Formation of apolipoprotein-specific high-density lipoprotein particles from lipid-free apolipoproteins A-I and A-II

Moira A. CLAY*†[1], Daniel A. CEHIC†‡, Diana H. PYLE*†, Kerry-Anne RYE†‡ and Philip J. BARTER*†‡

*The University of Adelaide, Department of Medicine, Royal Adelaide Hospital, Adelaide, South Australia 5000, Australia, †Lipid Research Laboratory, Hanson Centre for Cancer Research, Institute of Medical and Veterinary Science, Adelaide, South Australia 5000, Australia, and ‡The Cardiovascular Investigational Unit, Royal Adelaide Hospital, Adelaide, South Australia 5000, Australia

We have shown previously that apolipoprotein A (apoA)-I-containing high-density lipoprotein (HDL) particles are formed by the conjugation of lipid-free apoA-I with lipids derived from other lipoprotein fractions in a process dependent on non-esterified fatty acids, generated by the lipolysis of very-low-density lipoprotein (VLDL) or provided exogenously. In the present study, we show that this process is also able to generate HDL particles containing apoA-II (A-II HDL) and both apoA-I and apoA-II (A-I/A-II HDL). When lipid-free apoA-II was incubated with either VLDLs and lipoprotein lipase or LDLs and sodium oleate, a significant proportion of the apoA-II was recovered in the HDL density fraction. This was associated with the formation of several populations of HDL-sized particles with pre-β2 electrophoretic mobility, which contained phospholipids and unesterified cholesterol as their main lipid constituents. When both lipid-free apoA-I and lipid-free apoA-II were incubated with LDL and sodium oleate, both apolipoproteins were recovered in HDLs that contained phospholipids and unesterified cholesterol as their main lipids. Two populations of particles had diameters of 7.4 and 10.8 nm and pre-β2-migration; there was also a population of pre-β1-migrating particles of diameter 4.7 nm. ApoA-I and apoA-II were both present in the larger HDLs, whereas only apoA-I was present in the smaller particles. Immunoaffinity chromatography on an anti-(apoA-I)–Sepharose column revealed that 10–20 % of the apoA-II resided in particles that also contained apoA-I. The majority of the A-I/A-II HDL were present in a population of pre-β2 particles of 10.8 nm diameter. These results *in vitro* illustrate a potential mechanism for the formation of HDLs containing both apoA-I and apoA-II.

Key words: lipid-free apoliprotein A-I, lipid-free apoliprotein A-II, non-esterified fatty acids.

INTRODUCTION

Human population studies have clearly shown that the concentration of high-density lipoprotein (HDL) cholesterol is a powerful inverse predictor of premature coronary heart disease (CHD) [1,2]. The HDLs in human plasma are heterogeneous, consisting of several discrete subpopulations of particles that differ in size, density, shape (spherical or discoidal), electrophoretic mobility (pre-β and α) and composition. This heterogeneity has important implications in terms of the functions of HDLs [3,4]. Indeed, the apolipoprotein composition of HDLs appears to be one of the major determinants of their ability to protect against the development of atherosclerosis [5–9].

Evidence suggests that HDLs containing apolipoprotein A (apoA)-I are superior to those containing apoA-II as protectors against CHD [5,8,9]. This highlights the need to understand the origins and metabolism of different apolipoprotein-specific subpopulations of HDLs. There are three distinct subpopulations of these in human plasma [10,11]: one consisting of HDL particles containing both apoA-I and apoA-II (A-I/A-II HDL), one featuring HDL particles containing apoA-I without apoA-II (A-I HDL) and a minor subpopulation of HDL particles containing apoA-II without apoA-I (A-II HDL). A-I HDLs have been reported to originate as preformed particles secreted from the liver or intestine [12,13]. They might also be formed extracellularly either in plasma, where they are generated as redundant surface components shed from chylomicrons following hydrolysis of their triacylglycerol by lipoprotein lipase (LPL) [14,15], or in the interstitial space, where they are assembled from their individual constituents when lipid-free apoA-I recruits phospholipids and unesterified cholesterol from cell membranes [16–18]. Finally, our recent studies have shown that A-I HDL might be formed by the conjugation of lipid-free apoA-I with lipids derived from other lipoprotein fractions in a process dependent on the presence of non-esterified fatty acids (NEFA) [19]. The origins of A-II HDL and A-I/A-II HDL are unknown. In the present study, we investigate whether A-II HDL and A-I/A-II HDL are also formed by this last, NEFA-dependent process.

