COMMUNICATION

α-Helical Requirements for Free Apolipoproteins to Generate HDL and to Induce Cellular Lipid Efflux

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The structural requirement has been studied for apolipoproteins in their free form to interact with cells, to generate high density lipoprotein (HDL), and to cause cellular lipid efflux (*J. Biol. Chem. 266*, 3080-3086, 1991). It is shown that human apolipoprotein (apo) A-IV and apolipophorin III of *Manduca sexta* cause cholesterol efflux from cholesterol-loaded mouse peritoneal macrophages and reduce intracellularly accumulated cholesteryl ester as a result of forming HDL-like particles with cellular lipids, as do apoA-I, A-II and E. On the other hand, similar to apoC-III, reduced-and-carboxymethylated human apoA-II had no such effect. Thus, apolipoproteins seem to require at least four amphiphilic helical segments per molecule to express this function.
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Removal of cholesterol from cells is the initial step of cholesterol transport from peripheral tissues to the liver, and high density lipoprotein (HDL) has been shown to function as an efficient acceptor of cellular cholesterol *in vitro* (1-5). When incubated with human plasma, cellular cholesterol taken up appears first in a minor HDL subfraction, "pre-βHDL", and then in other HDL fractions (6).

We have demonstrated that free apolipoproteins (apo) A-I, A-II and E in solution interact with macrophages and generate HDL-like particles with pre-β electrophoretic mobility, resulting in the release of cellular cholesterol and in lower levels of cellular cholesteryl ester (7). The K_m values of the reaction seem to be consistent with the putative free apolipoprotein concentrations in plasma or in peripheral lymph, since those values are as low as 0.25% of the apoA-I concentration in plasma (7-9). Thus, this reaction may play a physiological role in the first step of cholesterol removal from cells and appears consistent with the finding that cellular cholesterol appears first in "pre-βHDL" (6).

This reaction was observed with apoA-I, A-II and E while apoC-III did not show such an effect (7) although the latter protein binds to lipids as strongly as do the three former apolipoproteins (10-12). It appears that some parameter related to apolipoprotein chain length, such as the number of amphiphilic helical segments (11,13-15), may be one of the determining factors for formation of HDL-like particles rather than the presence of specific domains that would be required for the interaction. In order to test this hypothesis, complementary experiments were carried out with a series of apolipoproteins, including human apoA-IV, reduced-and-carboxymethylated human apoA-II (CMapoA-II) (16), and insect apolipophorin III (apoLpIII) from *Manduca sexta* (17).

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Abbreviations: apo, apolipoprotein; apoLpIII, apolipophorin III; CM, reduced and carboxymethylated; HDL, high density lipoproteins; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate.

MATERIALS AND METHODS

ApoA-I, A-II and apoE were isolated from human plasma as previously described (10,12). ApoA-IV was isolated from fresh human plasma according to the method described by Weinberg and Scanu (18). The bottom fraction of the density 1.21 g/mL adjusted with NaBr was dialyzed against 5 mM Na-phosphate buffer, pH 7.4, containing 0.15 M NaCl, and then diluted with the same volume of the buffer, to which NaCl was added at 0.28 g/mL. The fraction was then mixed with 20% Intralipid (KabiVitrum Canada, Newmarket, Ontario) at a ratio of 3:1 (v/v) and incubated at 37°C for 1 hr. The lipid emulsion was isolated by centrifugation and delipidated with ethanol/diethyl ether (1:3, v/v). Protein was applied to a DEAE/cellulose column using a gradient from 50 to 90 mM Tris-HCl, pH 8.2, containing 8 M urea. The fraction containing apoA-IV was rechromatographed under the same condition to obtain pure apoA-IV, which was identified as a single band with a molecular weight of 45,000 in polyacrylamide gel electrophoresis in the presence of 1% sodium dodecylsulfate (SDS-PAGE). ApoLpIII was isolated from hemolymph of *Manduca sexta* (17) and kindly provided by Dr. Robert Ryan, Lipid and Lipoprotein Research Group, University of Alberta, Edmonton, Alberta, Canada. The protein migrated as a single band in SDS-PAGE. ApoA-II was reduced and carboxymethylated by iodine acetate in the presence of β-mercaptoethanol as previously described (16,19). The modified apoA-II migrated as a single band with an apparent molecular weight of 8700 in SDS-PAGE. All apolipoproteins were lyophilized and stored under argon at −75°C. Aqueous solutions of each protein were prepared in a manner as described previously (10, 12,17). Protein concentrations of the solution were determined by absorbance at 280 nm using the specific molar extinction coefficient for each protein.

