Human Apolipoprotein A-IV Binds to Apolipoprotein A-I/A-II Receptor Sites and Promotes Cholesterol Efflux from Adipose Cells*

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Cholesterol efflux was studied in cultured mouse adipose cells after preloading with low density lipoprotein cholesterol. Exposure to complexes containing human apolipoprotein A-IV and l-α-dimyristoylphosphatidylcholine (DMPC) as well as to human lipoprotein particles containing apolipoprotein A-IV but not apolipoprotein A-I and particles containing apolipoproteins A-IV and A-I showed that both artificial and native apolipoprotein A-IV-containing particles were able to promote cholesterol efflux at 37 °C as a function of time and concentration. The half-maximal concentration was found to be 0.3 × 10⁻⁶ M for apolipoprotein A-IV DMPC complexes. Binding experiments performed in intact cells at 4 °C with labeled apolipoprotein A-IV DMPC complexes showed the existence of specific binding sites, with a Kd value of 0.32 × 10⁻⁹ M and a maximal binding capacity of 223,000 sites/cell. By cross-competition experiments with labeled and unlabeled complexes containing apolipoprotein A-IV, A-I, or A-II, it appeared that all three apolipoproteins bind to the same cell-surface recognition sites. It is suggested that apolipoprotein A-IV, which is present in the interstitial fluid surrounding adipose cells in vivo at concentrations similar to those required in vitro for the promotion of cholesterol efflux, plays a critical role in cholesterol removal from peripheral cells.

When compared to other apolipoproteins, apolipoprotein A-IV (apoA-IV), first described in rat (1), is of particular interest as it appears mainly in a lipoprotein-free form after centrifugation or gel filtration of plasma. In human, the 46-kDa protein was shown to be a component of chylomicrons, very low density lipoproteins, and high density lipoproteins but to occur to a major extent in the lipoprotein-free fraction (2–7). Despite the fact that apoA-IV is mainly synthesized in the intestine (8) as a component of newly secreted chylomicrons (9), no specific function has been so far assigned to apoA-IV in human. However, it has been shown that human apoA-IV behaves as a potent cofactor of lecinthincholesterol acyltransferase (EC 2.3.1.43) (10, 11). Moreover, rat apoA-IV has been shown to promote cholesterol efflux from human skin fibroblasts, suggesting a recognition process (12). In agreement with this assumption, specific binding of rat apoA-IV to rat liver membranes was inferred from results that showed rat apoA-IV as being one of the ligands responsible for binding of rat HDL to rat hepatocytes in culture (13, 14). Specific binding of human apoA-IV to cultured endothelial cells from bovine aorta has been recently shown (15), and a common binding site for apoA-IV and apoA-I has been proposed (16).

Cultured Ob1771 adipose cells have previously been shown to accumulate cholesterol upon exposure to low density lipoproteins (LDL). The addition of apoA-I liposomes, but not apoA-II liposomes, promoted cholesterol efflux (17) despite the fact that both proteoliposomes were able to bind to the same cell-surface sites of intact cells (18). Since LpA-I particles, but not LpA-II particles (isolated by immunoaffinity chromatography from HDL), were able to promote cholesterol efflux, it was proposed that apoA-I plays the role of an agonist and apoA-II that of an antagonist (17, 18). In this study, we show that apoA-IV DMPC complexes promote cholesterol efflux from Ob1771 cells preloaded with cholesterol by LDL. ApoA-IV-containing particles (isolated by immunoaffinity chromatography from native plasma (LpA-IV) and separated into particles containing apoA-I (LpA-I) or not containing apoA-I (LpA-IV-A-I) are all effective in promoting cholesterol efflux. ApoA-IV appears to bind to the same sites present in intact cells which recognize apoA-I and apoA-II.

EXPERIMENTAL PROCEDURES

Materials—Culture media and bovine serum were products of Flow Laboratories, Inc. Na⁺/K⁺ was purchased from Amersham Corp. Recombinant human growth hormone (Genotropin, batch 60663) was a gift from AB Kabi. L-α-Dimyristoylphosphatidylcholine and all other chemicals were obtained from Sigma.

Cells—Characterization of the Ob1771 preadipocyte clonal line, a subclone of mouse Ob17 cells, has been previously reported (19). Cells were plated at 2 × 10⁵ cells/cm² in 35-mm dishes (for assays of cholesterol efflux) or in 24-multiwell plates (for assays of binding) and grown in Dulbecco's modified Eagle's medium supplemented with 6% bovine serum, 200 units/ml penicillin, 50 μg/ml streptomycin, 33 μM biotin, and 17 μM pantothenate (standard medium). At confluen,

[The text continues with more details about the experimental procedures and data analysis.]
exposed for 48 h to the same medium containing 100 μg/ml LDL. This led to the accumulation of unsaturated cholesterol only (17). After careful washing with lipoprotein-deficient standard medium, the cells were exposed at 37 °C to the same medium supplemented with various artificial or native lipoprotein particles as indicated. Cellular cholesterol content of the cells was measured by HPLC on duplicate dishes as described before (17) at various times as indicated. The results are expressed as micromoles of cellular cholesterol/milligram of cell protein.

