunodetection (Fig. 10, bottom left-hand panel). Non-specific association of apoE with the immunoprecipitate is shown in lane a for one of the apoE 3/3 samples only. The absence and presence of α_2M in respective samples are shown in the bottom right-hand panel. Consistent with the result described in the upper panel, significantly less apoE was found in the immunoprecipitate from apoE 4/4 subjects compared to that of subjects with an apoE 2/2 or apoE 3/3 phenotype. (The lighter concave band present in the left-hand panel of Fig. 3A was not present in this experiment as the immunoprecipitation was carried out in the absence of Protein A.)

In order to determine whether this effect of apoE isoform was specifically due to a difference in the interaction of apoE isoforms with α_2M, in vitro binding experiments were carried out with purified apoE3 and apoE4. In contrast to experiments described in Fig. 4, apoE isoforms were not radioiodinated, so as to reduce the possibility of introducing artefactual differences caused by variability in radiolabeling. ApoE which bound to α_2M and α_2M-MA was thus determined by immunodetection with ^{125}I-labeled anti-apoE antibody. Results in triplicate are shown in the upper panel of **Fig. 11**. Lanes a and b (containing α_2M and α_2M-MA, respectively, without added apoE) acted as controls. Although apoE was present in this commercial preparation of α_2M (as described in Fig. 3), no apoE band was visible at the level of sensitivity required in this experiment. ApoE4 bound less effectively than apoE3 to both forms of α_2M, and both apoE4 and apoE3 bound

