Desialylation of Human Apolipoprotein E Decreases Its Binding to Human High-Density Lipoprotein and Its Ability to Deliver Esterified Cholesterol to the Liver

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Apolipoprotein E (apoE) plays a significant role in the delivery of high-density lipoprotein (HDL) cholesterol to the liver via the apoB/E receptor. The roles of the apoE sialylation status in its association with HDL and in the reverse cholesterol transport (RCT) function of HDL have not been well defined. Furthermore, long-term ethanol treatment impairs apoE sialylation and leads to its decreased content in HDL. Therefore, we investigated the association of either sialo apoE (SapoE) or desialo apoE (DSapoE) with HDL and its effect on the RCT function of HDL. The dextran sulfate precipitation method showed that [^{125}I]DSapoE binding to HDL was 27.3% ($P < .02$) to 35.5% ($P < .001$) lower versus [^{125}I]SapoE. Scatchard analysis of the specific binding data showed that [^{125}I]SapoE had 11.2 times more affinity for HDL than [^{125}I]DSapoE based on size-exclusion chromatography (K_d = 89.7 *v* 1,010 nmol/L). Similarly, [^{125}I]HDL had 4.5 times more affinity for SapoE compared with DSapoE based on solid-phase binding (K_d = 21.9 *v* 104.4 nmol/L). Furthermore, esterified cholesterol uptake from reconstituted HDL particles (rHDLs) by HepG2 cells increased over basal uptake up to 153% when rHDLs contained SapoE, versus only 37% with DSapoE. Enzymatic resialylation of DSapoE completely restored its HDL-binding and RCT properties, identical to those of SapoE. It is therefore concluded that desialylation of apoE decreases its binding to plasma HDL, leading to an impaired RCT function.

APOLIPOPROTEIN E (apoE) is a polymorphic secretory sialylated glycoprotein synthesized by a wide variety of tissues in mammals, particularly the liver, brain, and peripheral tissues such as macrophages.[1-5] Newly synthesized apoE is highly sialylated, whereas 75% to 80% of plasma apoE is nonsialylated.[6] In normal plasma, apoE is distributed unevenly among the various lipoproteins,[7] with its highest content in high-density lipoprotein ([HDL] 50%), followed by very–low-density lipoprotein (VLDL) and intermediate-density lipoprotein (20%) and low-density lipoprotein ([LDL] 10%). One of the roles of apoE, via its association with HDL, is to promote the removal of cholesterol from peripheral tissues and to transport it to the liver for elimination,[8] a process called reverse cholesterol transport (RCT). The cholesterol in the HDL molecule is taken up by the liver by the apoB/E receptor either directly[9] or after transfer to apoB-containing lipoproteins[10] via cholesterol ester transfer protein. An alternate pathway has also been proposed, mediated by a putative HDL receptor.[11,12] However, it is envisioned that the HDL-apoE receptor–mediated pathway accounts for the delivery of some cholesterol from the peripheral tissues to the liver.[13]

We have previously shown that long-term ethanol treatment impairs hepatic sialylation of apoE.[14] We also showed that while the total plasma apoE did not change, the proportion of apoE associated with HDL decreased with a concomitant increase in VLDL-apoE.[15] These results in rats were confirmed in another laboratory[16] and extended in human alcoholics.[17] Thus, the ethanol-mediated alteration in the distribution of apoE between HDL and VLDL could affect the RCT function of HDL. We therefore hypothesize that sialic acid–containing apoE has a higher affinity for HDL than desialylated apoE, while the opposite may be true for VLDL.

In terms of the roles of sialic acid residues in apoE function, only two facts are well established: (1) apoE sialylation is not essential for its secretion,[3,18] and (2) both sialo (SapoE) and desialo (DSapoE) apoE-phospholipid complexes bind with equal affinity to the fibroblast LDL receptor.[19] However, the roles of sialic acid in the association of apoE with HDL or VLDL and in the RCT function of HDL have not been reported. In the present study, we investigated these aspects and demonstrate that desialylation of apoE markedly affects not only its binding to HDL but also the RCT function of HDL.

MATERIALS AND METHODS

Materials

The sources of the chemicals were as follows: Bolton-Hunter reagent, American Radiolabeled Chemicals (St Louis, MO); [^{3}H]cholesteryl-oleate ([CO] 79 Ci/mmol), Amersham (Arlington Heights, IL); bovine serum albumin, (BSA) and Biogel A0.5M and 15M, BioRad (Hercules, CA); Eagle's minimum essential medium (EMEM), Bio-Whittaker (Walkersville, MD); fetal calf serum (FCS), Life Technologies (Gaithersburg, MD); dextran sulfate (15 kd), Genzyme (Cambridge, MA); sodium decyl sulfate ($NaDecylSO_4$), Pfaltz and Bauer (Waterbury, CT); egg-yolk phosphatidylcholine (PC), CO, and agarose-bound neuraminidase, Sigma (St Louis, MO); and goat immunoglobulin G anti–human apoAI, apoAII, apoB, and apoE, International Immunology Corp (Marietta, CA). All other chemicals were of reagent grade.

Cell Culture

HepG2 cells were obtained from the American Type Culture Collection (HB-8065; Rockville, MD). Cells were maintained at 37°C in a 5% CO_2/air humidified atmosphere in EMEM supplemented with 1% nonessential amino acids, 2 mmol/L L-glutamine, 1% Pen-strep (Bio-Whittaker), and 10% (vol/vol) FCS according to the supplier's instructions. When needed, cells were subcultured and grown as monolayers in six-well culture dishes at 1×10^6 cells per well (4 mL EMEM) at 37°C

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in a 5% CO_2/air humidified atmosphere. Confluence of the cell layers was observed after 36 to 48 hours. All experiments were performed with confluent cells.