Low density lipoprotein (LDL) was labeled with [1,2-^{3}H]-cholesteryl oleate (45.4 Ci/mmol, Amersham, Arlington Heights, IL) as described (7,20) and then acetylated (7,21). Mouse peritoneal macrophages were obtained from ICR mice by peritoneal lavage, and approximately 2×10^6 cells were placed in each dish (3.5 cm). Cells adhering to the dishes were incubated with labeled acetylated LDL for 24 hr (and with the [*methyl*-^{3}H]choline chloride, Amersham, when phospholipids were also labeled), and then for another 24 hr without lipoprotein at 37°C (7). The cells were then exposed to the apolipoprotein solutions in the medium at 37°C for a certain period of time (7). The lipids were extracted from the medium and the cells, analyzed by thin-layer chromatography, and radioactivity in free and esterified cholesterol (and in each choline-containing phospholipid when labeled) was determined as previously described (7). Specific radioactivities of free and esterified cholesterol were determined for a few experimental points in each series of the experiment, based on quantitation of the lipids by gas chromatography. An average value was used for calculation of cholesterol levels of

other experimental points based on radioactivity (7). To determine the density of the peak for cholesterol, the medium was analyzed by sucrose density gradient ultracentrifugation as previously described (7).

RESULTS AND DISCUSSION

Figure 1 shows the change in cellular cholesterol and its efflux into the medium resulting from incubation of the cells with apoLpIII and apoE. The release of free cholesterol from cholesterol-loaded macrophages into the medium was demonstrated with both apolipoproteins. The reciprocal decrease in intracellular cholesterol was demonstrated for esterified cholesterol. In both cases, the rates were dose-dependent showing a saturation kinetics profile. K_m values obtained from double reciprocal plots were 1-1.5 $\times$ 10^{-6} M and 4 $\times$ 10^{-7} M for apoLpIII and apoE, respectively. The results were highly consistent with those previously reported for apoA-I, A-II and E (7). Figure 2 shows the release of free cholesterol into the medium as mediated by apoA-II, CMapoA-II, and by apoA-IV. Substantial efflux of free cholesterol was demonstrated with apoA-II consistent with previous work (7), but CMapoA-II did not cause cholesterol release from the cells. On the other hand, apoA-IV was shown to be capable of removing cellular cholesterol. Analysis of the medium in case of apoLpIII by density gradient ultracentrifugation showed peaks for free cholesterol, phosphatidylcholine and sphingomyelin all at the density of 1.1 g/mL (Fig. 3), consistent with the results previously obtained with apoA-I, A-II and E (7).

All these apolipoproteins contain amphiphilic α-helical segments of some 22-24 amino acid residues which are thought to be responsible for interactions with lipids (11, 13-16,22,23). Table 1 lists the apolipoproteins examined

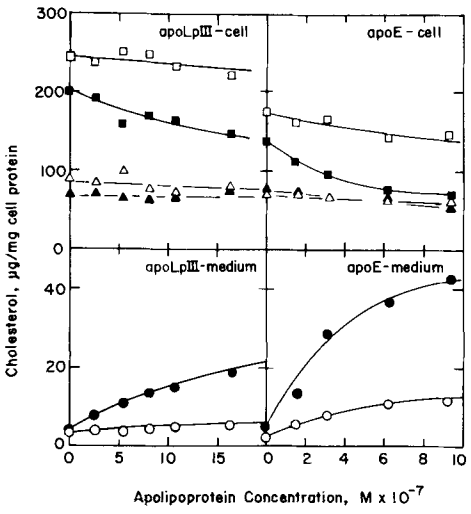

FIG. 1. Release of free cholesterol from macrophages and reciprocal decrease in intracellular cholesterol induced by apolipoproteins. Cholesterol-loaded macrophages were exposed to a solution of human apoE or apoLpIII of *Manduca sexta* for 6 hr (open symbols) or 24 hr (closed symbols). Free cholesterol in the medium (circles), and free and esterified cholesterol in the cells (triangles and squares, respectively) were determined as described in the Methods section (8). Cell protein per dish was 43.8 $\pm$ 5.0 μg for apoLpIII and 81.8 $\pm$ 10.3 μg for apoE.

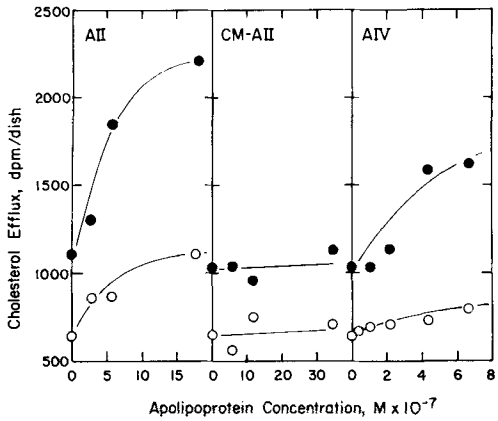

FIG. 2. Apolipoprotein-induced release of cholesterol from macrophages into the medium. ApoA-II, left; CMapoA-II, middle; apoA-IV, right. Cholesterol-loaded macrophages were incubated with apolipoproteins for 6 hr (open circles) or 24 hr (closed circles). Specific radioactivity of cholesterol is approximately 4000 dpm per μg of total cellular cholesterol. Cell protein per dish was 25.6 $\pm$ 2.9 μg.

