Effect of Particle Size and Temperature on the Conformation and Physiological Behavior of Apolipoprotein E Bound to Model Lipoprotein Particles†

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Received September 7, 1989; Revised Manuscript Received February 16, 1990

ABSTRACT: The effect of particle size and structural order/disorder of the lipid domains on the conformation and physiological behavior of lipid-associated apolipoprotein E (apoE) was evaluated. Circular dichroic (CD) spectra of apoE bound to large (LME) and small (SME) microemulsion particles, composed of dimyristoylphosphatidylcholine (DMPC) and cholesteryl oleate (CO), and to DMPC disks revealed that at 4 °C, where all of the lipid constituents were in an ordered state, apoE bound to LME displayed ~60% α-helicity, while apoE bound to SME and DMPC disks displayed 73% and 95% helicity, respectively. Over the temperature range 4–50 °C, encompassing the lipid thermal transitions, only apoE bound to LME demonstrated an abrupt change in its CD spectrum (decrease in α-helicity) in response to temperature. To determine the source of the abrupt CD change, the constants for dissociation (K_d) of apoE from the surface of the large and small microemulsion particles were determined at 4, 25, and 37 °C. These results demonstrated that at 4 °C, the K_ds for binding of apoE to the LME and SME were approximately equal; however, between 4 and 25 °C, there was a 5-fold increase in the K_d for binding of apoE to the LME, whereas the K_d for binding to the SME remained constant. The physiological effects of these differences in apoE secondary structure and equilibrium binding were examined by measuring the capacity of each apoE-containing particle to compete with LDL for binding to human fibroblasts, and by measuring the capacity of the apoE–microemulsion particles to suppress HMG-CoA reductase activity. This measurement revealed that all three model lipoproteins competed with ^{125}I-LDL for receptor binding. Microemulsions alone were incapable of suppressing reductase activity; however, in the presence of apoE, the microemulsions were bound and internalized, and their cholesterol component suppressed HMG-CoA reductase activity. These studies indicate the dependence of apoE conformation and lipid binding constant on the size and temperature of the lipid particle and suggest the value of the DMPC/CO/apoE system for introducing exogenous materials into cells via the B/E receptor.

Apolipoprotein E (apoE)[1] is known to mediate the binding of certain lipoproteins to the high-affinity cell surface receptor of fibroblasts in culture. ApoE-containing lipoproteins which bind to this receptor include cholesteryl ester rich very low density lipoprotein (CER-VLDL) (Gianturco et al., 1980; Hui et al., 1984) and HDL_c (Mahley & Innerarity, 1977; Innerarity & Mahley, 1978) derived from the plasma of cholesterol-fed rabbits and dogs, and a specific class of VLDL (HTG $VLDL_1$, Sf 100–400) from hypertriglyceridemic subjects (Gianturco et al., 1982). Disks formed from phospholipid vesicles and apoE have also been shown to bind to the receptor (Innerarity et al., 1979); however, the protein alone displays no binding (Innerarity & Mahley, 1978). Interestingly, normal $VLDL_1$ contain approximately the same total mass of apoE as HTG $VLDL_1$; however, these normal lipoproteins fail to bind to the receptor (Gianturco et al., 1982). Thus, it has been suggested that the specific three-dimensional conformation of apoE on the surface of lipoproteins is responsible for their varying capacities to bind to the receptor.

Recently, we have developed microemulsion models of cholesteryl ester rich lipoproteins containing dimyristoylphosphatidylcholine (DMPC) and cholesteryl oleate (CO). These microemulsions have been sized by column chromatography and fall into the CER-VLDL (~750-Å diameter) and HDL_c (~300-Å diameter) size ranges. Both particle populations have been examined by differential scanning calorimetry and found to exhibit two thermal transitions. The first transition, at 25–30 °C, has been assigned to DMPC in the surface shell and the second at ~42 °C, to CO in the core (Mims et al., 1986a). ApoE binds to the surface of the large (LME) and small (SME) microemulsion particles to form model lipoprotein complexes (Mims et al., 1986b).

In this study, the circular dichroic (CD) spectra of apoE bound to the large (apoE–LME) and small (apoE–SME)

† This work has been supported by Texas Heart Association Grant-in-Aid 88G-205 (M.P.M.), by Specialized Center of Research in Atherosclerosis Grant HL-27341, by Robert A. Welch Foundation Grant Q-837 (J.D.M.), and by a National Science Foundation Instrumentation Grant (PCM-8413751).

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[1] Abbreviations: apoE; apolipoprotein E (M_r 34 000) isolated from cholesteryl ester rich VLDL of hypercholesterolemic rabbits; CER-VLDL; cholesteryl ester rich very low density lipoproteins isolated from hypercholesterolemic rabbits; DMPC, L-α-dimyristoylphosphatidylcholine; CO, cholesteryl oleate; LME, large synthetic microemulsions (~750-Å diameter) containing DMPC and CO; SME, small synthetic microemulsions (~295-Å diameter) containing DMPC and CO; CD, circular dichroism; DMEM, Dulbecco's modified Eagle's medium; DSC, differential scanning calorimetry; BSA, bovine serum albumin; STI, soybean trypsin inhibitor; HDL_c, high-density lipoproteins isolated from the plasma of hypercholesterolemic dogs or humans; LPDS, lipoprotein-deficient serum; Tris, tris(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; TLC, thin-layer chromatography.

0006-2960/90/0429-6639$02.50/0

microemulsion particles and DMPC disks (apoE–DMPC) were examined to determine if the particle size or the structural order/disorder of the lipid domains affected the conformation of bound apoE. The dissociation constants for binding of apoE to the large and small microemulsion particles were also measured as a function of temperature. The complexes were then tested for binding to the apoB/E receptor of normal human fibroblasts, and for their capacity to suppress HMG-CoA reductase activity. The goal of this study was to examine the relationship between lipoprotein particle size and apoprotein secondary structure, and the capacity of apoprotein–lipid complexes to bind to the apoB/E receptor.

Materials and Methods

Materials. DMPC from Sigma (St. Louis, MO) was judged >99% pure by TLC in chloroform/methanol/water/acetic acid (65:25:4:1). Cholesteryl oleate from Sigma was judged >99% pure by TLC in hexane/diethyl ether/acetic acid (90:10:1). Both lipids were used without further purification. Dextran sulfate and Brij 96 were also obtained from Sigma. Dulbecco's modified Eagle's media, fetal calf serum, trypsin–EDTA solution, and gentamicin sulfate were purchased from GIBCO (Grand Island, NY). Sodium [^{125}I]iodide (carrier free) in NaOH was obtained from New England Nuclear (Boston, MA). Bovine serum albumin and Aquacide were obtained from Calbiochem (San Diego, CA). Tissue culture flasks and petri dishes were obtained from Falcon Plastics (Cockeysville, MD).