**Lipoproteins, Apolipoproteins, and Proteoliposomes—**High density lipoproteins (HDLs) and LDL were isolated from plasma of normo- lipemic human blood donors by sequential flotation ultracentrifugation: LDL between densities of 1.019 and 1.063 g/ml and HDLs between 1.15 and 1.21 g/ml by adding solid KBr. Lipoprotein-defic- ient serum was obtained as previously described (17). ApoA-I and apoA-II were isolated from HDLs as outlined before (20). ApoA-IV was isolated from human lipoprotein-deficient serum as described (21).

Complexes containing apolipoproteins and DMPC were prepared by the cholate dialysis procedure as described for apo-I liposomes by Chen and Albers (22) at a phosphatidylcholine:protein molar ratio of 150:1. The Stokes radius of the complexes was estimated to be 10 ± 10 Å. Labeling of apolipoproteins with 125I was performed by the method of Hunter and Greenwood (23); specific radioactivities ranged from 200 to 400 cpm/ng protein.

The isolation of lipoprotein particles containing apoA-IV (LpAIV) was performed at 4 °C by immunooaffinity chromatography. First, a fraction of 0.65 M ammonium sulfate (precipitate) from rabbit immunosorbers directed against human apoA-IV was passed over a cyanogen bromide-activated Sepharose 4B column to which human apoA-IV had been previously coupled. Second, after elution of anti- apoA-IV antibodies with a solution of 0.2 M glycine buffered with HCl at pH 3 followed by concentration using an Amicon PM-10 membrane, immunosorbers were prepared by coupling the specific antibodies (30 mg) to cyanogen bromide-activated Sepharose 4B (5 mg/g of gel) (24). Antibody coupling, immunosorbers were equilibrated with phosphate-buffered saline (pH 7.4) containing 0.01% EDTA (buffer A). For isolation of LpAIV particles, 5 ml of human native plasma were run over the immunosorbent column specific for apoA-IV; the sample was applied at a slow rate. After binding of all apoA-IV-containing particles, the gel was washed extensively with buffer A, and nonspecifically bound material was eluted with 0.5 M NaCl. The retained fractions were collected as LpAIV particles, and an aliquot was saved for the subsequent isolation by immunooaffinity chromatography on an anti-apo-I column (see below). To dissociate and collect the retained fraction, 20 ml of 3 M NaSCN were applied to the anti-apo-I column. To minimize contact between apoA-IV-containing particles and the dissociating agent, immunosorbers were constructed with a layer of Sephadex G-25 below the immunosorbers portion. This bottom layer allowed immediate separation of the particles from the dissociating agent. For isolation of lipoprotein particles containing apo- A-I (LpArv:Ai) or not containing apoA-I (LpArv:Aa), LpAIV particles were run over an immunooaffinity column specific for human apoA-I as described above. The unretracted fraction (LpAIV:Aa) was collected, whereas the retained fraction (LpAIV:Aa) was eluted as described above. After collection, both fractions were extensively dialyzed against buffer A and concentrated under reduced pressure. The various apolipoproteins were then measured in both fractions using specific enzyme immuno- noassays (25); full characterization of these various lipoprotein particles will be published elsewhere.

**Binding Assays—**Binding of labeled apolipoprotein-DMPC complexes to intact Ob1771 cells was performed for 2 h at 4 °C as described previously (17). Nonspecific binding was determined by measuring the amount of radioactivity when incubations were carried out in the presence of 100-fold excess of the corresponding unlabeled lipoprotein. Specific binding varied from 30 to 40% for apoA-I, from 20 to 30% for apoA-II, and from 15 to 20% for apoA-IV (see Fig. 3). The ratios of bound to free 125I-apolipoprotein-DMPC complexes were plotted against bound complexes according to the method of Scatchard (26).

**RESULTS**

Following cholesterol preloading of adipose cells by LDL, DMPC complexes containing apoA-IV were then assayed for their ability to promote cholesterol efflux as a function of time at 37 °C. Complexes containing apoA-I or apoA-II were used in parallel. As shown in Fig. 1, both apoA-IV-DMPC and apoA-I-DMPC complexes were able to promote cholesterol efflux from adipose cells, whereas as already reported (17, 27), no efflux was observed in the presence of apoA-II-DMPC complexes.