EXPERIMENTAL

Isolation of lipoproteins, apolipoproteins and LPL

Plasma was obtained from normal volunteers (plasma triacylglycerol 1–3 mM), who had fasted for 12 h. Very-low-density lipoproteins (VLDLs) were isolated in the supernatant, after ultracentrifugation at a density of 1.006 g/ml at 100000 rev./min (approx. 291000 ***g***) for 16 h at 10 °C in a Beckman 100.4 rotor using a Beckman TL-100 Tabletop ultracentrifuge. HDLs ($1.063 < d < 1.21$ g/ml) and LDLs ($1.019 < d < 1.055$ g/ml) were isolated from human plasma (Transfusion Service, Royal Adelaide Hospital, Australia) by sequential ultracentrifugation in a Beckman L8-70 M ultracentrifuge as described previously [20]. Human apoA-I and apoA-II were purified to homogeneity

Abbreviations used: apoA, apolipoprotein A; CHD, coronary heart disease; HDL, high-density lipoprotein; A-I (or A-II or A-I/A-II) HDL, HDL particles containing apoA-I (or apoA-II or both apoA-I and apo-AII) respectively; LPL, lipoprotein lipase; NEFA, non-esterified fatty acids; TBS, Tris-buffered saline; (V)LDL, (very)-low-density lipoprotein.

[1] To whom correspondence should be addressed (e-mail maclay@camtech.net.au).

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following delipidation of HDLs [21] and chromatography on a column of Q-Sepharose Fast Flow (Pharmacia Biotechnology AB, Uppsala, Sweden) as described previously [22]. Lipoproteins and lipid-free apolipoproteins were dialysed against Tris-buffered saline [TBS; 0.01 M Tris buffer, pH 7.4, containing 0.15 M NaCl, 0.01 % (w/v) Na_2-EDTA and 0.02 % (w/v) NaN_3] before use in incubations. LPL was purified from bovine milk as described previously [23] and dialysed against TBS before use in incubations.

Experimental conditions and processing of samples

In the first series of experiments, mixtures of lipid-free apoA-II, VLDL, LPL and TBS were incubated for up to 3 h at 37 °C in stoppered tubes in a shaking water bath. The incubations also contained BSA, essentially fatty-acid-free (Sigma, St. Louis, MO, U.S.A.) to a final concentration of 3 % (w/v) and 100 units heparin/ml (Sigma) of incubation mixture to stabilize LPL activity.

In the second series of experiments, lipid-free apoA-II (in the absence or presence of lipid-free apoA-I) was supplemented with sodium oleate (99 % pure; Sigma), LDL and TBS and incubated for up to 2 h at 37 °C, in stoppered tubes in a shaking water bath. After the initial incubation period, the mixtures were supplemented with BSA (3 %, w/v) and the incubation was continued for an additional hour. A fresh suspension of sodium oleate (2 mg/ml in TBS) was prepared for each experiment by vortex-mixing. Details of the individual incubations are described in the legends to the Figures and Tables.

When the incubations were complete, any apolipoproteins that had been incorporated into new HDL particles were isolated as the HDL density fraction of 1.1–1.25 g/ml by sequential ultracentrifugation in a Beckman TL-100 Tabletop ultracentrifuge, with two spins at the lower density and a single spin at the higher density. Aliquots of the HDL density fraction were subjected to immunoaffinity chromatography, agarose gel electrophoresis and non-denaturing PAGE (gradient gels), and assayed for lipids and apolipoproteins, as described below.