Lipoprotein Isolation

Human VLDL ($d < 1.006$ g · mL^{-1}) and HDL ($1.063 < d < 1.21$ g · mL^{-1}) were isolated from fresh pooled plasma by sequential flotation.[20] All lipoprotein preparations were extensively dialyzed against saline-EDTA (154 mmol/L NaCl and 0.25 mmol/L EDTA) and stored at 4°C. All preparations were used within 1 week of isolation. Analysis of the chemical composition of each lipoprotein was performed with the following kits and methods: cholesterol, Sigma diagnostic kit no. 352 according to the method of Allain et al[21]; triglycerides, Sigma diagnostic kit no. 339 according to the method of McGowan et al[22]; and phospholipids, according to the method of Bartlett.[23] All protein determinations were performed according to the method of Bradford[24] with BSA as a standard.

Purification of apoAI

Human HDL was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis ([SDS-PAGE] 8% to 12%, 20 × 20 cm). ApoAI was eluted from a cut-out strip of the gel, corresponding to 28 kd, after overnight incubation at 4°C in 50 mmol/L sodium phosphate buffer (pH 9.0), 100 mmol/L NaCl, and 100 mmol/L $NaDecylSO_4$. The final isolated apoAI was found to be pure based on the appearance of a single 28-kd band on SDS-PAGE and a positive Ouchterlony band with anti-human apoAI, but not with anti-apoB, anti-apoE, and anti-apoAII.

Purification of apoE

ApoE was purified from delipidated human VLDL by heparin-sepharose chromatography [25] The purity of apoE was always verified by SDS-PAGE and by a positive Ouchterlony band with anti-human apoE antibodies but not with anti-apoAI, anti-apoAII, and anti-apoB. The sialic acid content of apoE was measured according to the method of Horgan.[26] Throughout this report, this apoE fraction will be termed sialo apoE (SapoE).

Desialylation of SapoE

Desialylated apoE (DSapoE) was obtained according to a previously published procedure.[27] Briefly, 0.1 mg SapoE was treated with 0.1 U agarose-bound neuraminidase in 100 mmol/L sodium acetate (pH 5.5), 5 mmol/L calcium chloride, and 1 mmol/L benzamidine (2.0 mL final vol) for 1 hour at 37°C, followed by overnight incubation at 4°C. Desialylation was monitored by measurement of sialic acid, and DSapoE integrity was verified by SDS-PAGE.

Preparation of Resialylated apoE From DSapoE

Enzymatic resialylation of DSapoE to obtain resialylated apoE (RSapoE) was performed according to the procedure of Want et al.[28] A rat liver Golgi-rich fraction was used as the source of sialyltransferase and was prepared according to the method of Leelavathi et al.[29] Briefly, the assay (in a final vol of 500 μL) included 50 μg neuraminidase-treated human apoE (DSapoE), 10 mmol/L imidazole (pH 7 0), 2.5 mmol/L $MgCl_2$, 20 μmol/L [^{14}C]cytidine monophosphate–*N*-acetylneuraminic acid ([CMP-NANA] 12.35 Ci/mmol), 20 μg/mL aprotinin, and 1 mmol/L benzamidine. Triton X-100 was omitted to prevent solubilization of membrane-bound Golgi proteins. The reaction was initiated by the addition of 400 μg protein from the rat liver Golgi-rich fraction (specific activity, 2 nmol NANA/mg protein/h) and continued for 1 hour at 37°C. The reaction mixture was then centrifuged at 10,000 × *g* for 1 hour at 4°C to pellet the Golgi fraction. The incorporation of labeled sialic acid into apoE-containing supernatant was verified by trichloroacetic acid (TCA) precipitation on an aliquot as follows: 30 μL of the supernatant was applied to 2.4-cm Whatman (Clifton, NJ) filter discs (GF/C) and washed extensively with ice-cold 10% TCA to remove non–apoE-bound [^{14}C]CMP-NANA, and the filters were then rinsed with ice-cold ethanol, dried, immersed in 4 mL UltimaGold scintillant (Packard, Meriden, CT), and counted for radioactivity.

Labeling

Iodination. SapoE, DSapoE, BSA, apoAI, VLDL, and HDL (100 μg protein) were labeled with the Bolton-Hunter reagent[30] according to the manufacturer's instructions. After labeling, the samples were dialyzed extensively until radioactivity in the dialysis buffer (25 mmol/L ammonium bicarbonate, 0.3 mmol/L EDTA, and 0.1% 2-mercaptoethanol) was at the background level. The protein purity and concentration were then checked by SDS-PAGE and densitometry For apoE, it was found that 92% of the radioactivity was precipitable in 10% (wt/vol) TCA and 90% of the label was retained on an immunoaffinity anti-human apoE column. The specific activity was 1.12×10^5 dpm/μg for both forms of apoE. The specific activity of other labeled materials was as follows: 3.52×10^5 dpm/μg for apoAI, 3.48×10^5 dpm/μg for BSA, 2.64×10^5 dpm/μg protein for HDL, and $4\,82 \times 10^5$ dpm/μg protein for VLDL.

HDL labeling with [^{3}H]cholesteryl-oleate. This procedure was essentially based on the method used by Brown and Goldstein [31] A 125-μL volume of human HDL (1 mg protein) was incubated in a mixture containing 100 μL [^{3}H]cholesteryl-oleate in dimethyl sulfoxide (5.5×10^6 dpm/mg HDL protein) and 275 μL 10-mmol/L Tris hydrochloride (pH 8.0), 154 mmol/L NaCl, and 0.3 mmol/L EDTA for 2 hours at 37°C followed by overnight incubation at 4°C, after which the sample was dialyzed extensively against the same buffer and stored on ice until use. After dialysis, the specific activity was 3.52×10^6 dpm/mg HDL protein (64% labeling efficiency).

Binding Studies of [^{125}I]apoE With HDL Using Dextran Sulfate Precipitation

The indicated amounts of [^{125}I]apoE and HDL were incubated for 1 hour at 37°C in phosphate-buffered saline ([PBS] pH 7.4) containing 0.01% Triton X-100 in a final volume of 20 μL. HDL was precipitated from the incubation mixture by dextran sulfate precipitation.[32] Briefly, after this incubation period, HDL precipitation was performed for 15 minutes at room temperature at a final concentration of 0.65% dextran sulfate and 0.2 mol/L $MnCl_2$, followed by centrifugation at 10.000 × *g* for 10 minutes. The pellet and supernatant were separated, and the radioactivity was measured in a Beckman (Fullerton, CA) Gamma 5500 counter. The amount of [^{125}I]apoE in the pellet fraction in the absence of HDL was negligible.