A rapid decrease in cellular cholesterol content occurred (~0.5 μg/min/mg of cell protein) and reached a minimal value within 2 h. This minimal value was similar to that observed in control cells before exposure to LDL cholesterol. This suggests the existence in adipose cells of a nonmobilizable pool of cholesterol, whereas apoA-I-DMPC or apoA-IV-DMPC complexes were able to modulate the efflux of a mobilizable pool of cholesterol.

To determine that, during the incubation time for cholesterol efflux, no significant degradation was taking place, efflux was studied with apoA-1-DMPC and apoA-IV-DMPC complexes which were subjected to trichloroacetic acid precipitation after incubation. More than 96% of the radioactivity remained precipitable in 10% trichloroacetic acid.

In a second set of experiments, increasing the concentration of apoA-IV-DMPC complexes over a fixed time period of 5 h led to cholesterol efflux in a dose-dependent manner: the concentrations of apoA-IV-DMPC complexes required to bring a half-maximal and a maximal effect were 14 and 50 μg/ml, respectively (Fig. 2).

The isolation from native plasma of lipoprotein particles containing apoA-IV (LpAIV) was made possible by immuno- affinity chromatography. Sequential use of specific antibodies directed against apoA-IV and apoA-I allowed the separation of lipoprotein particles also containing apoA-I (LpAIV:Aa) or deprived of apoA-I (LpAIV−Aa). The results of Fig. 2 indicate that LpAIV, LpAIV:Aa, and LpAIV−Aa were all active in promoting cholesterol efflux, with concentrations required to bring a half-maximal effect of 20, 7, and 7 μg/ml, respectively; the concentration required to bring a maximal effect was 50 μg/ml for the various lipoprotein particles.

To see whether cholesterol removed from the cells was recovered in apolipoprotein A-IV-DMPC complexes, medium was analyzed after cholesterol efflux. Medium was either passed over an anti-apoA-IV immunooaffinity column or ultra- centrifuged at a density of 1.21 g/ml. After immunooaffinity chromatography, more than 95% of the cholesterol was re- covered with the bound fraction, and more than 98% of the

**FIG. 1.** Kinetics of cholesterol efflux from cholesterol-preloaded Ob1771 cells in presence of various apolipoprotein- DMPC complexes. Cholesterol preloading of Ob1771 cells was performed at 37 °C as described under "Experimental Procedures." After careful washing, cells were exposed to 100 μg/ml apoA-1-DMPC (●), apoA II DMPC (▲), or apoA-IV DMPC (○) complexes. The determi- nation of cholesterol content was performed on duplicate dishes by HPLC (17) at the indicated times. The mean values from duplicate dishes were reported (±SD; n = 2) and are representative of two independent experiments performed on two independent series of cells. The cholesterol content (mean ± range) of Ob1771 cells continuously exposed to lipoprotein-deficient serum was 12 ± 2 μg/ mg of cell protein.
FIG. 2. Dose-response curves of cholesterol efflux from cholesterol-preloaded Ob1771 cells in presence of artificial and native apoA-IV-containing particles. Cholesterol preloading of Ob1771 cells was performed as described for Fig. 1 using a different series of cells. After 5 h of exposure to increasing concentrations of apoA-IV:DMPC complexes (A), LpAIV (A), LpAIV-AI (O), and LpAIV:A, (O), duplicate dishes were carefully washed and assayed separately for cholesterol content. The mean values from duplicate dishes are reported (<±15% from the mean). Note that the same batch of plasma was used to prepare the various native apoA-IV-containing particles. The cholesterol content (mean ± range) of Ob1771 cells continuously exposed to lipoprotein-deficient serum was 11 ± 2 ng/ml of cell protein.

cholesterol was found in the d < 1.21 g/ml fraction after ultracentrifugation (data not shown). Thus, cholesterol removed from the cells appeared to be tightly associated with the lipoprotein particles present in the incubation medium.

The results of Figs. 1 and 2 support an interaction between apoA-IV and intact Ob1771 cells. Therefore, binding experiments were performed with labeled apoA-IV:DMPC complexes. As shown in Fig. 3, a saturation curve was obtained after incubating the cells for 2 h at 4 °C. The percentage of nonspecific binding averaged 20 ± 5% (mean ± range of two experiments); a Scatchard plot (Fig. 3, inset) allowed the determination of a Kd value of 0.32 × 10^{-6} M and a Bmax value of 171 ng of apoA-IV bound per mg of cell protein (223,000 sites/cell; see Table I).