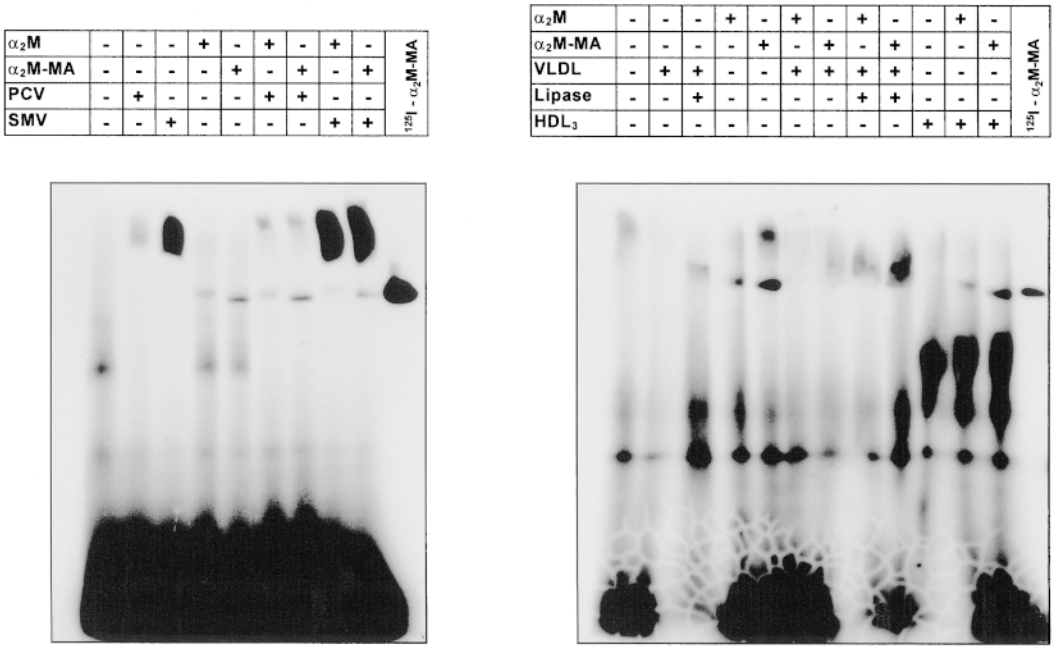

Fig. 8. Effect of phospholipid vesicles, VLDL, and HDL_3 on the binding in vitro of ^{125}I-labeled apoE3 with native and methylamine-treated α_2M. Left-hand panel: ^{125}I-labeled apoE3 (1.3 μg) was incubated for 3 h at 37°C with different combinations of α_2M (20 μg), α_2M-MA (20 μg), phosphatidylcholine vesicles (65 μg) and/or sphingomyelin vesicles (65 μg) in a final volume of 120 μl. ^{125}I-labeled apoE3 complexes were separated on a non-denaturing gradient gel and were visualized by radiography. The control lane on the far right contained ^{125}I-labeled α_2M. Right-hand panel: ^{125}I-labeled apoE3 (1.3 μg) was incubated for 3 h at 37°C with different combinations of α_2M (50 μg), α_2M-MA (50 μg), VLDL (2 mmol triglyceride) with or without bacterial lipase (100 units), and/or HDL_3 (20 μg protein), in a final volume of 120 μl. The control lane on the far right contained ^{125}I-labeled α_2M-MA.

less effectively to α_2M compared to α_2M-MA (consistent with data in Fig. 4 for ^{125}I-labeled apoE). Under the conditions and ligand concentrations of this experiment (see legend to Fig. 11), apoE isoforms were about 2-times more effective in their binding to α_2M-MA than to α_2M, and binding of apoE3 was about 2-times more effective than that of apoE4 (Fig. 11, bottom panel). In a separate experiment, apoE2 was found to bind to an equal extent as apoE3 to α_2M (data not shown).

DISCUSSION

Evidence has been obtained in the present study for the presence of apoE in human plasma bound to α_2M. Two-dimensional non-denaturing gradient gel electrophoresis of human plasma resulted in the separation of an apoE-containing complex intermediate in size between LDL and HDL (18.5 nm in diameter), which co-migrated with plasma α_2M (Fig. 1). Isolation of α_2M by gel filtration chromatography, electroelution, or immunoprecipitation resulted in co-isolation of apoE (Figs. 2 and 3). Furthermore, commercial preparations of α_2M (isolated by metal-chelate chromatography) were consistently found to contain detectable amounts of apoE. The physiological association of apoE with α_2M in plasma was supported by in vitro experiments, showing that apoE bound to α_2M in a time- and concentration-dependent manner (Fig. 4).

We have found that the binding of apoE to α_2M does not depend on the presence of lipid molecules (Fig. 5) and is apparently non-covalent in nature. This conclusion is supported by the finding that: *1*) under non-reducing conditions, SDS gel electrophoresis caused the majority of apoE to dissociate from isolated α_2M, and *2*) ingestion of an oral fat load caused an apparent dissociation of apoE from α_2M, as evidenced by a reduction in the amount of apoE co-migrating with α_2M (Fig. 7). Plasma concentration of TRL apoE increases and HDL apoE decreases after the ingestion of a fat-rich meal, due in large part to transfer of apoE from HDL to TRL (41). Evidence presented in Fig. 7, demonstrates that all HDL-sized particles devoid of apoB or apoA-I participate in this transfer, including apoE associated with α_2M. Apparently, the affinity of apoE binding to TRL is greater than the affinity of apoE binding to α_2M and other HDL-sized particles, consistent with the finding that VLDL, but not HDL_3, inhibited the in vitro association of ^{125}I-labeled apoE3 with both α_2M and α_2M-MA (Fig. 8). ApoE associated with α_2M is thus readily transferable and is apparently part of a metabolically active pool of apoE associated with HDL-sized particles.