Microemulsion and Vesicle Preparation. Large microemulsion particles were prepared as described previously (Mims et al., 1986a) by sonication and column chromatography. Small microemulsion particles were prepared in essentially the same manner; DMPC and CO were dissolved in 2-propanol in a 1:1 weight ratio (approximately 40 mg of total lipid). The solvent was removed, and the lipids were resuspended in 3.5 mL of 10 mM diethyl malonate, 0.15 M NaCl, and 1 mM EDTA, pH 7.4 at 60 °C, and sonicated for 1 h using a Heat Systems sonifier (W-350) equipped with a microtip at 40% power (140 W). The temperature was maintained at 55–60 °C throughout the sonication procedure. After sonication, the lipid mixture was centrifuged for 30 min at 18000*g* and 25 °C. After centrifugation, the cloudy fraction was carefully withdrawn so as not to disturb the titanium pellet. The crude microemulsion preparation was subjected to gel filtration over a Sepharose CL-2B column (1.6 × 90 cm); fractions which eluted within roughly the same size range as LDL were combined and concentrated by Aquacide treatment. To remove any possible contaminating DMPC vesicles, the concentrated preparation was placed at 4 °C for several hours or overnight. DMPC vesicles, which were unstable at this temperature, fused and formed larger multilamellar vesicles which were removed by centrifugation for 30 min at 18000 rpm, 15 °C. To further ensure that no vesicles contaminated the small microemulsion preparation, the density of the sample was adjusted to 1.03 g/mL with KBr, and the sample was spun for 18 h at 35000 rpm, 15 °C, in a Beckman SW 50.1 rotor. At this density, which was intermediate between that calculated for the small microemulsion and DMPC vesicles, the vesicles sank to the bottom of the tube while the microemulsion particles floated to the top where they were easily harvested. The SME recovered in this way was subjected to a second ultracentrifugal step at d = 1.03 g/mL, harvested from the top of the tube, and dialyzed against an appropriate buffer before use in experiments. The yield of SME particles following the ultracentrifugal steps was approximately 25%; these particles were stable for several weeks. DMPC vesicles were prepared by sonication as described previously (Mims et al., 1988).

Electron Microscopy. Formvar- and carbon-coated copper grids were pretreated with 0.1% bovine serum albumin. Small microemulsions were then applied to the grids and negatively stained with 2.0% phosphotungstic acid, pH 7.4. Grids were examined with a Jeol 200CX electron microscope. Magnifications were calibrated by using a Fulam diffraction grating replica. The average particle radius of each sample was determined from measurement of 100 individual particles, and these results are recorded in Table I.

Preparation of CER-VLDL. Lipoproteins were obtained from the plasma of adult female New Zealand white rabbits maintained on a 2% cholesterol diet (ICN Biochemicals). Rabbits were maintained on the diet for 4–6 weeks after which blood was collected in 1% EDTA from the central artery of the ear. Cellular components were sedimented by low-speed centrifugation, and Trasylol (Mobay Chemical Co.) was added immediately to inhibit apoprotein proteolysis. CER-VLDL was obtained by ultracentrifugation for 18 h at plasma density (55000 rpm, 4 °C). The whitish supernatant was removed and respun through buffered saline. The lipoproteins were again ultracentrifuged at 4 °C for 18 h to remove residual albumin. The lipoprotein fraction was removed by decantation, applied to a 1.6 × 90 cm column of Sepharose CL-2B, and eluted at a flow rate of 7 mL/h. The desired fractions were combined, treated with Trasylol, concentrated, and stored under nitrogen at 4 °C until use.

Preparation of ApoE. ApoE was isolated from the plasma of rabbits maintained on 2% cholesterol diets by a modification of the method of Roth et al. (1977). This protein had a molecular weight of 34000 as determined from SDS gel electrophoresis and was judged pure by its migration as a single band.

Lipid and Protein Quantitation. The chemical composition of the microemulsion and vesicle preparations was determined by a combination of methods. Phospholipid content was measured via the phosphorus assay of Bartlett (1959) using a multiplication factor of 21.87 for DMPC. Cholesteryl ester was determined by using an enzymatic assay kit from Calbiochem. Protein was determined by the method of Lowry (1951).

Radioiodination of LDL and ApoE. ^{125}I-Labeled LDL and apoE were prepared by the iodine monochloride procedure (Bilheimer et al., 1972). The final specific activity was <450 cpm/ng for LDL and <350 cpm/ng for apoE. In every case, >98% of the ^{125}I radioactivity was precipitable by incubation with 10% w/v trichloroacetic acid.

Preparation of ApoE–DMPC Complexes. DMPC vesicles prepared as described above were incubated with apoE in a final DMPC:apoE weight ratio of 2:1 at 25 °C for 2 h. In order to separate the apoE–DMPC complex from lipid-free apoE, the density of the samples was adjusted to 1.15 g/mL with KBr, and the samples were centrifuged for 24 h at 40000 rpm in a Beckman SW 50.1 rotor. At this density, apoE–DMPC discoidal complexes floated to the top of the tube where they were harvested by suction with a pasteur pipet; lipid-free apoE sank to the bottom of the tube. ApoE–DMPC complexes were dialyzed against appropriate buffer before use in experiments. When prepared in this manner, the complexes had a DMPC:apoE weight ratio of 5.5:1.

Preparation of ApoE–Microemulsion Complexes. Microemulsion complexes prepared as described above were incubated with apoE in a final DMPC:apoE weight ratio of 5:1 for 2 h at 30 °C. ApoE–LME complexes were separated from

lipid-free apoE by centrifugation at d = 1.006 g/mL, 4 °C, for 24 h at 40000 rpm in a Beckman SW 50.1 rotor and harvested from the top of the tube. ApoE–SME complexes were isolated by adjusting the density of the sample to 1.15 g/mL and centrifuging the sample at 4 °C for 24 h at 40000 rpm in a Beckman SW 50.1 rotor. The apoE–SME complex was harvested from the top of the tube and dialyzed against appropriate buffer before use in experiments. Phospholipid and cholesterol assays were performed on all particle preparations before use in experiments.

Circular Dichroism Measurements. Circular dichroic spectra were measured in a Jasco Model 500A spectropolarimeter equipped with a thermostatted cell holder. All measurements were made in a quartz cell (path length = 0.1 mm). Because of the contribution of lipids to the measured CD spectra of the apoE–DMPC and apoE–microemulsion complexes, and the possibility of light scattering by the large microemulsion complexes, spectra were measured for the apoE complexes and for the vesicles and microemulsions alone. Samples of large and small microemulsions and vesicles were prepared and divided into two equal aliquots. To one aliquot was added the appropriate amount of apoE as described above; the other aliquot served as the lipid control. ApoE complexes and their protein-free counterparts were treated identically in the ultracentrifugal isolation procedure, and all samples were dialyzed against 0.15 M NaCl, pH 7.0, prior to CD measurements. Phosphorus assays were performed on each sample, and the phosphorus concentrations of control and apoE complex pairs were adjusted to the same value with saline. Samples were allowed to equilibrate at each temperature for 10 min before spectra were recorded. To improve the signal to noise ratio, 16 scans were recorded and averaged for each of the protein-bound and protein-free samples at each temperature. Spectra of apoE bound to the surface of each of the particles were obtained by subtracting the spectrum of the apoE complex from its protein-free counterpart at each temperature.