To explore the possibility that apoA-IV binds to the same sites which recognize apoA-I and apoA-II (17), competition experiments were performed in which binding of either of the three labeled apolipoproteins was performed at 4 °C in the presence of the two other unlabeled apolipoproteins. The results of Fig. 4 show clearly that apoA-I (or apoA-II) -DMPC complexes competed with binding of 125I-labeled apoA-IV-DMPC complexes to intact cells (Fig. 4A). In a similar way, apoA-I (or apoA-IV) -DMPC complexes competed with binding of 125I-labeled apoA-II-DMPC complexes (Fig. 4B), whereas apoA-IV-DMPC complexes (but not apoE-DMPC complexes) competed for binding of 125I-labeled apoA-1-DMPC complexes (Fig. 4C). To address the magnitude of apolipoprotein transfer between labeled and unlabeled particles during competition experiments at 4 °C, cells were exposed for 2 h at 4 °C to incubation medium containing labeled apoA-II-DMPC complexes in the absence or presence of increasing amounts of unlabeled apoA-IV-DMPC complexes.

![Table I](https://example.com/table.png)

**Table I** Parameters of binding and cholesterol efflux of various apolipoprotein complexes

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Binding</th>
<th>Cholesterol efflux</th>
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<tbody>
<tr>
<td></td>
<td>Kd (μM)</td>
<td>Bmax (sites/cell)</td>
</tr>
<tr>
<td>apoA-IV</td>
<td>0.32</td>
<td>223,000</td>
</tr>
<tr>
<td>apoA-II</td>
<td>0.2</td>
<td>100,000</td>
</tr>
<tr>
<td>apoA-I</td>
<td>1</td>
<td>193,000</td>
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* From Ref. 27.

**Fig. 4.** Competitive inhibition of binding of 125I-labeled apolipoprotein-DMPC complexes to Ob1771 cells. Ob1771 cells were incubated for 2 h at 4 °C in the presence of 0.6 μM labeled apoA-IV-DMPC complexes and increasing concentrations of unlabeled apoA-IV-DMPC (■), apoA-II-DMPC (■), or apoA-I-DMPC (■) complexes as indicated (A); 0.1 μM labeled apoA-II-DMPC complexes and increasing concentrations of apoA-IV-DMPC (■), apoA-II-DMPC (■), or apoA-I-DMPC (■) complexes (B); and 1 μM labeled apoA-I-DMPC complexes and varying concentrations of apoA-IV-DMPC (■), apoA-I-DMPC (■), or apoE-DMPC (C) complexes (C). 100% binding corresponds to 105, 20, and 80 ng/mg of cell protein for labeled apoA-I, apoA-II, and apoA-IV, respectively. The results are representative of two independent experiments performed on two different series of cells. The mean values of duplicate dishes are reported (<±10% from the mean).
Incubation medium was passed over an anti-apoA-IV immunoaffinity column. There was actually no increase of labeled apoA-II in the fraction retained on the column when apoA-IV was present over that retained nonspecifically when apoA-IV was absent (data not shown). This indicates that no labeled apoA-II was actually transferred to apoA-IV-DMPC complexes. Moreover, experiments (Fig. 4C) showing the absence of competition of apoE-DMPC complexes with respect to binding of labeled apoA-I-DMPC complexes, also support the absence at 4 °C for 2 h of a significant transfer of apolipoproteins between various lipoprotein particles. The concentrations of unlabeled apolipoproteins which were required to prevent 50% of binding of the other labeled apolipoproteins were found to be 0.2 × 10^{-8} M for apoA-IV, 0.3-0.6 × 10^{-8} M for apoA-II, and 0.6 × 10^{-8} M for apoA-I. These values were within the range of those calculated from the binding isotherms of labeled apolipoproteins, which were found to be 0.32 × 10^{-8} M for apoA-IV (Fig. 3), 0.2 × 10^{-6} M for apoA-II, and 10^{-6} M for apoA-I (17). The data of Table I summarize the binding parameters of the three apolipoprotein-DMPC complexes to intact adipose cells at 4 °C and their ability to promote cholesterol efflux at 37 °C. The maximal number of binding sites/cell was 223,000 for apoA-IV, 100,000 for apoA-II, and 193,000 for apoA-I.

DISCUSSION

This study demonstrates the ability of human apoA-IV to promote cellular efflux from cholesterol-preloaded Ob1771 cells. This cellular model appears rather unique as adipose cells, in vitro as well as in vivo, accumulate cholesterol mainly in an unesterified form. Providing that cholesterol accumulation takes place in the presence of LDL, no cholesterol in an unesterified form is present (31). However, in the absence of LDL, cholesterol may be transferred from rat HDL, when complexed with dioleoylphosphatidylcholine or its nonhydrolyzable ether analog, dioleylphosphatidylcholine, was more efficient than apoA-I in promoting cholesterol efflux from cholesterol-preloaded human skin fibroblasts (12); apoE complexes were also active. This is in agreement with the parameters of cholesterol efflux promoted from cholesterol-preloaded cells by the same particles. The result, coupled with that showing a close relationship between the presence of apoAI/apoA II receptor sites and the promotion of cholesterol efflux (36), is consistent with a requirement for HDL receptor sites in this process.

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