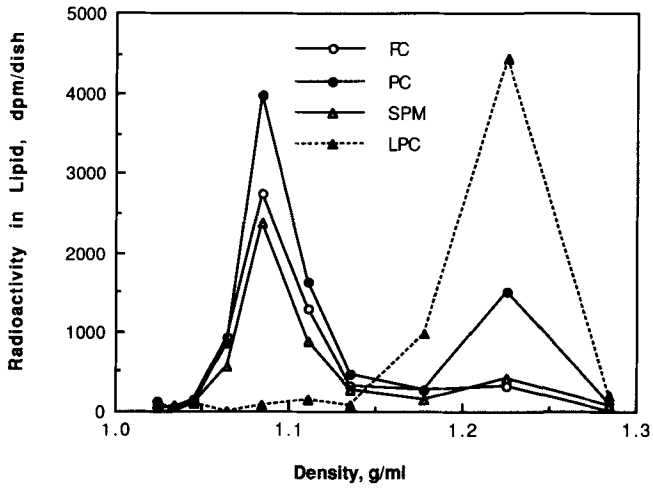

FIG. 3. Density gradient ultracentrifugation of the medium containing apoLpIII after incubation with macrophages labeled with [^{3}H]cholesterol and [^{3}H]choline. Cell protein per dish was 15.3 $\pm$ 1.1 μg. Symbols: free cholesterol, (○); phosphatidylcholine, (●); sphingomyelin, (△); lysophosphatidylcholine (▲).

TABLE 1

Cellular Cholesterol Efflux Induced by Free Apolipoproteins and Their Number of Amphiphilic Helices

Apolipoprotein	Number of amino acid residues	Number of helices[a] (reference)	Cholesterol release from macrophages[b]
ApoA-IV	376	≈ 12 (22)	+
ApoE	299	8 (15)	+
ApoA-I	243	6 (14)	+
ApoLpIII	166	5 (23)	+
ApoA-II	77 × 2	≈ 4 (11)	+
CMapoA-II	77	≈ 2	−
ApoC-III	79	≈ 2 (13)	−

[a] Number of amphiphilic helical segments of 20 to 24 amino acids per molecule as predicted (references).

[b] Capability of apolipoproteins to generate HDL and to cause cellular lipids efflux upon interaction with cholesterol-loaded macrophages.

and gives the putative number of such segments in each molecule. The data suggest that apolipoproteins with more than four such segments are capable of removing cellular cholesterol by generating HDL-like particles, whereas those with two or less such segments can not.

ApoC-III and CMapoA-II both contain at most only two amphiphilic helical segments. ApoC-III is in a random structure and does not self-associate in aqueous solution (10,12,24). However, it binds to the lipid surface as strongly as do other apolipoproteins whereby an amphiphilic helical structure is induced (10–12). Human apoA-II is a disulfide-linked homodimer and its carboxymethylation generates two identical monomers which differ from the original monomer only by the modified cystein at residue 6. CMapoA-II strongly self-associates in aqueous solution, thereby inducing a helix, and binds to the lipid surface (16). However, neither generate HDL-like particles upon interaction with macrophages.

Most vertebrate apolipoproteins except apoB, including those examined in this study, are likely to belong to a gene family under the same evolutionary tree (25,26) having common structure of multi-segments of amphiphilic helices. However, sequence homology is not strikingly high among them, so that it seems difficult to postulate that these apolipoproteins interact with the cells *via* a specific domain. ApoA-I, A-II and A-IV complexed with phospholipid reportedly bind to the same membrane "receptor site" and promote cholesterol efflux from adipocytes (27). This may argue against specific interactions between these apolipoproteins and membrane protein(s). Furthermore, cholesterol removal has been demonstrated with insect apoLpIII from *Manduca sexta*, which is poorly homologous to human apolipoproteins in terms of amino acid sequence (28), but is similar in function to human apolipoproteins in regard to physicochemical properties (28,29) based on its amphiphilic helical segments (23). It is therefore unlikely that any specific domain, such as a recognition site for a cell membrane protein, could be required for interaction of free apolipoproteins with cholesterol-loaded macrophages to generate HDL-like lipoproteins under removal of cellular cholesterol.

Thus, it seems that lipid-protein interactions mediated by amphiphilic helices are mainly responsible for generation of HDL upon interaction with the cell surface. However, not all the parameters for lipid-protein interaction of apolipoproteins directly correlate with this. ApoA-I, A-II, A-IV and E spontaneously generate disc-like particles with certain phospholipids such as dimyristoyl phosphatidylcholine (27,30–32), but CMapoA-II does not form typical "discs" even with this phospholipid (33). However, apoC-III does form the disc-like particles with dimyristoyl phosphatidylcholine although it has a peptide chain of about the same length as dose CMapoA-II, and both are incapable of causing cellular lipid efflux (34). The apparent dissociation constants of apoA-I, A-II, C-II and C-III are all in the same order when they are measured for the surface of phospholipid/triglyceride microemulsion having a diameter of 26 nm (10), while the dissociation constant of apoE is significantly higher (12). Therefore, we cannot completely rule out a more specific interaction between the membrane and certain apolipoproteins as a contributing mechanism for the generation of HDL.

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