Electrophoretic analysis

Gradient gel electrophoresis

Aliquots of lipid-associated apolipoproteins were electrophoresed for 3000 V·h on 3–40 % non-denaturing polyacrylamide gradient gels (Gradipore, Sydney, Australia), stained with Coomassie Blue G-250 and destained with acetic acid [24]. The Stokes diameter of the particles was calculated with reference to standards in a high-molecular-mass electrophoresis calibration kit (Pharmacia Biotechnology AB), which were supplemented with 1 mg/ml ovalbumin (4.66 nm Stokes diameter).

Agarose gel electrophoresis

Aliquots of lipid-associated apolipoproteins were subjected to agarose gel electrophoresis, as described previously [19]. Each gel included one track of purified lipid-free apoA-II or apoA-I as a marker of pre-β-migrating particles and one track of HDLs (density fraction 1.063–1.21 g/ml) isolated from human plasma as a marker of α-migrating particles.

Two-dimensional gel electrophoresis

In selected experiments, particles were separated on the basis of charge by agarose gel electrophoresis (first dimension), and then on the basis of size by non-denaturing polyacrylamide gradient gel electrophoresis (second dimension) [19]. Duplicate samples of the new HDL particles were subjected to agarose gel electrophoresis, as described above. The duplicate lanes were excised from the agarose gels and annealed on to the same gradient gel, with a lane of high-molecular-mass standards separating the two agarose strips; the origin of the agarose strips was always aligned with the outer edge of the gradient gel. The gradient gel was electrophoresed, as described above, and transferred to nitrocellulose membranes [19]. To compare the distribution of apoA-I with that of apoA-II in the new HDL particles, half of the membrane was immunoblotted for apoA-I and half for apoA-II, as described previously [19].

Chemical analyses

All assays were performed on a Cobas-Fara centrifugal analyser (Roche Diagnostics, Zurich, Switzerland). Concentrations of total cholesterol, unesterified cholesterol and phospholipids were measured using enzymic kits (Boehringer Mannheim, Mannheim, Germany). The concentration of cholesteryl esters was calculated as the difference between the concentrations of total and unesterified cholesterol. Concentrations of apoA-I and apoA-II were measured immunoturbidometrically as described previously [19], using antisera raised in sheep against human apoA-I (see below) or apoA-II (Boehringer Mannheim). The assay was standardized using appropriate dilutions of either lipid-free apoA-I or apoA-II, purified from human plasma as described above.

Immunoaffinity chromatography

Raising of antiserum against apoA-I

Antiserum against purified human apoA-I was raised in sheep by the subcutaneous injection of mixtures of 1.5 ml of antigen (500 mg of protein) and 3 ml of Freund's Complete Adjuvant. The sheep received booster injections of mixtures of 1.5 ml of antigen (1 mg of protein) and 3 ml of Freund's Incomplete Adjuvant at 4-week intervals. The antiserum was purified by affinity chromatography on CNBr-activated Sepharose 4B (Pharmacia), coupled with either human albumin (Sigma) or purified human apoA-II. The antibodies were precipitated with ammonium sulphate at 40 % saturation and dialysed extensively against TBS before use. ApoA-I antibody preparations were monospecific for human apoA-I, as judged by immunoblots against purified human apoA-I, apoA-II and albumin.

Preparation of anti-(apoA-I) immunoaffinity columns

Antiserum against human apoA-I was covalently coupled with CNBr-activated Sepharose 4B at a ratio of 1:1 (v/v) by following the manufacturer's instructions (Pharmacia). Before use, the high-affinity binding sites on the anti-(apoA-I)–Sepharose column were blocked with purified human apoA-I (500 μg/ml of gel) for 1 h at room temperature. After blocking, any unbound apolipoproteins were eluted with TBS, and apolipoproteins that had bound with low affinity were eluted with 0.1 M acetic acid, pH 2.7. The loading capacity of the anti-(apoA-I)–Sepharose column was 200–300 μg of apoA-I/ml.