Differential Scanning Calorimetric Measurements. Differential scanning calorimetry (DSC) was performed on all particles using a Microcal MC-2 scanning calorimeter. All data were collected as heating scans at a heating rate of 30 °C/h.

ApoE Binding Experiments. Binding experiments were performed in 10 mM diethyl malonate buffer (pH 7.4), containing 0.15 M NaCl and 0.5 M sucrose. The incubation mixture (1.0 mL) contained a constant amount of DMPC (500 μM) and various amounts of ^{125}I-apoE (0.4–10 μM). After incubation at 4, 25, or 37 °C for 2 h, the mixtures were transferred to a Beckman TLA 100.3 rotor thermostated and centrifuged at respective temperatures in a Beckman TL-100 ultracentrifuge (48000 rpm, 3.3 h at 4 °C; 40000 rpm, 4 h at 25 °C; 36000 rpm, 4.4 h at 37 °C). The top fraction (400 μL) was removed from each tube by using a syringe with a flat-tip needle and then counted to determine the protein concentration. Phospholipid concentration in the top and bottom fractions was determined by the method of Bartlett (1959); cholesteryl oleate was determined by using an enzymatic test kit (Boehringer Mannheim). All lipids were found in the top fraction. The lipid-bound apoE concentration was determined by subtracting the background of free apoE concentration in the top fraction (determined by centrifugation of apoE in lipid-free solutions) from the total apoE measured in the top fraction of the ultracentrifuged apoE/lipid mixture. A comparison between sucrose and KBr for adjusting the density of the incubation mixture was performed at 25 °C using the SME. The K_d values obtained with either sucrose or KBr differed by less than 15%.

Human LDL and Lipoprotein-Deficient Serum (LPDS). LDL and lipoprotein-deficient plasma were isolated from the plasma of healthy human subjects by ultracentrifugation (Goldstein et al., 1983). The lipoprotein-deficient plasma was converted to LPDS by incubating it at 4 °C for 24 h with thrombin (10 US units/mL). The resulting clot was removed by centrifugation and the LPDS sterilized by passage through a Millipore filter (0.45 μm). The protein concentration of the LPDS was adjusted to 50 mg/mL by dilution with normal saline and frozen at −70 °C until use.

Cells in Culture. Normal fibroblasts were derived from a preputial specimen from a normal infant. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 3.7 mg/mL $NaHCO_3$, and 50 μg/mL gentamicin sulfate and used between the seventh and twentieth passages. The cultures were maintained in a humidified atmosphere of 5% CO_2 in air at 37 °C. Confluent monolayers were harvested with a solution of 0.5% trypsin/0.02% EDTA in Hank's base and reseeded in 75 cm^2 flasks for cell maintenance, or in 60- or 35-mm petri dishes (4.0×10^5 or 1.0×10^5 cells, respectively per dish) for use in experiments. Four days after the cells had been plated into petri dishes, they were washed twice with DME media containing 10% LPDS; then 2 mL of DME media containing 10% LPDS was added. The experiments were begun 24 h after the cells had been in media containing 10% LPDS.

Assays for Binding, Internalization, and Degradation. The procedures of Goldstein et al. (1983) were used for binding (4 and 37 °C), internalization (37 °C), and degradation (37 °C) assays. Competition assays at 37 °C were initiated by the addition of DMEM media, 10% LPDS, 3 μg/mL ^{125}I-LDL, and varying amounts of unlabeled lipoproteins or model complexes to cells growing in petri dishes. All solutions were warmed to 25–35 °C before being added to the cells. The incubations were performed in a CO_2 incubator for 5 h. After 5 h, the medium was removed to determined proteolytic degradation by analysis of [^{125}I]monoiodotyrosine. An aliquot of the media was placed in 15-mL conical plastic tubes and chilled to 4 °C; BSA solution (2 mg/mL in 0.05 mM Tris, pH 7.4) was added as a carrier, and the protein was precipitated with trichloroacetic acid. After incubating at 4 °C for 30 min, the protein was pelleted by centrifugation; the clear supernatant was treated with hydrogen peroxide and then extracted with chloroform to remove free iodine. An aliquot of the aqueous phase was counted in a γ counter to determine proteolytic degradation.

High-affinity binding was determined after washing the cell monolayers at 4 °C with three aliquots of cold Tris-buffered saline containing 2 mg/mL BSA. Each dish was then incubated twice for 10 min at 4 °C with the same BSA-buffered saline solution followed by one rapid wash with ice-cold Tris-buffered saline with no BSA. Each dish then received an aliquot of 4 mg/mL dextran sulfate in 50 mM NaCl/10 mM HEPES at pH 7.4 and was placed on a rotary shaker for 1 h at 4 °C. Aliquots were removed from each dish and counted to determine high-affinity binding. The cells were then washed with Tris-buffered saline and dissolved by incubation overnight at 4 °C in 0.1 N NaOH. An aliquot of the cell suspension was counted to determine the amount of ^{125}I-LDL which had been internalized by the cells, and another aliquot was used to determine the content of cellular protein by the Lowry procedure. Binding studies at 4 °C were performed in the same manner, except that the DMEM media

contained 25 mM HEPES (pH 7.4) instead of bicarbonate, and 0.5 μg/mL of ^{125}I-LDL was used. Cells were preincubated at 4 °C in the media for at least 30 min. All solutions were prechilled to 4 °C prior to addition to the cells, and all additions and incubations were performed in a 4 °C cold room.

HMG-CoA Reductase Assay. The procedures of Goldstein et al. (1983) were used for HMG-CoA reductase activity in cultured cells. Cells were incubated in 10% LPDS for 24 h prior to beginning the experiments. The experiment was initiated by the addition of 2 mL of DMEM medium containing 10% LPDS and lipoprotein or microemulsion complexes. The incubations were performed in a CO_2 incubator for 24 h. After 24 h, the medium from each dish was discarded, and the cells were washed twice with 2 mL of cold Tris-buffered saline. The cells were scraped with a rubber policeman into 1 mL of cold Tris-buffered saline and transferred to a plastic microcentrifuge tube. The cell suspension was centrifuged at 1000 rpm for 1 min and the supernatant discarded. The cell pellet was frozen in liquid nitrogen and kept at −190 °C until the assay was performed.

Frozen cell pellets were dissolved in 0.1 mL of 50 mM phosphate buffer containing 5 mM dithiothreitol, 1 mM EDTA, and 0.25% Brij 96. The suspension was vortexed to resuspend the pellet, and incubated at 37 °C for 15 min to disrupt the cell membrane. The microsomal suspensions were spun in the microfuge for 1 min, and the tubes were put on ice.