Binding Studies of [^{125}I]apoE With HDL or VLDL Using Biogel A0.5M Size-Exclusion Chromatography

The indicated amounts of [^{125}I]apoE species were incubated with HDL (1 or 12 5 μg protein) or VLDL (2.5 μg protein) in PBS (10 mmol/L potassium phosphate, pH 7.4, and 154 mmol/L NaCl) containing 10% glycerol for 1 hour at 37°C. At the end of this incubation period, the reaction mixture was loaded on top of a 1 × 22-cm Biogel A0.5M column preequilibrated with PBS (pH 7.4). The separation of free and lipoprotein-bound [^{125}I]apoE was performed isocratically with PBS (pH 7.4) at room temperature (flow rate, 0.3 mL/min). Fractions (0.3 mL) were collected and counted for radioactivity. While HDL- or VLDL-bound [^{125}I]apoE was eluted in the void volume (~5 mL), free [^{125}I]apoE was trapped in the column. The authenticity of each fraction was verified by SDS-PAGE.

Binding Studies of apoE With [^{125}I]HDL, [^{125}I]VLDL, [^{125}I]apoAI, or [^{125}I]BSA Using Solid-Phase Binding Assays

A solid-phase binding assay technique was developed to quantify the association of either form of apoE with iodinated ligands (HDL, VLDL, apoAI, and BSA) based on previously reported similar procedures.[33,34] For the binding assay, 96-well polystyrene microtitration plates (PRO-BIND Assay Plates, Falcon, Becton-Dickinson, Lincoln Park, NJ) were initially coated with SapoE or DSapoE (10 μg/mL) in 150 mmol/L NaCl and 50 mmol/L Tris, pH 7.6 (TBS) containing 5 mmol/L $CaCl_2$ for 18 hours at 4°C. Unoccupied sites were blocked with TBS containing 4% BSA, 1% gelatin, and 0.05% Tween 20 (TBGT) at room temperature for 1 hour. The wells were then washed three times with TBGT buffer to remove unbound apoE. Six serial dilutions (100 μL/well) of iodinated ligands in binding buffer (10 mmol/L $NaPO_4$, pH 7.5, 100 mmol/L NaCl, and 0.1% gelatin) were added and the reactions were incubated for 16 hours at 4°C. After four washes with binding buffer, each well was washed thrice with 100 μL 1% Triton X-100, and these washes were combined and counted in a Beckman Gamma 5500 counter to account for the amount of apoE-bound iodinated ligand radioactivity. Each concentration point was assessed in triplicate. The concentration range for the iodinated ligands was as follows: VLDL, 65.1 fmol/L to 203.4 pmol/L; HDL, 3.8 pmol/L to 11.9 nmol/L; apoAI, 11.0 pmol/L to 34.4 nmol/L; and BSA, 4.0 pmol/L to 12.4 nmol/L. A control experiment with iodinated apoE species was performed to determine whether the sialylation state of apoE quantitatively affected its binding to the microplate well surface. Binding was measured by counting the remaining radioactivity after overnight incubation, blocking, and washing as described earlier. Quantitation of apoE association with various radiolabeled ligands (apoAI, HDL, or VLDL) was performed using the software package StatMost (DataMost Corp, Salt Lake City, UT). The experimental data were analyzed according to the following form of the binding isotherm,

$$dpm_{spec} = \frac{dpm_{max} \times [L]}{K_D + [L]}, \qquad \text{Eq 1}$$

where dpm_{spec} is the specifically bound radioactivity, dpm_{max} is the maximum specifically bound radioactivity at saturation, [L] is the free labeled ligand molar concentration, and K_D is the dissociation constant. For each amount of added labeled ligand, dpm_{spec} was calculated by subtracting the bound radioactivity found in wells coated only with blocking buffer from the bound radioactivity found in wells coated with SapoE or DSapoE. Since this value reflected only a maximum of 6% of the initial radioactivity added, [L] was considered to be equal to the total concentration of radiolabeled ligand added.

Preparation of Reconstituted HDL Particles

The reconstituted HDL particle (rHDL) complexes were prepared by sodium cholate dispersion according to previously reported methods.[35-38] A stock lipid solution containing CO (2.04 mg/mL), [^{3}H]CO (61.1×10^6 dpm/mL), and egg-yolk PC (54 mg/mL) was prepared in chloroform and stored at −20°C. A typical preparation of rHDL was prepared as follows: 90 μL stock lipid solution was added to a 5-mL polypropylene (PP) conical tube, dried under N_2, resuspended in 0.4 mL dispersion buffer (10 mmol/L Tris hydrochloride, pH 8.0, 154 mmol/L NaCl, 1 mmol/L NaN_3, and 0.01% EDTA) by vortexing for 2 minutes, and incubated for 1 hour at 4°C. Subsequently, 83.8 μL sodium cholate (30 mg/mL) was added, and after vortexing for 1 minute, the solution was incubated again for 1 hour at 4°C. Under these conditions, the molar ratio for PC/sodium cholate was 1/1 and the molar ratio for PC/CO was 20/1. Various combinations of apolipoprotein solutions in PBS containing 100 μg apoAI with or without apoE were lyophilized in 5-mL PP test tubes to which 48.4 μL [^{3}H]CO/CO/PC/sodium cholate solution was added. Each sample mixture was then incubated for 1 hour at 4°C and dialyzed extensively against dispersion buffer after adjusting the volume to 0.5 mL with dispersion buffer. The labeling efficiency was found to be 82% to 90%. The homogeneity and size of the reconstituted lipoprotein complexes were assessed by size-exclusion chromatography on Biogel A15M and by negative-staining electron microscopy.[39] Furthermore, to prove that the amounts of SapoE and DSapoE bound by rHDL were similar to the corresponding values found with native HDL, a comparative binding experiment was performed with both apoE species and either native HDL or rHDL using the dextran sulfate precipitation method. On Biogel A15M, rHDL was found to elute as a single peak. Electron microscopic analysis of our rHDL preparation showed the characteristic disc-shaped structures (average diameter, 15 nm) similar to those found in the literature for rHDL.[35,40-42]