Isolation of apolipoprotein-specific HDLs by immunoaffinity chromatography

Aliquots of the HDL density fraction, which had been isolated from incubations containing lipid-free apoA-I, lipid-free apoA-II, LDL and sodium oleate, were rotated with anti-(apoA-I)–Sepharose for 1 h at room temperature in sealed Polyprep chromatography columns (0.8 cm × 4 cm; Bio-Rad, Hercules,

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CA, U.S.A.). HDL particles that did not bind to the columns were washed off with TBS (8 column vol.). HDL particles that had bound to the columns were eluted with 6 column vol. of 0.1 M acetic acid, pH 2.7, and were neutralized immediately with 1 M Tris (pH 11.0). Bound HDL particles were generally eluted in 1.5–2 column vol. of acetic acid.

RESULTS

Formation of new HDL particles during incubations of lipid-free apoA-II, VLDL and LPL

Purified, lipid-free apoA-II (final concentration 0.4 mg/ml) was supplemented with VLDL (final concentration 2 mM triacylglycerol) and incubated at 37 °C for 3 h in the absence or presence of bovine milk LPL. After the incubation, the VLDLs were removed by ultracentrifugation and the incorporation of lipid-free apoA-II into new lipid-associated particles was assessed by the appearance of apoA-II in the HDL density fraction of 1.07–1.25 g/ml. In control incubations, which did not include LPL, apoA-II was not recovered in the 1.07–1.25 g/ml density fraction. When LPL was present in the incubations, 16% of the apoA-II was recovered in the HDL fraction. When the HDL fraction was subjected to non-denaturing polyacrylamide gradient gel electrophoresis, several subpopulations of HDL-sized particles were apparent. However, after incubation in the presence of LPL there were multiple peaks present with Stokes diameters of 6.3, 6.5, 6.7, 7.4 and 8.0 nm (Figure 1). In the absence of LPL, there were no bands present on the gel (results not shown).

Formation of HDL particles during incubations of lipid-free apoA-II with sodium oleate and LDL

Purified, lipid-free apoA-II (0.6 mg/ml incubation mixture) was supplemented with LDL (313 μg of apoB/ml incubation mixture) and incubated at 37 °C for 2 h in the absence or presence of sodium oleate (final concentration 1.2 mM). During the last hour of the incubation, fatty-acid-free BSA (3%, w/v) was added to bind to the sodium oleate. After the incubation, the lipid-free apoA-II was separated from lipid-associated apoA-II in the ultracentrifuge. A significant proportion of the apoA-II (10–15%) was recovered in the HDL density fraction of 1.1–1.25 g/ml, following the incubation of lipid-free apoA-II with LDL and sodium oleate.

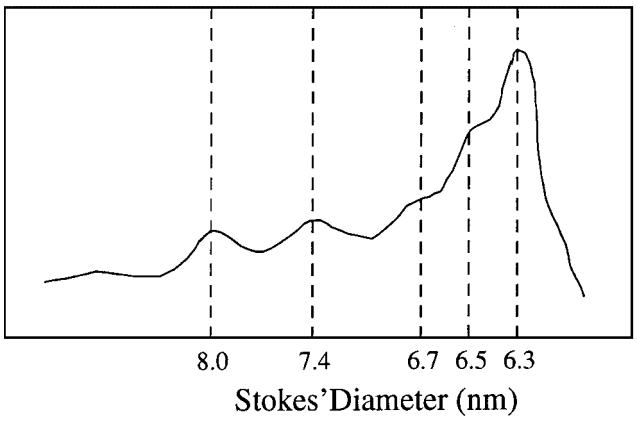

Figure 1 Size distribution of new HDL particles formed during incubation of lipid-free apoA-II with VLDL and LPL