Our results have demonstrated that the binding of apoE with α_2M was dependent on the native versus activated state of α_2M. In experiments with both labeled and unlabeled apoE (Figs. 4 and 11), α_2M activated with methylamine (α_2M-MA) was found to have a 2-fold greater capacity to bind apoE compared to α_2M. α_2M-MA also had a greater affinity to bind apoE compared to α_2M (K_D = 1.18 and 2.23 μm, respectively). Activation of purified α_2M (or

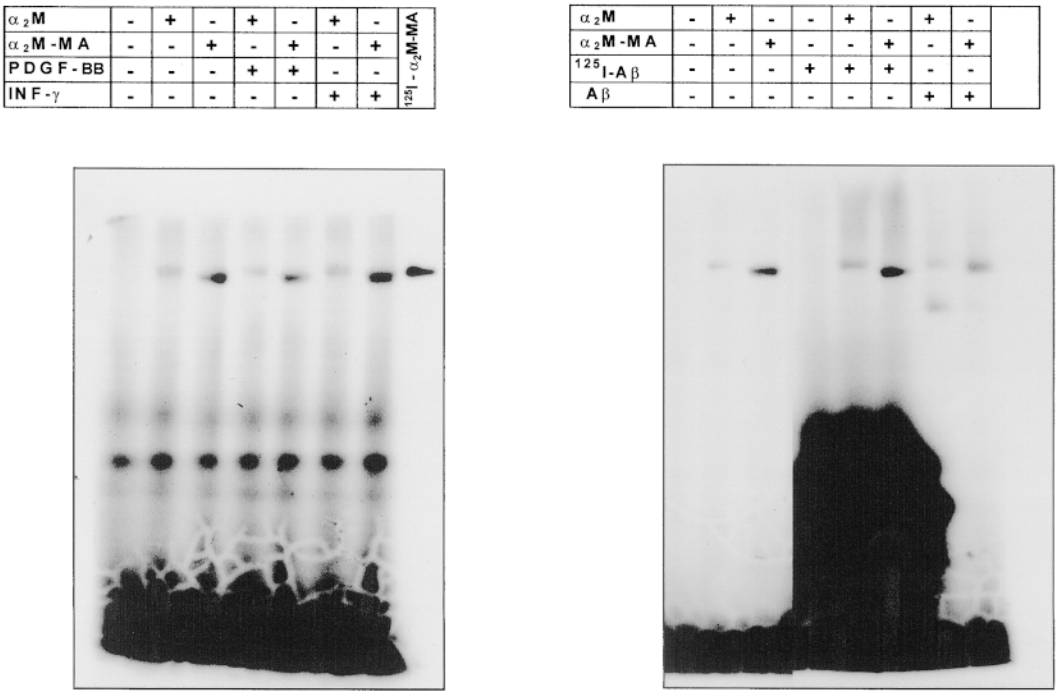

Fig. 9. Effect of platelet-derived growth factor (PDGF-BB), interferon-γ (INF-γ) and β-amyloid protein (Aβ) on the in vitro binding of ^{125}I-labeled apoE3 with native and methylamine-treated α_2M. Left-hand panel: ^{125}I-labeled apoE3 (0.5 μg) was incubated for 3 h at 37°C with different combinations of α_2M (50 μg), α_2M-MA (50 μg), PDGF-BB (5 μg) and/or INF-γ (5 μg) in a final volume of 120 μl. ^{125}I-labeled apoE3 complexes were separated on a non-denaturing gradient gel and were visualized by radiography. The control lane on the far right contained ^{125}I-labeled α_2M. Right-hand panel: ^{125}I-labeled apoE3 (0.7 μg) was incubated for 3 h at 37°C with different combinations of α_2M (50 μg), α_2M-MA (50 μg), and Aβ (7 μg) in a final volume of 120 μl. Binding of ^{125}I-labeled Aβ (1.2 ng) to α_2M and α_2M-MA is shown in the middle lanes as indicated.