Esterified Cholesterol Uptake Assay

An adequate volume of dialyzed rHDL was mixed with EMEM (4-mL final vol) and added as a sterile solution (by filtration through 0.22-μm sterile filters) to each culture dish containing confluent HepG2 cells ($\sim 1 \times 10^6$ cells). An aliquot (0.1 mL) of the incubation medium was analyzed for the total esterified cholesterol radioactivity added to each dish. The uptake of labeled esterified cholesterol by the cells was determined at the indicated time intervals by counting the cell-associated radioactivity as follows. The cell medium was completely removed, and after thorough washing of the cells three times with PBS to remove the medium radioactivity, the cells were dissolved in 1 mL PBS containing 0.1% SDS and 1% Triton X-100. The dissolved cells were mixed with 4 mL UltimaGold scintillation cocktail (Packard) and analyzed for radioactivity in a Beckman LS-6500 liquid scintillation spectrometer.

RESULTS

Composition of HDL and VLDL

Human HDL ($1.063 < d < 1.21$) is 51.9% protein by weight[43] and is heterogeneous since it is composed of mainly two populations of HDL particles of different molecular size, 360,000 d (mainly HDL_2) and 175,000 d (HDL_3),[7] with a protein composition of 41% and 55% (wt/wt), respectively.[44] Therefore, the proportion of the two HDL subpopulations is 22% (HDL_2) and 78% (HDL_3), which led us to assume a reasonable average protein molecular weight for HDL of 112,000 d. We necessarily used total HDL for the binding assay because that is how it exists physiologically in the plasma. VLDL ($d < 1.006$) has an average molecular weight of 45×10^6 d,[7] and its protein composition is 7.7% (wt/wt).[43] Therefore, we assumed a protein molecular weight for VLDL of 3.465×10^6 d. The protein and lipid composition of HDL and VLDL purified in our laboratory agrees very well with the values found in the literature[43,45] (Table 1), supporting our assumptions for molecular weight.

Preparation of DSapoE

DSapoE was obtained after neuraminidase treatment of heparin-affinity–purified VLDL-apoE (SapoE) as described in the Methods. No sialic acid was detected in DSapoE, while SapoE had 0.9 mol sialic acid/mol protein. DSapoE was found to have the same electrophoretic properties as SapoE (Fig 1), indicating that apoE integrity was preserved after neuraminidase treatment. This is further confirmed by the complete restoration of both its binding and RCT properties after resialylation, as shown later.

Table 1. Protein and Lipid Composition (% weight) of Human HDL and VLDL

Parameter / Data source	HDL (1 063 < d < 1.21)			VLDL (d < 1.006)		
	a	b	c	a	b	c
Protein	56 3	50-55	51.9	10.2	5-10	7.7
Total cholesterol	15.0	15-16	17.9	15 2	15-25	21.6
Triglyceride	3.7	3	8.0	57.9	50-65	49.9
Phospholipid	25.0	20-25	22.7	16.7	15-20	18.6

NOTE. Data sources are as follows: a, the present study; b, Soutar et al[45]; c, Chapman.[44]

Association of Iodinated SapoE and DSapoE With HDL Monitored by Dextran Sulfate/$MnCl_2$ Precipitation

Preliminary experiments indicated that the yield of labeled HDL in the HDL pellet reached a plateau when HDL protein in the assay exceeded 30 μg (Fig 2). In addition, the presence in the assay of up to 1,000 times excess nonspecific protein, BSA, did not affect the amount of radioactive apoE bound to HDL.

We analyzed apoE binding to HDL as a function of each species of apoE concentration. [^{125}I]DSapoE consistently bound less efficiently to HDL than [^{125}I]SapoE. The significant decrease in binding was 27.3% ($P < .02$) to 35.5% ($P < .001$) (Fig 3).

Association of Iodinated SapoE and DSapoE With HDL or VLDL Monitored by Biogel A0.5M Size-Exclusion Chromatography

A preliminary experiment was performed with [^{125}I]HDL to validate this method. Chromatography of 270 μg [^{125}I]HDL protein yielded greater than 90% of the label and HDL protein in the void volume (Fig 4A), as shown by overlapping radioactivity and protein elution profiles. Significantly, the same elution profile was observed when only a small amount of [^{125}I]HDL (1 μg) was chromatographed and monitored for radioactivity (data not shown). Similarly, VLDL was also eluted in the void volume (data not shown). In contrast, less than 15% of the label was recovered in the void volume when [^{125}I]SapoE alone was chromatographed (Fig 4B). Even this may be due to the self-association properties of apoE in the absence of other proteins or lipids,[46,47] leading to high–molecular-weight complexes that elute in the void volume. The remaining [^{125}I]SapoE was retained on the column.

To estimate the specific binding parameters of SapoE and DSapoE with HDL and VLDL, a series of Scatchard experiments were performed (Fig 5). Analysis of these specific binding data (total binding − nonspecific binding) with the LIGAND program showed that SapoE exhibited an 11.2-fold greater affinity than DSapoE with respect to interaction with HDL. On the other hand, SapoE showed only a 2.7-fold higher affinity for VLDL than DSapoE. Furthermore, SapoE showed a 3.2-fold greater affinity for HDL compared with VLDL. In contrast, DSapoE showed a 1.3-fold greater affinity for VLDL compared with HDL.