Lipid-free apoA-II (0.4 mg/ml incubation mixture) was incubated at 37 °C for 3 h in the presence of VLDL (2 mM VLDL triacylglycerol), LPL, heparin (100 units/ml incubation mixture) and fatty-acid-free BSA (3%, w/v). After incubation, lipid-associated apoA-II was isolated by ultracentrifugation as the HDL density fraction of 1.07–1.25 g/ml and electrophoresed on a 3–40% non-denaturing gradient gel, as described in the Experimental section. The gel was stained with Coomassie Blue G-250, and the distribution profile of the HDLs was obtained by laser densitometric scanning of the gel.

The appearance of apoA-II in the HDL density range was dependent on the concentration of LDL, sodium oleate and lipid-free apoA-II in the incubation mixture (Figure 2). When lipid-free apoA-II (0.6 mg/ml incubation mixture) was incubated with either increasing amounts of LDL and a constant concentration of sodium oleate (1.2 mM) (Figure 2A) or a constant amount of LDL (313 μg of apoB/ml incubation mixture) and concentrations of sodium oleate in excess of 0.4 mM (Figure 2B), there was a progressive increase in the apoA-II recovered in the HDL fraction. If neither the LDL nor sodium oleate was included in the incubations, less than 3% of the apoA-II was recovered in the HDL density fraction. When mixtures containing a fixed concentration of both LDL (313 μg of apoB/ml incubation mixture) and sodium oleate (1.2 mM) were incubated with increasing amounts of lipid-free apoA-II, there was also a progressive increase in the amount of apoA-II recovered in the HDL density range (Figure 2C). The incorporation of lipid-free apoA-II into lipid-associated particles was also dependent on the length of the incubation before adding BSA. Between 0–2 h of incubation, there was a progressive increase in the recovery of apoA-II in the HDL density range (Figure 2D). However, after 2 h of incubation, there was no further incorporation of apoA-II into the HDL density range.

Consistent with the formation of apoA-II–lipid complexes during the incubations described above was the appearance of discrete subpopulations of HDL-sized particles, as judged by non-denaturing polyacrylamide gradient gel electrophoresis (Figure 3). When lipid-free apoA-II was incubated in the absence of either sodium oleate or LDL, there were no HDL-sized particles evident (results not shown). When all the constituents were present, there were two main populations of particles present with Stokes diameters of 7.8 and 7.4 nm and three minor subpopulations with diameters of 11.4, 10.2 and 8.4 nm. In order to characterize these particles further, their electrophoretic mobility was determined by agarose gel electrophoresis, relative to that of α-migrating human plasma HDL, which was given an R_F value of 1.0. Previous work has shown that lipid-free apoA-II migrates with a pre-β1 mobility [17,25,26]; in these studies, lipid-free apoA-II had an R_F value of 0.54 relative to human HDL. As found in our previous study [19], we have classified particles with R_F values of 0.5–0.75 as being pre-β1-migrating, particles with R_F values of 0.76–0.9 as being pre-β2-migrating and particles with R_F values of 0.91–1.05 as being α-migrating. The new apoA-II-containing HDL particles had an R_F value of 0.81, as determined by agarose gel electrophoresis, and were therefore pre-β2-migrating particles.

The composition of the newly formed A-II HDL is shown in Table 1; lipid constituents are expressed per mol of apoA-II. Phospholipids were the main lipid constituent, with smaller amounts of unesterified cholesterol and cholesteryl esters also present. There were neither detectable NEFA (results not shown) nor triacylglycerols in the A-II HDL.

Formation of HDL particles during incubations of lipid-free apoA-I and lipid-free apoA-II with LDL and sodium oleate

Most of the apoA-II in human plasma is accommodated in HDL particles containing both apoA-I and apoA-II (A-I/A-II HDL).