α_2M in plasma) by addition of PC-PLC also caused an increase in apoE binding to α_2M (Figs. 5 and 6). Previous studies have shown that various cytokines and growth factors (e.g., TGF-β1, NGF-β, PDGF, bFGF, and TNF-α) bind to α_2M-MA with greater affinity than to native α_2M (43). TGF-β2 is the only growth factor studied to date that binds native and α_2M-MA with equal affinity, while IL-1β is capable of binding to only α_2M-MA (17). These molecules do not bind to α_2M through its well-characterized proteinase-trapping mechanism, whereby α_2M peptide bonds are cleaved and exposed thiol ester bonds are subsequently broken to allow for covalent linkage of α_2M to the trapped proteinase (44). Instead, these cytokines and growth factors bind: *1*) non-covalently and reversibly to different forms of α_2M, *2*) covalently by thiol-disulfide exchange, or *3*) covalently at the exact instant that thiol ester bonds are exposed by a trapped proteinase (43). As we have found that the majority of apoE binding to α_2M is non-covalent and reversible, we hypothesize that the first of these mechanisms is most likely to apply to apoE. The observation that apoE has a greater capacity to bind to α_2M-MA than to α_2M raises the possibility that apoE may facilitate the plasma clearance of activated α_2M. One can speculate that the association of apoE, which has a high affinity for heparan sulfate proteoglycans (HSPG) (2), could be involved in the initial liver cell binding of α_2M (which is not a heparin-binding protein (45)), allowing α_2M to be brought into close proximity for interaction with the α_2M/LRP receptor. We have estimated that apoE is associated with approximately 1 in every 150 molecules of α_2M in circulating plasma. Thus, although only a small proportion of total plasma α_2M molecules (less than 1%) are associated with apoE, this may represent a very metabolically active pool of α_2M. The effect of apoE on α_2M and α_2M-MA interaction with HSPG and the α_2M/LRP receptor is currently under investigation.

Additional evidence for the non-covalent association of apoE with α_2M was provided by the observation that in vitro incubation of plasma with sphingomyelinase (SM-ase), but not other phospholipases, caused a significant reduction in apoE associated with α_2M (Fig. 6). We have interpreted this result as evidence that SM-ase led to the formation of lipid products which combined to form larger lipid complexes or, alternatively, induced lipoproteins to fuse and/or aggregate (46). These large lipid particles preferentially bound apoE, which was non-covalently bound to plasma α_2M. They were, however, too large to enter and hence be detected in 3–24% sizing gels. Evidence for the preferential binding of apoE to lipid vesicles is provided by results in Fig. 8, showing that sphingomyelin vesicles, and to a lesser extent phosphatidylcholine vesicles, were able to inhibit the in vitro association of ^{125}I-labeled apoE3 with α_2M. PC-PLC treatment of plasma also resulted in the transfer of apoE from smaller to larger lipoproteins. PC-PLC, however, caused α_2M in plasma to become "activated," as shown (Fig. 5) by the change in