Association of Iodinated HDL, VLDL, apoAI, and BSA With SapoE or DSapoE in a Solid-Phase Binding Assay

The binding of various iodinated ligands onto immobilized SapoE or DSapoE was monitored as described in the Methods. A control experiment showed no significant difference between the amount of [^{125}I]SapoE and [^{125}I]DSapoE immobilized to the well surface. It is reasonable to assume that this monitoring technique mirrors the physiological binding of apoE to HDL (or VLDL), since identical studies have been conducted to examine the physiological binding of apoE or apoJ to β-amyloid

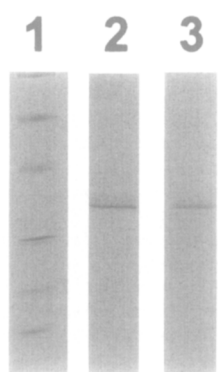

Fig 1. SDS-PAGE analysis of SapoE and DSapoE. Aliquots of apoE after heparin-sepharose purification (SapoE, lane 2) and after desialylation (DSapoE, lane 3) were analyzed on a 4%-20% gradient SDS-PAGE. Lane 1: molecular weight markers (from top) 97, 66, 45, 30, 20.1, and 14.4 kd.

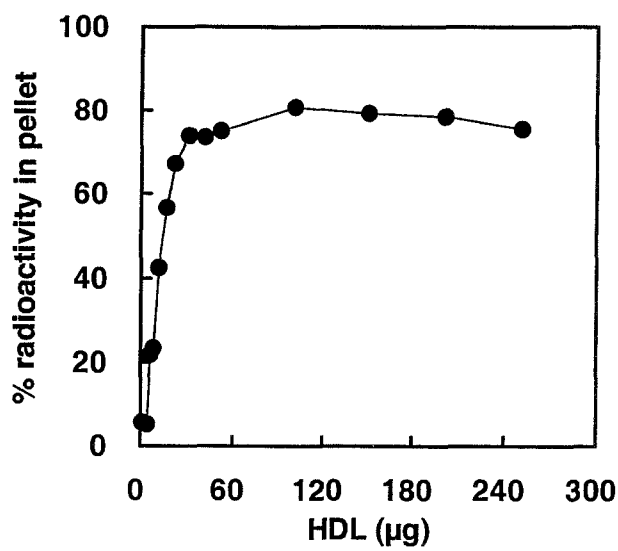

Fig 2. Concentration dependence of human HDL precipitation by dextran sulfate and $MnCl_2$. Twenty microliters containing a constant amount of human [^{125}I]HDL (1.25 μg) and increasing concentrations of nonradioactive human HDL in PBS were precipitated by addition of 180 μL precipitation solution (0.65% dextran sulfate, 0.2 mmol/L $MnCl_2$, 0.01% Triton X-100, and 0.154 mol/L NaCl final concentrations in the assay). After inubation at room temperature for 15 minutes, the reaction mixtures were centrifuged at 10,000 × *g* for 10 minutes. The supernatant was carefully removed, and radioactivity in the pellet was measured to account for the amount of precipitated [^{125}I]HDL. Results are expressed as a percentage of the [^{125}I]HDL initial radioactivity added to the incubation mixture.

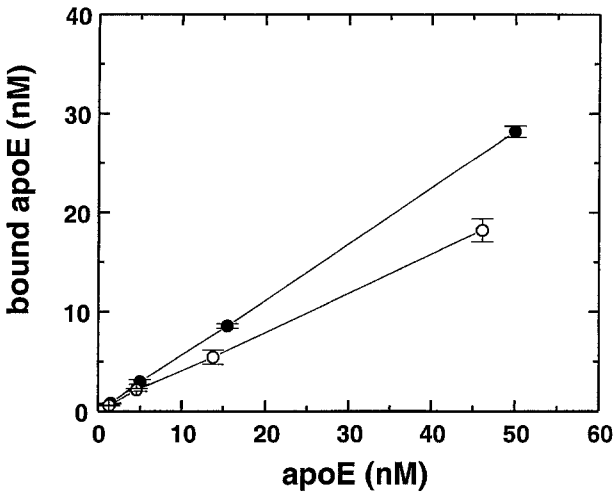

Fig 3. DSapoE binds less efficiently to HDL than SapoE as demonstrated by dextran sulfate/$MnCl_2$ precipitation. Indicated amounts of [^{125}I]SapoE (●) [^{125}I]DSapoE (○) and 30 μg HDL protein were incubated in 20 μL PBS for 1 hour at 37°C, followed by determination of HDL-associated radioactivity as described in Fig 2. Data are the average of 3 independent measurements.

peptide.[33,34] A quantitative analysis of the results according to Equation 1 is summarized in Table 2. [^{125}I]HDL bound five times more efficiently ($P < .0001$) with SapoE versus DSapoE. Similar experiments with [^{125}I]VLDL as the ligand showed that the binding affinity for SapoE was only twofold better ($P < .005$) compared with DSapoE. ApoAI also exhibited a higher affinity for SapoE versus DSapoE. However, it must be pointed out that the affinity of apoAI for SapoE and DSapoE was only 10% and 30%, respectively, of the corresponding values for HDL. In contrast, BSA did not show any significant binding on either form of apoE.

Esterified Cholesterol Uptake by HepG2 Cells From rHDL Particles

In an attempt to evaluate whether an alteration in the association of apoE with HDL influences the ability of HDL to deliver esterified cholesterol to the liver, we determined the uptake by human HepG2 liver cells of labeled esterified cholesterol from rHDL particles of well-defined lipid and apoprotein content.

It should be pointed out that these esterified cholesterol uptake experiments were performed with rHDL particles prepared by the established cholate dialysis method consisting of constant amounts of apoAI, cholesteryl ester, and PC in a molar ratio of 1.6:12.5:250, respectively. This ratio is known to yield maximum cholesterol efflux from monocytic cells.[48] The only variable was the incorporation of SapoE or DSapoE. Whenever apoE was incorporated into the rHDL particle, the apoAI:apoE molar ratio was also kept constant at a physiological level of 11:1.[4] It is significant that the values for the binding of SapoE and DSapoE species to rHDL (50% and 43% binding to 30 μg rHDL protein, respectively) were quantitatively similar to the corresponding values with native HDL (52% and 44% binding to 30 μg HDL protein, respectively; Fig 3). Each rHDL particle was tested for esterified cholesterol uptake at least at three different concentrations.