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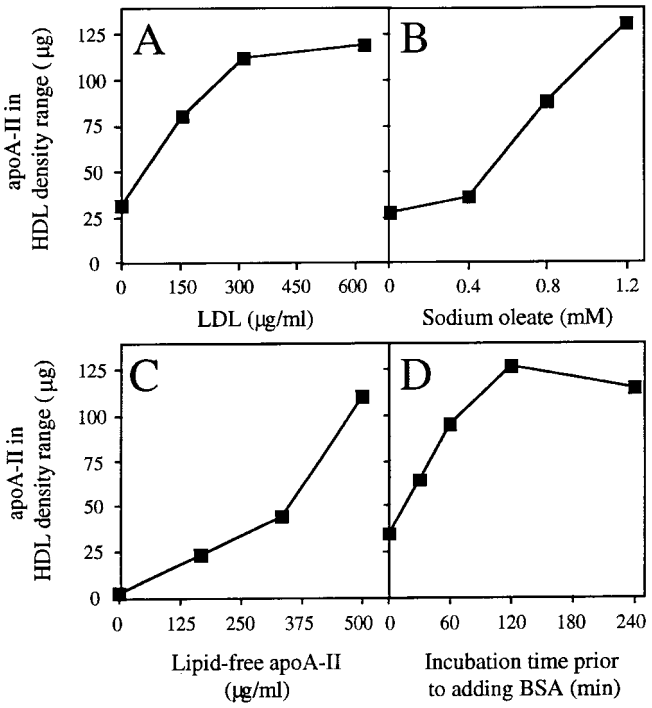

Figure 2 Concentration and time-dependence of recovery of apoA-II in the HDL density fraction after incubating lipid-free apoA-II with LDL and sodium oleate

Lipid-free apoA-II (0.6 mg/ml incubation mixture) was incubated at 37 °C for 3 h in the presence of increasing amounts of LDL and 1.2 mM sodium oleate (**A**) or increasing amounts of sodium oleate and 313 μg of LDL apoB/ml incubation mixture (**B**). In addition, mixtures of LDL (313 μg of apoB/ml incubation mixture) and 1.2 mM sodium oleate were incubated with increasing amounts of lipid-free apoA-II (**C**). During the last hour of the incubation, fatty-acid-free BSA (3%, w/v) was added to the mixture to bind the sodium oleate. In another series of experiments, lipid-free apoA-II (0.6 mg/ml incubation mixture) was incubated with 1.2 mM sodium oleate and LDL (313 apoB of μg/ml incubation mixture) for increasing amounts of time before adding BSA for an additional hour (**D**). After the incubation, lipid-associated apoA-II was isolated by ultracentrifugation as the HDL density fraction of 1.1–1.25 g/ml and the concentration of apoA-II determined as described in the Experimental section.

We hypothesized that the process in which A-I HDL [19] and A-II HDL (Figures 2 and 3) were formed from lipid-free apolipoproteins in incubations with sodium oleate and LDL might also result in the formation of A-I/A-II HDL. To investigate this, lipid-free apoA-I (1.2 mg/ml incubation mixture) was mixed with lipid-free apoA-II (0.12 mg/ml incubation mixture) and incubated at 37 °C for 3 h in the presence of LDL (313 μg of apoB/ml incubation mixture) and 1.2 mM sodium oleate. In six separate experiments, 2–3 % of the apoA-I (2–3 mg) and 32–50 % of the apoA-II (3–5 mg) were recovered in the HDL density range (1.1–1.25 g/ml) after the incubation. The amount of apoA-II that was associated with lipid increased progressively as the concentration of lipid-free apoA-II in the initial incubation mixture was increased (Figure 4, upper panel). Concomitantly with the increase in lipid-associated apoA-II was a decrease in lipid-associated apoA-I (Figure 4, lower panel). This decrease in apoA-I recovery is consistent with previous reports that show that lipid-free apoA-II displaces apoA-I from HDLs [27,28].