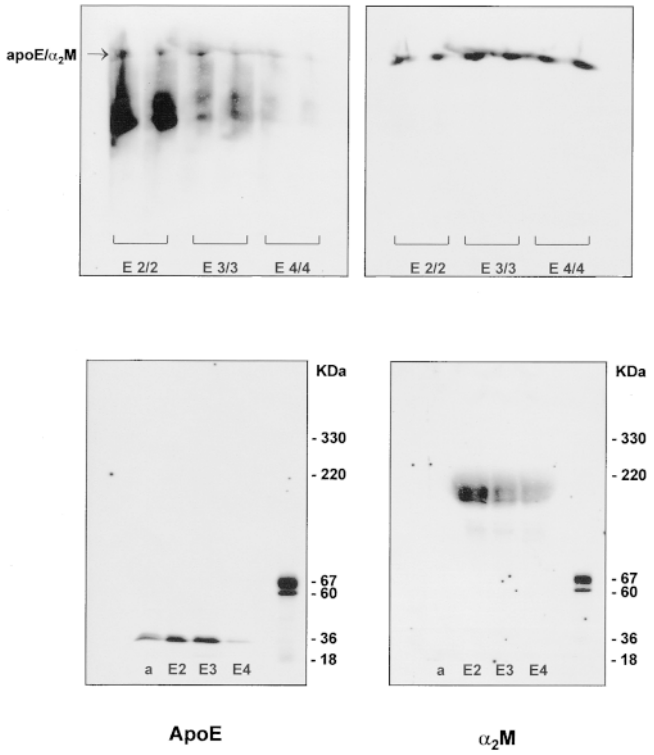

Fig. 10. Association of apoE with α_2M in plasma from individuals with different apoE phenotypes. Upper panels: plasma was obtained from two subjects with an apoE 2/2 phenotype, two subjects with an apoE 3/3 phenotype, and two subjects with an apoE 4/4 phenotype, having plasma HDL apoE concentrations of 2.5 and 2.3, 1.9 and 1.3, and 1.0 and 1.0 mg/dl, respectively. Plasma samples (200 μl) were separated by agarose gel electrophoresis, and gel slices containing pre-β_2-migrating material were excised and reseparated together on a non-denaturing gradient gel. ApoE and α_2M were detected by immunoblotting with ^{125}I-labeled antibodies (left- and right-hand panels, respectively); apoE associated with α_2M is indicated by an arrow. Lower panels: plasma (500 μl) from subjects with an apoE 2/2, 3/3, or 4/4 phenotype were immunoprecipitated with non-specific anti-human-IgG antibodies or with anti-human-α_2M antibodies. Immunoprecipitates were separated by SDS polyacrylamide gradient-gel electrophoresis and apoE and α_2M were detected by immunoblotting (left- and right-hand panels, respectively; non-specific immunoprecipitation of apoE by anti-human-IgG antibodies is shown for apoE3 sample only: labeled "a"). Migration of ^{125}I-labeled molecular mass markers is indicated on the right.

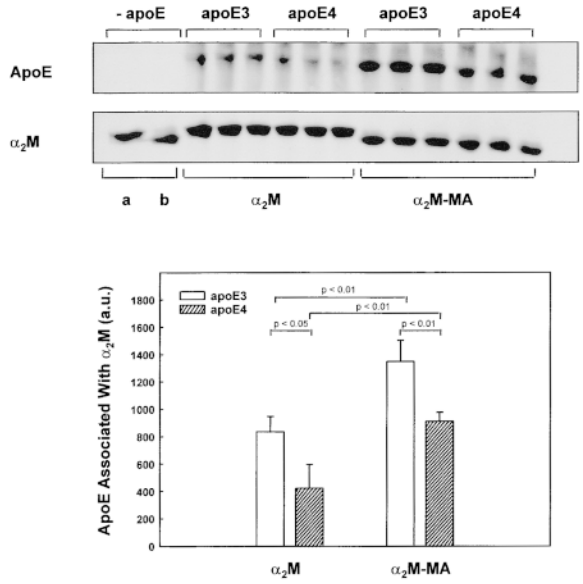

Fig. 11. Association of apoE3 and apoE4 with native and methylamine-treated α_2M in vitro. ApoE3 or apoE4 (5 μg) were incubated with α_2M or α_2M-MA (50 μg) for 3 h at 37°C. ApoE associated with α_2M or α_2M-MA was separated from unbound apoE by non-denaturing gel electrophoresis. ApoE migrating with α_2M or α_2M-MA (top gel) and α_2M or α_2M-MA themselves (bottom gel) were detected by immunoblotting with ^{125}I-labeled antibodies. Control lanes "a" and "b" contained α_2M or α_2M-MA, respectively, without added apoE. ApoE associated with α_2M or α_2M-MA was quantitated by densitometric scanning. Results (means ± SD, n = 3) are shown in the lower histogram and have been compared statistically by Student's *t*-test.

electrophoretic migration of isolated α_2M treated with PC-PLC. This resulted in more apoE to be associated with α_2M (Fig. 6), consistent with the concept that activation of α_2M results in increased binding of apoE to α_2M. Activation of α_2M by a phospholipase (rather than a protease) has not been previously reported, and the reason why PC-PLC (but not other phospholipases) can activate α_2M remains unclear. The activation of α_2M by PC-PLC suggests a role for α_2M in the clearance and regulation of tissue phospholipase C activity.