At the highest apoprotein concentration tested, the basal uptake rate for labeled esterified cholesterol from rHDL particles containing only apoAI was significantly increased (127% to 153%) when SapoE was incorporated into rHDL particles, whereas DSapoE incorporation into rHDL caused only a 26% to 37% increase in esterified cholesterol uptake (Fig 6A). Esterified cholesterol uptake from various rHDL particles by HepG2 cells showed saturation kinetics, reaching the maximum at about 300 μg added rHDL protein in the incubation medium (Fig 6B). Again, it was found that esterified cholesterol uptake was increased up to 127% in the presence of SapoE, whereas it was increased only up to 25% in the presence of DSapoE, even at the saturation level of rHDL particles.

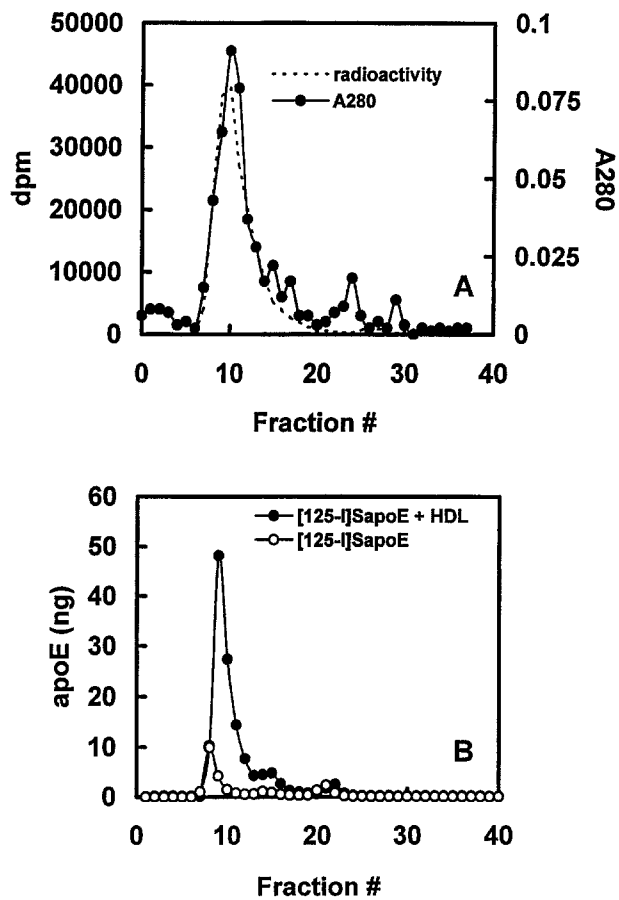

Fig 4. Elution profiles of [^{125}I]HDL and [^{125}I]apoE on a Biogel A0.5M size-exclusion chromatography column. (A) [^{125}I]HDL. Native HDL (270 μg) and [^{125}I]HDL (346,700 dpm) were mixed in 300 μL PBS (pH 7.4) containing 10% glycerol and loaded on a Biogel A0.5M column equilibrated with PBS. The mobile phase was PBS. Fractions (0.3 mL) were collected, and the absorbance at 280 nm (—●—) and radioactivity (····) were measured. A control experiment with blue dextran indicated that HDL elutes in the void volume. (B) [^{125}I]apoE. The 2-following assay mixtures (20 μL in PBS, pH 7.4, containing 10% glycerol) were analyzed on Biogel A0.5M columns: (●) 1.25 μg [^{125}I]SapoE and 12.5 μg HDL protein and (○) 1.25 μg [^{125}I]SapoE. The mobile phase was PBS. Fractions (0.3 mL) were collected and radioactivity was measured. Results are expressed as ng labeled apoE per fraction based on the specific activity of [^{125}I]SapoE.