A solution (50 μL) containing 25 mM dithiothreitol, 100 mM glucose 6-phosphate, 15 mM NADP, and 0.75 unit of glucose-6-phosphate dehydrogenase was pipetted into 16 × 125 capped tubes on ice. Sixty microliters of the microsomal preparation supernatant was added to each tube. The samples were placed in a 37 °C water bath for 5 min; then 20 μL of 260 μM [^{14}C]HMG-CoA (4600 cpm/μL) was added to start the assay. After 2 h, 20 μL of 60 mM [^{3}H]mevalonic acid lactone carrier (7000 cpm/μL) dissolved in 2.5 N HCl was added. The reaction was allowed to continue at 37 °C for 15 min to ensure complete lactonization of the mevalonic acid. This was followed by the addition of 1 mL of water and 1 g of anhydrous sodium sulfate; the mixture was extracted twice with 10 mL of diethyl ether. The extracts were combined, and the ether was evaprorated under a stream of nitrogen. The residue was dissolved in 60 μL of chloroform/methanol (2:1 v/v), spotted on silica gel TLC plates along with a standard of [^{3}H]mevalonolactone, and placed in a tank containing benzene/acetone (1:1). The standard was located by scraping 3-cm regions of the standard lane. A section of each lane extending 1 cm above and 1 cm below the standard band was scraped and placed in a vial containing 10 mL of Scintiverse E. Each sample was subjected to double-label scintillation counting.

Results

Production of Small Microemulsion. Several methods for the production and stabilization of SME particles have appeared in the literature. One of the most reproducible, that of Ginsberg et al. (1982), required 5 h of sonication followed by several centrifugal steps. In order to minimize sonication time and the possibility of lipid oxidation or degradation, we selected a protocol similar to that used for preparing large microemulsion particles (Mims et al., 1986a). In this scheme, DMPC and CO were dissolved in 2-propanol in a 1:1 weight ratio. The solvent was removed, and the lipids were resuspended in buffer and sonicated for 1 h at 55–60 °C. The crude microemulsion preparation was subjected to gel filtration over a Sepharose CL-2B column (1.6 × 90 cm). The column

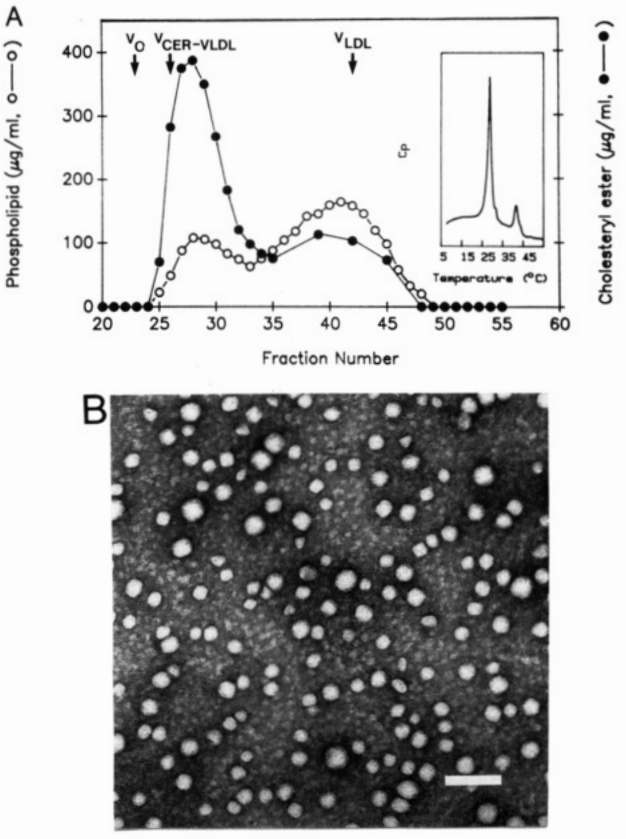

FIGURE 1: (A) Elution profile of small (SME) and large (LME) microemulsions of DMPC/CO over a 1.6 × 90 cm column of Sepharose CL-2B at 23 °C. Elution conditions: 7.2 mL/h; 2.4 mL/fraction. Open circles represent DMPC concentration in micrograms per milliliter; closed circles represent CO concentration in micrograms per milliliters. Inset: DSC thermogram of the small microemulsion (SME). Endotherms at 28 and 42 °C correspond to thermal transitions in the phospholipid surface shell and cholesteryl ester core, respectively. (B) Electron micrograph of small microemulsion particles isolated by gel filtration chromatography as shown in (A). Micrograph is at 72075×. Bar = 0.1 μm.

elution profile (Figure 1A) demonstrated two size populations of microemulsion particles. The larger microemulsion emerging in the first peak had a size similar to that of rabbit CER-VLDL, while the smaller microemulsion in the second peak eluted at a volume similar to that of human LDL. Fractions 36–45 in the second peak (SME) were combined, concentrated, and subjected to two ultracentrifugal washes to remove any contaminating DMPC vesicles. After the washing steps, the DMPC:CO weight ratio was 1.1:1. Electron micrographs (Figure 1B) of the small microemulsion complexes showed a population of spherical particles with an average diameter of 295 ± 64.5 Å. A differential scanning calorimetric thermogram of this material (inset to Figure 1A) indicated that the thermal behavior of these particles was qualitatively similar to that of the large microemulsion, demonstrating two endotherms: one at ~28 °C and a second at 42 °C. Binding of apoE to SME (as with LME) had little effect on the temperature of either DSC transition (data not shown). Table I summarizes the thermal behavior of the large and small microemulsions, of DMPC vesicles, and of their complexes with apoE.

Circular Dichroism. The CD spectra of apoE bound to disks, LME, and SME were examined over the temperature range 4–50 °C, to determine if particle size or the order/disorder of the lipid domains had an effect on the conformation of the protein. Chen and Kane (1975) have shown that the component lipids of LDL exhibit multiple Cotton effects in

Table I: Physical and Chemical Properties of Native and Model Lipoproteins Containing ApoB and/or ApoE

particle	diameter (Å)	% composition			T_M (°C)
		phospholipid	cholesteryl ester	protein	
LDL	200–220[a]			20.0	
CER-VLDL	750–1050[a]	13.5	68.3	3.3	
apoE-LME	525–1100[b]	19.6	78.6	1.8	27.8 (DMPC) 44.0 (CO)
apoE-SME	208–520[c]	48.8	46.8	4.4	25.0 (DMPC) 42.0 (CO)
apoE-DMPC	(110 × 60)[d]	82.2		17.8	24.6 (DMPC)

[a] Mims and Morrisett (1988). [b] Mims et al. (1986a). [c] This report. [d] M. P. Mims, unpublished results.

the UV region. Except for phospholipid, these Cotton effects showed reversible thermal dependence, becoming increasingly negative at lower temperatures. In general, most lipids exhibit very small CD bands between 210 and 230 nm. Below 210 nm, however, both cholesterol and cholesteryl esters exhibit minima at ~208 nm and maxima at 197–198 nm which become negative below ~195 nm. Thus, in order to obtain the spectrum of the bound apoE, it was necessary to make corrections in the spectra of the apoE–microemulsion complexes for contributions by cholesteryl ester. These corrections were made by subtracting the CD spectrum of the microemulsion alone from that of the apoE–microemulsion complex.