We have found that subjects with an apoE 4/4 phenotype had less apoE associated with α_2M than subjects with an apoE 3/3 or apoE 2/2 phenotype (Fig. 10). These apoE 4/4 individuals also had lower total and HDL apoE concentrations (as is characteristic of the general population) and they had less plasma apoE associated with HDL-sized particles. As was the case in the postprandial situation, the amount of apoE associated with α_2M thus tended to be proportional to the amount of apoE associated with all HDL-sized particles (Fig. 10). It has been shown in a number of studies that apoE4 has a greater affinity for VLDL than apoE3 or apoE2 (11, 47, 48). The preferential association of apoE4 for VLDL is dependent on the specific interaction of amino- and carboxyl-terminal domains of the apoE molecule, which is, in turn, dependent on the presence of a salt bridge between amino acid residues arginine 61 and glutamic acid 255 (49). We postulate that the preferential association of apoE4 for VLDL can explain why less apoE is associated with α_2M or, alternatively, the structural characteristics of apoE4 itself can directly affect its interaction with α_2M. In vitro evidence was in fact obtained to support both of these possibilities. First, when ^{125}I-labeled apoE3 was incubated with α_2M in the presence of VLDL, the association of ^{125}I-labeled apoE3 with α_2M was almost completely inhibited, suggesting that the affinity of apoE for VLDL is greater than its affinity for α_2M (as discussed earlier). Although a comparative experiment with ^{125}I-labeled apoE4 was not carried out, it is likely that the relative affinity of apoE for VLDL, as determined by the phenotype of apoE, affects the availability of apoE for interaction with α_2M. At the same time, in vitro binding experiments with purified apoE isoforms in the

absence of VLDL showed that the direct binding of apoE4 to α_2M was less than that of apoE3 (Fig. 11). Charge or conformational differences in apoE isoforms can thus have a direct effect on their binding to α_2M. ApoE4 also binds less strongly than apoE3 to the microtubule-associated protein tau, which is a major component of neurofibrillary tangles in patients with Alzheimer's disease (50). It would be of interest to determine whether substitution of arginine 61 or glutamic acid 255 increases the affinity of apoE4 for α_2M, in the same way that modification of these residues reduces affinity of apoE4 for VLDL (49). Increased or decreased affinity of apoE4 for these complexes may help to explain the increased risk of coronary and Alzheimer's disease in individuals with an apoE4 phenotype (8, 9).

Binding of apoE to α_2M was found not only to be affected by apoE phenotype and by lipid complexes having a high affinity for apoE, but also by ligands known to bind to α_2M. Results of experiments shown in Fig. 9 demonstrated that certain ligands such as PDGF-BB and Aβ, but not INF-γ, had the potential to interfere with apoE binding. This was evident for α_2M-MA more so than α_2M. Several studies have investigated the competitive binding of different ligands to α_2M (43, 51), although the physiological relevance of these interactions remains to be determined. Of particular interest, is the possible interaction of α_2M, Aβ, and apoE. Increased deposition of Aβ is one of the principal neuropathological features of Alzheimer's disease (52). α_2M is a carrier protein for Aβ (23) and has been found to accumulate in the cortex and hippocampus of patients with Alzheimer's disease (53). At the same time, apoE binds to Aβ, and apoE4 binds faster and with a different pH dependence than apoE3 (54). Both apoE and α_2M have therefore been implicated in the perturbed metabolism of Aβ in Alzheimer's disease, and their direct interaction may be of pathophysiological significance.

In conclusion, the present study has provided evidence for the non-covalent association of apoE with α_2M in human plasma. Our results have demonstrated that: *1*) activated α_2M has a greater capacity to bind apoE than native α_2M; *2*) various proteins or lipoproteins known to bind apoE or α_2M can potentially affect the interaction of apoE with α_2M; and *3*) association of apoE with α_2M or α_2M-MA is dependent on apoE phenotype. Although the functional significance of apoE binding to α_2M has not yet been determined, the present results suggest that a physiological interaction exists between apoE and α_2M that may be of relevance to the plasma metabolism of these molecules, or may be indicative of a cellular function for apoE bound to α_2M.

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