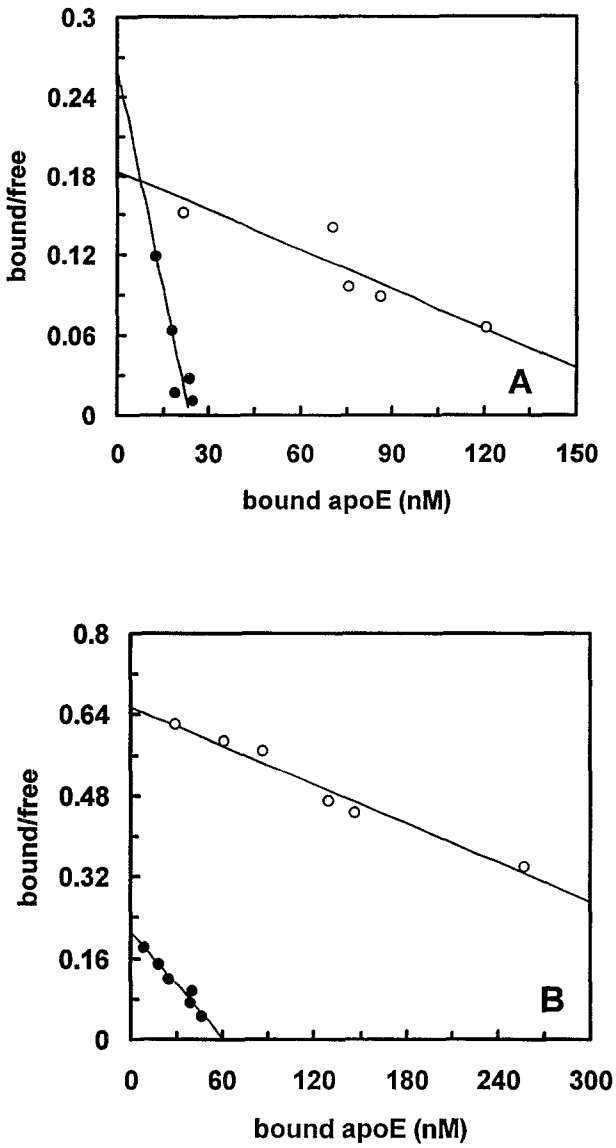

Fig 5. Scatchard analysis of the association of apoE-HDL and apoE-VLDL by the Biogel A0.5M size-exclusion chromatography method. (A) 0.1-1.0 μg [^{125}I]SapoE (●) or [^{125}I]DSapoE (○) was incubated with HDL (1 μg protein) in 20 μL PBS (pH 7.4) containing 10% glycerol for 1 hour at 37°C. (B) 0.05-1.0 μg [^{125}I]SapoE (●) or [^{125}I]DSapoE (○) was incubated with VLDL (2.5 μg protein) in 30 μL PBS (pH 7.4) containing 10% glycerol for 1 hour at 37°C. Each reaction mixture was then analyzed by size-exclusion chromatography as described in Fig 4. The amount of HDL- or VLDL-bound apoE was measured by determining the amount of radioactivity in the void volume. Nonspecific binding was estimated in the presence of a 20-fold excess of cold apoE, and was found to be 10% and 15% for SapoE and DSapoE, respectively. The binding parameters as determined with the LIGAND program using a single-binding-site model were as follows: SapoE-HDL, K_D = 90 ± 35 nmol/L, B_{max} = 24 ± 4 nmol/L; DSapoE-HDL, K_D = 1,010 ± 250 nmol/L, B_{max} = 186 ± 31 nmol/L; SapoE-VLDL, K_D = 287 ± 33 nmol/L, B_{max} = 61 ± 4 nmol/L; DSapoE-VLDL, K_D = 785 ±69 nmol/L, B_{max} = 514 ± 34 nmol/L.

Table 2. Binding Parameters for the Association of [^{125}I]HDL, [^{125}I]VLDL, and [^{125}I]apoAI With SapoE or DSapoE (solid-phase binding assay)

Labeled Ligand	K_D (nmol/L)	
	SapoE	DSapoE
HDL	21.9 ± 1 1	104.4 ± 3.9
VLDL	22.7 ± 5.7	46.6 ± 4.7
ApoAI	180 5 ± 1.5	322.1 ± 12.2

NOTE. Various concentrations of [^{125}I]HDL, [^{125}I]VLDL, [^{125}I]apoAI, or [^{125}I]BSA were incubated in microtiter wells coated with either SapoE or DSapoE (10 μg/mL) overnight at 4°C. Bound radiolabeled ligands were detected after thorough washing of the wells with binding buffer (150 mmol/L NaCl and 50 mmol/L Tris, pH 7.6) Each concentration point was assessed in triplicate. [^{125}I]BSA did not show any significant binding. Data for the other labeled ligands were analyzed according to Eq 1 by nonlinear regression with the StatMost program.

Reversibility of Desialylation of SapoE on its HDL Binding and RCT Function

To verify whether desialylation of SapoE by neuraminidase treatment affected its physical integrity and physiological function, we tested whether resialylation of DSapoE restored its HDL binding properties and RCT function. DSapoE (50 μg, 1.47 nmol) was resialylated with the rat liver Golgi-rich fraction as the source of sialyltransferase and with [^{14}C]CMP-NANA as the sialic acid precursor. The incorporation of [^{14}C]CMP-NANA into DSapoE showed that 0.58 mol sialic acid was incorporated per mol apoE protein. RSapoE binding to HDL, monitored by the dextran sulfate precipitation method, was 90% of the SapoE binding to HDL (47% and 52% binding to 30 μg HDL protein, respectively; Fig 3). Similarly, esterified cholesterol uptake from rHDL particles containing RSapoE by HepG2 cells was quantitatively similar to the uptake by rHDL particles containing SapoE (Fig 6B).

DISCUSSION

Human apoE is a 34-kd *O*-glycosylated protein consisting of a single polypeptide with the oligosaccharide chain attached to threonine residue 194.[18] It is synthesized by the liver and peripheral tissues.[4,49,50] Newly secreted apoE is incorporated into lipoproteins and mediates their catabolism by specific hepatic B/E cell receptors[51,52] and extrahepatic B/E cell receptors.[52] It is well established that newly synthesized apoE from both the liver and peripheral tissues (eg, macrophages) is highly sialylated, while 75% to 80% of plasma apoE is nonsialylated.[6] Although sialylation is not obligatory for secretion of apoE, its exact role in the metabolism of apoE has not been thoroughly delineated. The only evidence available thus far is that there is no apparent difference in the receptor binding activity of SapoE and DSapoE as measured by the competition of apoE-phospholipid complexes for LDL binding to fibroblasts.[19]

The present study provides direct evidence as to how the sialylation state of apoE regulates its association with HDL and VLDL. We have shown that apoE binding to HDL is significantly decreased after desialylation of SapoE (Fig 3). The results of Scatchard analysis (Fig 5) demonstrated a dramatic decrease (11.2-fold) in the binding affinity of apoE for HDL, due to the loss of sialic acid, whereas the association of apoE