A further complication arose for the large microemulsion particles which scattered light. Four effects of particle size on the CD spectrum were possible: (1) absorption flattening caused when particles closest to the light source prevent light from reaching those further away; (2) dispersion distortion which arises from light-scattering effects; (3) differential absorption; and (4) differential scattering (Urry, 1972; Mao & Wallace, 1984). Since the large microemulsion particles were spherical, and thus symmetric, and since the LME alone did not rotate light except at wavelengths where cholesteryl oleate was known to absorb (Chen & Kane, 1975), we have estimated the contributions of possibilities 3 and 4 to the apoE–LME spectrum to be negligible. Possibilities 1 and 2, absorbance flattening and light scattering, would cause attenuation of the CD signal and result in an underestimate of the secondary structure present (Duysens, 1956; Bustamante et al., 1983). In order to examine the contribution of these effects to the apoE–LME spectra, we measured the UV and CD spectra of two proteins which do not interact with the LME, bovine serum albumin (BSA) and soybean trypsin inhibitor (STI), in the presence and absence of the microemulsion. These measurements were made at the same protein and microemulsion concentrations and in the same path-length cell used in the first experiments. The spectra obtained for BSA and STI (250 μg of protein/mL) were almost identical with those obtained in the presence of the microemulsion when the lipid contributions were subtracted out. On the basis of these results, we concluded that under the conditions used in this study, absorption flattening and light scattering did not contribute to the CD spectra obtained. A representative set of data is shown in Figure 2 for the LME, apoE–LME, and the bound apoE difference spectra.

The results of the thermal CD study for apoE bound to DMPC disks, LME, and SME particles are shown in Figure 3. The α-helicities shown in this figure were calculated from the raw data which were corrected for lipid contributions. Specific ellipticities were calculated on the basis of the equation:

$$[\theta] = \theta^\circ M/10lc \qquad (1)$$

where θ° is the observed ellipticity in degrees, M is 115 for apoE (mean residue weight), l is the cell path length in centimeters, and c is the concentration of protein in grams per milliliter. Helicity was calculated from the relation (Chen et al., 1972)

$$\alpha = \frac{[\theta]_{222} + 2340}{30\,300} \qquad (2)$$

On the basis of these calculations, apoE bound to DMPC disks or to SME was found to be highly helical (95% and 73%, respectively, at 4 °C). ApoE bound to LME was less helical (60% at 4 °C). Over the temperature range 4–50 °C, the α-helicity of apoE bound to disks and SME declined monotonically by about 10–15%. ApoE bound to LME, however, demonstrated an abrupt decrease (from ~60% to 49%) in helicity between 18 and 25 °C (near the LME surface transition). The helicity then remained constant at 45–50% from 25 to 50 °C. ApoE alone displayed ~37% helicity over the entire temperature range (data not shown). The temperature-dependent changes in the apoE–LME CD spectra were reversible. When the apoE–LME and LME samples were

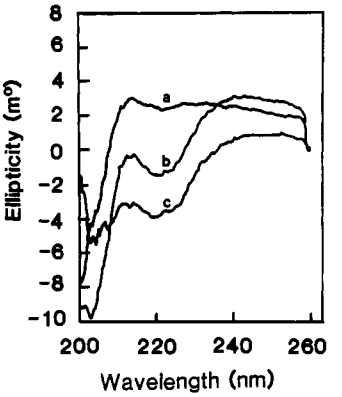

FIGURE 2: Circular dichroic spectra of (a) LME and (b) apoE–LME and (c) the difference spectrum between the two.

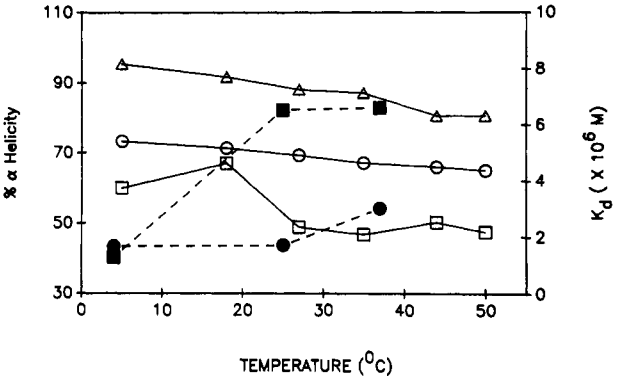

FIGURE 3: Temperature dependence of α-helicity of apoE bound to LME (□), SME (○), and DMPC disks (△). Helicity was computed from the CD spectra as described under Results. ApoE concentrations were 250 μg/mL for apoE–LME and apoE–SME measurements and 375 μg/mL for apoE–DMPC measurements. Closed symbols represent the K_ds measured for apoE binding to SME (●) and LME (■) at 4, 25, and 37 °C.

Table II: Constants for Dissociation of ApoE from Small and Large Microemulsions Containing DMPC and CO[a]

temp (°C)	K_d (×10⁶ M) small microemulsion	K_d (×10⁶ M) large microemulsion
4	1.68 ± 0.13	1.29 ± 0.31
25	1.70 ± 0.30	6.53 ± 0.22
37	3.02 ± 0.42	6.60 ± 0.28

[a] Average number of phospholipid molecules per apoE (N) = 1040 for both LME and SME complexes.

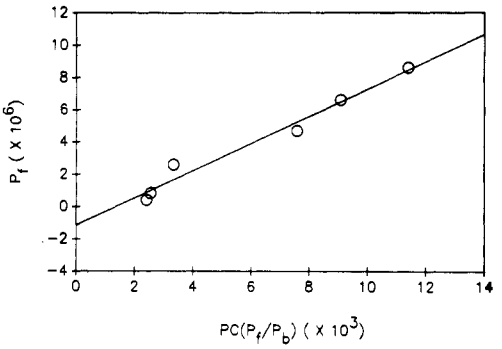

FIGURE 4: Binding curve for the association of apoE with SME at 4 °C.

cooled from 50 to 18 °C, a difference spectrum identical with the original 18 °C spectrum was obtained. Reheating the samples to 35 °C resulted in a difference spectrum identical with that obtained initially. Similar reversibility results were obtained for the apoE–SME and apoE–disk complexes.

ApoE Binding to the Microemulsion Particles. A series of studies was undertaken to determine the effect of particle size and temperature on the binding of apoE to the microemulsion particles. These studies were conducted at 4, 25, and 37 °C for both the large and small microemulsion particles. The results, summarized in Table II, were analyzed according to the model of Tajima et al. (1983) which assumes reversible binding. A representative set of data (Figure 4) is linearized according to the equation:

$$P_f = [PC](P_f/P_b)N - K_d \quad (3)$$

where P_f and P_b are the concentrations of free and bound protein, respectively, [PC] is the concentration of phospholipid, N is the binding saturation level, and K_d is the dissociation constant of apoE from the microemulsion surface. The dissociation constants reported in Table II were obtained as the ordinate intercept of the least-squares regressed line. These data show that for the small microemulsion, the dissociation constant is ~1.7 μM at 4 and 25 °C and increases slightly to 3.0 μM at 37 °C. In contrast to these results, apoE binds LME with a K_d at 25 and 37 °C (~6.6 μM) that is 5 times greater than that measured at 4 °C (1.3 μM).

Receptor Binding. (A) Competition Studies. ApoE-DMPC, apoE–SME, and apoE–LME, along with CER-VLDL and LDL, were tested for their capacity to compete with ^{125}I-LDL for binding by the B/E (LDL) receptor of fibroblasts. The lipoproteins and model particles were tested at protein concentrations which ranged from 0.17 to 8.4 μg/mL at 4 °C, and from 1 to 50 μg/mL at 37 °C; however, the results for apoE–SME and apoE–LME in Figure 5 are reported in terms of lipid-bound protein concentration rather than total protein concentration. Lipid-bound protein refers to the portion of the total apoE which remains bound to the lipid particle (P_b in eq 3); only apoE which is bound to a lipid particle has the potential to be recognized by the receptor. Lipid-bound apoE concentration, P_b, was calculated from eq 3 by substituting $P_T - P_b$ for P_f (where P_T is equal to the total concentration

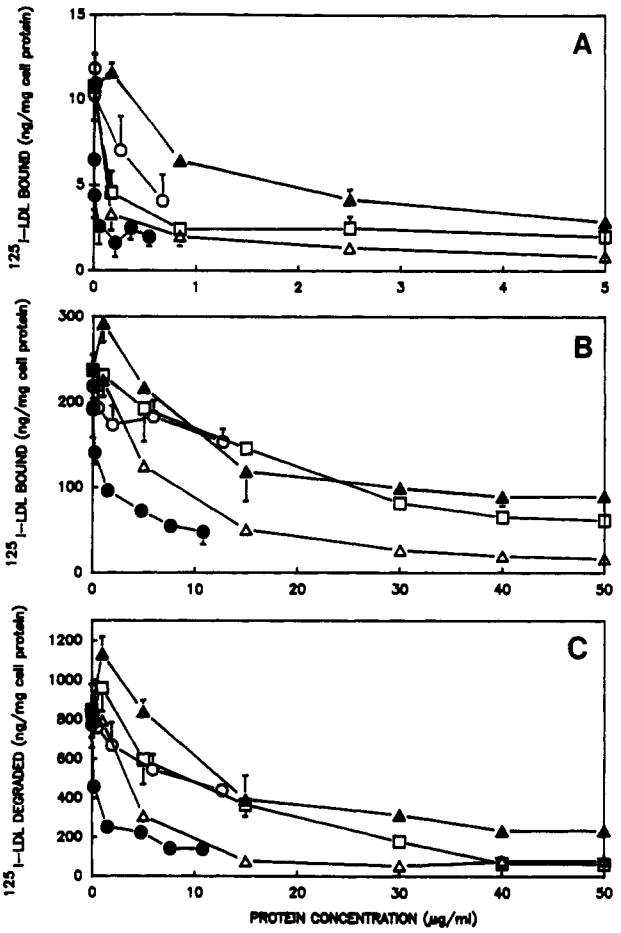

FIGURE 5: Competition between ^{125}I-LDL and various native and model lipoproteins for the B/E receptor of normal human fibroblasts. (A) Binding at 4 °C; lipid-bound apoE concentrations for the apoE–LME curve are 0.35, 1.4, 17, 67, 260, and 670 ng; lipid-bound apoE concentrations for the apoE–SME curve are 0.27, 6.4, 53, 210, 360, and 540 ng. (B) Total uptake at 37 °C; lipid-bound apoE concentrations for apoE–LME are 0.012, 0.048, 0.510, 1.92, 5.90, and 12.7 μg/mL; lipid-bound apoE concentrations for apoE–SME were 0.009, 0.21, 1.49, 4.76, 7.60, and 10.8 μg/mL. (C) Degradation at 37 °C. Symbols represent CER-VLDL (□), LDL (▲), apoE–LME (○), apoE–SME (●), and apoE–DMPC disks (△). Each point represents the mean of triplicate dishes of cells. Competition experiments were performed 3 times with three different preparations of microemulsion particles and disks. Where no error bars appear, the experimental error was smaller than the symbol itself.

of apoE) and solving for P_b using the phospholipid concentration ([PC]), and the K_d and N values determined at 4 and 37 °C in the binding studies. In accord with previous studies (Gianturco et al., 1980; Innerarity et al., 1979), the results demonstrated that CER-VLDL and apoE-DMPC disks competed very effectively with LDL for receptor binding. When the results obtained with the apoE–LME and apoE–SME complexes were plotted in terms of lipid-bound protein concentration, these particles also appeared to compete effectively with LDL for binding to the receptor. At both 4 and 37 °C, apoE–SME appeared to compete more effectively than apoE–LME for receptor binding. All of the differences observed at 37 °C for total uptake were also reflected in degradation measurements (Figure 5C). Control experiments in which microemulsion particles alone (without apoE) were added demonstrated that the lipid particles alone were not capable of competing for receptor binding (data not shown).

(B) HMG-CoA Reductase Studies. ApoE-LME, apoE-SME, LDL, and CER-VLDL were tested for their capacity to suppress HMG-CoA reductase activity in human skin fibroblasts. The results shown in Figure 6 demonstrate that both