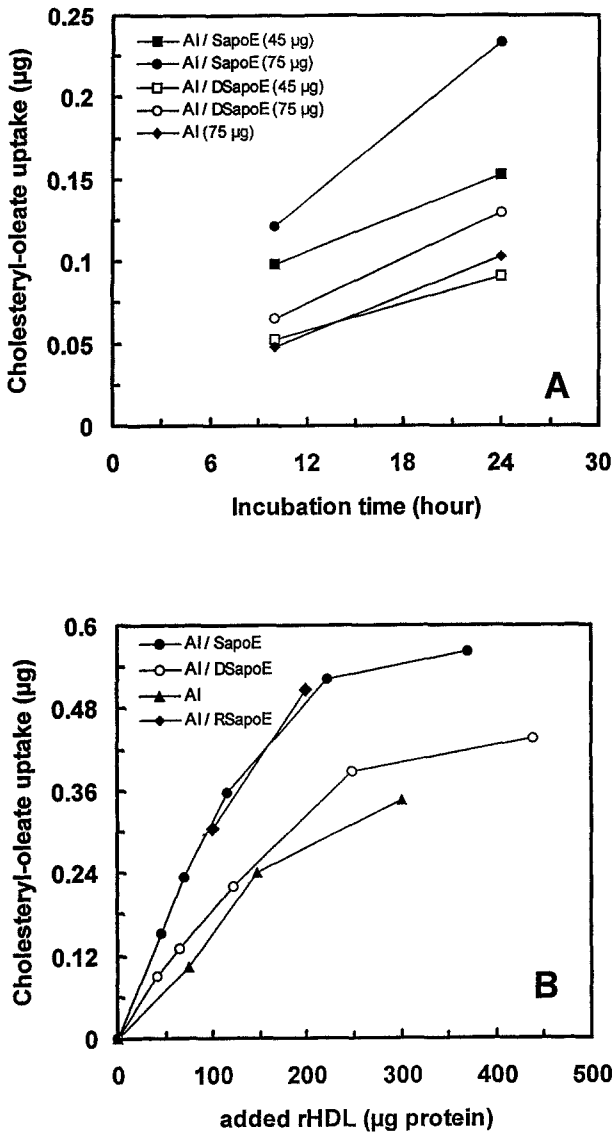

Fig 6. Effect of SapoE or DSapoE in rHDL on cholesterol uptake by HepG2 cells. In all experiments, ~1 × 10^6 confluent HepG2 cells were incubated at 37°C with the indicated rHDL particles in EMEM in a 5% CO_2/air humidified atmosphere. After the indicated incubation period, the culture medium was removed and the cells were washed thrice extensively with PBS and then dissolved in 1 mL PBS containing 0.1% SDS and 1% Triton X-100. Finally, the solubilized-cell radioactivity was measured. (A) Time-dependent uptake. The amount of rHDL added is expressed as µg protein. (B) Concentration-dependent uptake after 24 hours of incubation. rHDL abbreviations: AI/SapoE, [^{3}H]CO,CO:PC:apoAI:SapoE; AI/DSapoE, [^{3}H]CO,CO:PC: apoAI:DSapoE; AI, [^{3}H]CO,CO:PC:apoAI; AI/RSapoE, [^{3}H]CO,CO:PC: apoAI:RSapoE.

with VLDL was mildly affected by the loss of sialic acid (2.7-fold decrease). These observations have been confirmed by independent solid-phase binding studies (Table 2). Thus, the decreased apoE concentration in plasma HDL after chronic ethanol exposure[15-17] is likely due to ethanol-mediated impaired sialylation of apoE. Our previous and ongoing studies on this aspect indicate that ethanol inhibits the hepatic synthetic rate of sialyltransferase by downregulation of its mRNA.[53,54] The reason for the concomitant increase of DSapoE in VLDL after chronic alcohol consumption[55,56] could be explained by the fact that DSapoE showed higher affinity for VLDL than HDL (Fig 5B).

The physiological significance of protein glycosylation has been investigated in a number of biological systems. The pattern of sugars in glycoproteins governs how the cells interact with hormones, antibodies, and other cells. There are compelling studies wherein oligosaccharide moieties have been shown to act as signals for protein clearance from the blood,[57] for directing lysosomal enzymes to their target organelles,[58] for targeting proteins destined for lysosomes,[59] for biological functioning of glycoproteins,[60-63] or for cell-cycle regulation.[64-66] In terms of the lipoproteins, only a few examples are known for a role of glycosylation in specific function. For instance, LDL receptor activity is diminished in altered *O*-glycosylated LDL receptor.[67] More recently, Remaley et al[68] elegantly showed that *O*-linked glycosylation is not necessary for secretion of apoAII but does modify the association of apoAII to HDL. As another example, the present study shows how an alteration in the glycosylation state of apoE affects its association with HDL and VLDL.

The uptake by HepG2 cells of esterified cholesterol from rHDL containing apoAI as the only apoprotein is consistent with the putative HDL receptor–mediated process.[11,12] The observed 127% increase in this basal uptake by SapoE incorporation could account for the apoE receptor–mediated uptake (Fig 6B). In contrast, the incorporation of DSapoE into rHDL seems to produce only a marginal stimulation of esterified cholesterol uptake. However, when the sialylated state of apoE is restored by resialylation (DSapoE → RSapoE) followed by reconstitution of rHDL with RSapoE, esterified cholesterol uptake is at the same level as when SapoE is incorporated into rHDL (Fig 6B). Thus, it can be concluded that in our experimental conditions, desialylation of SapoE by neuraminidase does not affect its structural integrity, and enzymatic resialylation of DSapoE completely restores its physiological function when associated with HDL. The exact function of sialic acid in this process remains to be clarified. For example, it is not clear whether sialylation of apoE has a positive association effect on HDL three-dimensional structure that would facilitate recognition of the particle by the apoE receptor on the liver cell surface.

The physiological existence of the sialylated and desialylated species of apoE in the plasma is well established. We recognize the presence of apoE predominantly in the nonsialylated form in the plasma compartment, while newly synthesized apoE is highly sialylated. In one pathological condition, namely alcoholism, the distribution of the two species is perturbed in the liver and plasma. Therefore, the homeostasis of the two species can be regulated, although the mechanism involved has yet to be understood, particularly the exact mechanism of apoE desialylation. Our study provides insights on the consequence of apoE desialylation in cholesterol metabolism.

It is known that the hydrophobic primary sequence at the C-terminal end provides apoE with lipid binding properties.[46]

Our results show that the presence of sialic acid is required for this binding to be specific and functional. Alternatively, sialic acid (net negative charge) could be directly involved in the interaction with choline (net positive charge) on the head group of phospholipid, since PC is the predominant phospholipid in HDL. Based on these studies, it is reasonable to conclude that the glycosylation state of apoE plays an important role in its association with HDL, which in turn may affect its function in RCT.

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