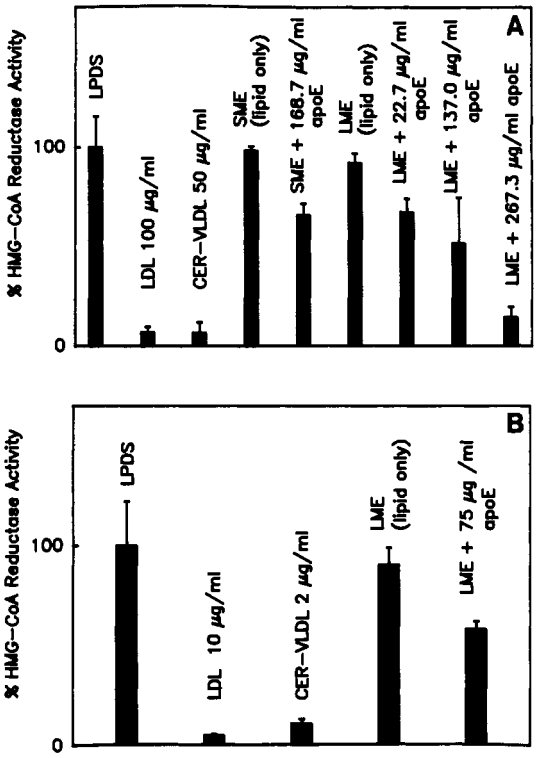

FIGURE 6: Comparison of the capacities of native lipoproteins and apoE–microemulsion complexes to suppress HMG-CoA reductase activity. (A) Total cholesteryl oleate concentration was 1.2 mg/mL for SME, apoE–SME, LME, and all three apoE–LME points. Total and lipid-bound apoE concentrations for apoE–SME were 168.7 and 32.8 μg/mL, respectively. Total apoE concentrations for the three apoE–LME experiments were 22.7, 137.0, and 267.3 μg/mL. The corresponding lipid-bound apoE concentrations for the apoE–LME experiments were 1.25, 5.34, and 7.75 μg/mL, respectively. One hundred percent activity (LPDS value) was equal to 39.2 pmol min^{-1} (mg of cell protein)$^{-1}$. (B) Total cholesteryl oleate concentration was 0.1 mg/mL for LME and apoE–LME. Total and lipid bound apoE concentrations for apoE–LME were 75 and 0.76 μg/mL, respectively. One hundred percent activity (LPDS value) was equal to 119.8 pmol min^{-1} (mg of cell protein)$^{-1}$.

LDL and CER-VLDL are capable of suppressing HMG-CoA reductase activity. LME and SME complexes were tested at identical cholesteryl oleate concentrations (1.2 mg/mL, Figure 6A), and the LME complex was also tested at a cholesteryl oleate concentration of 0.1 mg/mL (Figure 6B). At a cholesteryl oleate concentration of 1.2 mg/mL, the SME alone demonstrated little if any capacity to suppress the reductase; however, addition of 168.7 μg/mL apoE to the SME, corresponding to 32.8 μg/mL lipid-bound apoE, resulted in a 34% suppression of reductase activity. At an identical cholesteryl oleate concentration, the LME alone did not significantly suppress enzyme activity; however, addition of 22.7, 137.0, and 267.3 μg of apoE/mL (equivalent to 1.24, 5.34, and 7.75 μg of lipid-bound apoE, respectively) to the LME suppressed reductase activity by ~33, 48, and 86%, respectively (Figure 6A). At lower concentrations, LDL (10 μg/mL) and CER-VLDL (2 μg/mL) also effectively suppressed HMG-CoA reductase (Figure 6B). At a cholesteryl oleate concentration of 0.1 mg/mL, the LME alone did not suppress HMG-CoA reductase activity; however, addition of 75 μg of apoE/mL to the LME (corresponding to 0.76 μg/mL lipid-bound apoE) suppressed reductase activity by ~42%.

Discussion

Results obtained in these studies indicate that the conformation of apoE can be affected by the size of the particle to which it is bound, and possibly by the order/disorder of the lipid constituents. At 4 °C, all of the lipid constituents of the DMPC disks, LME, and SME are in an ordered state (Ginsberg et al., 1982; Mims et al., 1986a); thus, the effect of microemulsion particle size on the apoprotein conformation should be most evident. ApoE bound to LME displays ~60% helicity at 4 °C, while apoE bound to SME displays 73% helicity. This difference appears to be due primarily to particle size, since the protein:phospholipid ratio is the same for both particle preparations, and the K_ds for apoE binding to the particle surface are almost identical. The helicity of apoE bound to the DMPC disk is greater than that observed for apoE bound to either of the microemulsion particles, possibly reflecting a difference in the mechanism whereby apoE binds to the disk. ApoE binding to the microemulsion primarily involves interaction of the protein with the phospholipid polar head groups. In contrast, apoE binding to DMPC disks is mediated primarily through interaction of the protein with the phospholipid acyl chains. Thus, the conformation of apoE on the discoidal particle might be significantly different from its conformation on the spherical microemulsion. Indeed, using different lipid complexes and canine apoE, Chen et al. (1984) have also found that the helicity of an apoE–egg PC complex is greater than that of an apoE–ME complex.

The unusual temperature dependence of the apoE–LME CD spectrum is puzzling. Each of the particles, apoE–DMPC, apoE–SME, and apoE–LME, has a thermal transition in the range 4–50 °C; however, only apoE–LME demonstrates an abrupt change in its CD spectrum in response to temperature. This behavior could reflect either a change in the conformation of apoE bound to the microemulsion surface or an increase in the K_d for apoE binding to the LME surface. An increase in K_d would result in an increased ratio of unbound/bound apoE and would be reflected in the CD spectrum by a loss of α-helicity. Our binding results suggest that the second possibility is the more likely, since the K_d for apoE binding to the LME surface increases dramatically with increasing temperature between 4 and 25 °C (Figure 3). Why the surface thermal transition might result in decreased binding of apoE to LME, but not to SME, is not clear. This phenomenon may be related to differing surface curvatures of these different sized particles. Packing of DMPC headgroups on the surface of the LME particle is undoubtedly tighter than on the surface of the smaller, more highly curved SME particle. Looser DMPC packing on the SME surface could permit greater interaction of the protein with lipid, and result in tighter binding of apoE to the SME surface than is possible in the more closely packed LME surface. Above the thermal transition, the effective volume occupied by each phospholipid molecule is increased, the surface is less ordered, and the molecules are more mobile. Combined with the more restricted packing of DMPC molecules on the LME surface, the changes induced by the surface thermal transition might reduce interaction between DMPC molecules and apoE, resulting in a shift in the binding equilibrium. The DSC thermogram of the apoE–LME complex shows a single, broad endotherm in the region of the surface transition; no overlapping endotherm representative of the dissociation of surface-bound apoE is present. However, if apoE is not bound tightly to the LME surface, the energy released in dissociation might be small and hidden under the broad endotherm of the phospholipid transition.

Differences in the secondary structure of apoE on the surface of different model particles, and in the equilibrium binding of apoE to the surface of these particles, might be reflected in the physiological behavior of the protein. We have

examined this possibility by testing each particle for apoE-mediated binding to the B/E receptor of fibroblasts. Such a comparison is not entirely straightforward, however, since as we have shown above in binding studies, only a fraction of the apoE in solution is bound to the microemulsion particles and is thus potentially receptor-active. On the basis of the K_d values obtained in the binding experiments, and the particle phospholipid concentrations, the proportion of apoE bound to the microemulsion surface was calculated. A further complication arises in these interpretations, however, when one considers that more than one apoE molecule may bind to the surface of a microemulsion particle. On the basis of average size of the microemulsion particles (SME = 295 Å diameter, LME = 750 Å in diameter), the surface area of the DMPC molecule (50.2 Å^2 at 4 °C, 60.8 Å^2 at 37 °C) (Mims et al., 1986a), and the average number of phospholipid molecules per apoE molecule at saturation (1040) determined in the binding experiments, an average of 5446 at 4 °C (4496.5 at 37 °C) molecules of DMPC per SME particle and 35 202 at 4 °C (29 064 at 37 °C) per LME particle can be calculated. These values correspond to a maximum of ~5 (5.23 at 4 °C; 4.3 at 37 °C) apoE molecules on the SME surface and ~34 (33.8 at 4 °C, 27.9 at 37 °C) apoE on the LME surface at saturation. At apoE concentrations below saturation, fewer apoE molecules may be bound to the particle surface. Such considerations made it nearly impossible to perform direct binding studies in which there is a strict requirement for a fixed correspondence between apoE and particle concentrations over the entire range of protein concentrations tested.

Such a rigorous correlation might not be required in competition studies, however, if certain criteria are met. If one assumes that every apoE molecule on the microemulsion surface is independent, and that the binding of one apoE, and the simultaneous sequestering of the other apoE molecules on the particle, does not significantly alter the lipid-bound (or receptor-active) apoE concentration of the incubation medium, then competition experiments can be considered valid. The supposition of the independence of surface-bound apoE molecules is probably best made at less than 50% saturation of the particle surface so that there is less likelihood of physical contact between the apoE molecules, and cooperative behavior by the receptors (Funahashi et al., 1989). Indeed, Funahashi et al. (1989) have recently shown that when three or fewer apoE molecules (out of a possible seven) were bound to triglyceride-rich emulsions (260-Å diameter), little or no cooperativity was observed. Since only a very small fraction of the receptor-active apoE added to the dishes is bound to the cells, it is probably valid to assume that binding of a particle and the resulting removal of all of its associated apoE from the available pool have little effect on the overall active apoE concentration.

In this study, competition experiments were performed such that the protein:phospholipid ratio was constant at every point for the microemulsion complexes. Therefore, at each different protein concentration, a different number of apoE molecules could be bound to the microemulsion particle. At 4 °C, the average number of apoE molecules per particle was always less than one for the apoE–SME complex; thus, a constant relationship was maintained between protein concentration and particle concentration, and the results at each point could be compared. In the case of the apoE–LME complex, the first 3 points (Figure 5A) corresponded to less than 1 apoE per particle, and the next 3 to 1.75, 3.3, and 5 apoE per particle. On the basis of the above criteria, however, the apoE–LME competition curve may be considered valid, since even at the highest protein concentration, the particle surface saturation was <20%. Comparison of the apoE–SME and apoE–LME results suggests that at 4 °C, the apoE–SME complex competed more effectively with LDL for binding to the apoB/E receptor than did the apoE–LME particle. ApoE–SME also appeared to be more effective than apoE–DMPC in competing for receptor binding.

At 37 °C (Figure 5B,C), both apoE–microemulsion complexes also appear to compete effectively against ^{125}I-LDL for binding to the receptor. The lipid-bound apoE concentrations for apoE–SME range from 9 ng/mL to 10.8 μg/mL; the first 3 points of the competition curve represent <1 apoE per particle, and the last 3 1.28, 1.55, and 1.77 apoE per particle. At 37 °C, apoE–SME appears to compete much more effectively for receptor binding than any of the other particles tested. The lipid-bound apoE concentrations range from 12 ng/mL to 12.7 μg/mL for the apoE–LME complex; however, only the first point of the competition curve corresponds to <1 apoE/particle, with the highest protein concentration corresponding to 13.4 apoE/particle. All of the apoE–LME points represent less than 50% surface saturation, and no abrupt decrease in ^{125}I-LDL binding is observed which would suggest cooperative behavior. Again at 37 °C, apoE bound to the small microemulsion appears be a better ligand for the apoB/E receptor than apoE bound to large microemulsion particles. This apparent difference in the receptor affinities of apoE bound to the small and large particles may reflect a difference in the conformation of the protein; however, it may also reflect a difference in the size of the microemulsion particles themselves. It is possible that the small microemulsion particles, which are closer in size to LDL than the large particles, may have access to a population of receptors which are sterically inaccessible to the large microemulsion particles. Thus, even if apoE bound to either particle had the same receptor affinity, in competition studies this would be reflected in an apparent superior capacity of the apoE–SME particles to compete with ^{125}I-LDL. Comparison of the final points in the competition curves of CER-VLDL and LDL, however, demonstrates that the large CER-VLDL displaces more ^{125}I-LDL than LDL itself, suggesting that the large size of the CER-VLDL does not hinder its access to the receptors. Since the LME particles are in the same size range as CER-VLDL, the differences in the competition curves of apoE–SME and apoE–LME may in fact be due to differences in the conformation of the bound protein. Although rabbit CER-VLDL has been reported to compete more effectively than LDL for ^{125}I-LDL binding to the LDL receptor (Gianturco et al., 1980), we consistently observed this only at 4 °C, and not at 37 °C. Although this result is rather unexpected, it does not alter the interpretation of the apoE–DMPC, apoE–SME, or apoE–LME data.

HMG-CoA reductase studies were undertaken to show that the apoE–microemulsion particles were capable not only of binding to the apoB/E receptor but also of regulating intracellular enzymes in the same way as the native lipoproteins LDL and CER-VLDL do. Neither the large nor the small microemulsion alone was capable of suppressing enzyme activity; thus, if nonspecific uptake of the microemulsion particles occurred, the internalized cholesteryl ester was not in a pool which could participate in regulation of HMG-CoA reductase. When apoE was added to the microemulsion complexes in the incubation medium, significant reduction of HMG-CoA reductase activity was observed. In the case of the large particles, increasing the apoE concentration while holding the lipid concentration constant at 1.2 mg of cholesteryl oleate/mL

produced increasing suppression of the enzyme. At a cholesteryl oleate concentration of 0.1 mg/mL and a lipid-bound apoE concentration of 0.76 μg/mL, the apoE–LME complex also effectively suppressed HMG-CoA reductase. Direct comparison of the results for the small and large apoE–microemulsion particles is difficult since binding and internalization of each large particle delivers 6–8 times more cholesteryl ester to the cell than the small particle does. Nevertheless, both apoE–microemulsion complexes appeared to mimic the action of LDL and CER–VLDL on the enzyme, demonstrating that the microemulsion complexes are valuable models for the study of intracellular lipoprotein metabolism.

ACKNOWLEDGMENTS

We thank Dr. John S. Olson and Dr. David Via for helpful discussion during preparation of the manuscript and Dr. John Guyton and Mr. Keith Klemp for their assistance with the experiments involving electron microscopy. In addition, we thank Drs. Sandra Gianturco and William Bradley for performing the initial fibroblast experiment, demonstrating the feasibility of the model lipoprotein–cell interaction studies.

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