The new HDL particles formed during incubations of lipid-free apoA-I and lipid-free apoA-II with LDL and sodium oleate were subjected to two-dimensional agarose–polyacrylamide gradient gel electrophoresis to determine the distribution of apoA-I and apoA-II between newly formed HDL subpopulations

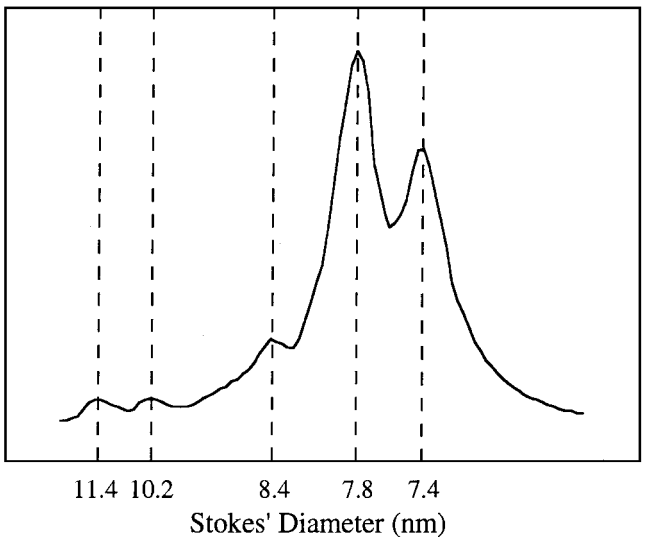

Figure 3 Size distribution of new HDL particles formed during incubation of lipid-free apoA-II with LDL and sodium oleate

Lipid-free apoA-II (0.6 mg/ml incubation mixture) was incubated at 37 °C for 3 h in the presence of 1.2 mM sodium oleate and LDL (313 μg of apoB/ml incubation mixture). During the last hour of the incubation, fatty-acid-free BSA (3% w/v) was added to the mixture to bind the sodium oleate. After the incubation, lipid-associated apoA-II was isolated by ultracentrifugation as the HDL density fraction of 1.1–1.25 g/ml and electrophoresed on a 3–40% non-denaturing polyacrylamide gradient gel, as described in the Experimental section. The gel was stained with Coomassie Blue G-250 and the distribution profiles of the HDLs were obtained by laser densitometric scanning of the gel.

Table 1 Composition of new HDL particles formed during incubation of lipid-free apoA-II with sodium oleate and LDL

Lipid-free apoA-II (0.6 mg/ml incubation mixture) was incubated at 37 °C for 3 h in the presence of 1.2 mM sodium oleate and LDL (313 μg of apoB/ml incubation mixture). During the last hour of the incubation, fatty-acid-free BSA (3%, w/v) was added to the mixture to bind the sodium oleate. After the incubation, lipid-associated apoA-II was isolated by ultracentrifugation as the HDL density fraction of 1.1–1.25 g/ml and its composition was determined as described in the Experimental section. PL, phospholipids; UC, unesterified cholesterol; CE, cholesteryl esters; TG, triacylglycerols. Each value represents the mean of triplicate determinations.

	Stoichiometry (mol/mol)			
Experiment	PL/ApoA-II	UC/ApoA-II	CE/ApoA-II	TG/ApoA-II
I	5.7	1.9	1.9	0
II	8.4	3.3	2.3	0
III	5.7	2.3	2.1	0

of different size and electrophoretic mobility. Figure 5(A) shows apoA-I and apoA-II immunoblot analysis of the two-dimensional gels. The apoA-I that was recovered in the HDL fraction was present as three main populations of particles. Two of the populations had a pre-β2 electrophoretic mobility and diameters of 10.8 and 7.4 nm, whereas the third had pre-β1 mobility and a diameter of 4.1 nm. ApoA-II coincided with apoA-I in the two larger populations of particles, whereas the smaller particles contained only apoA-I.

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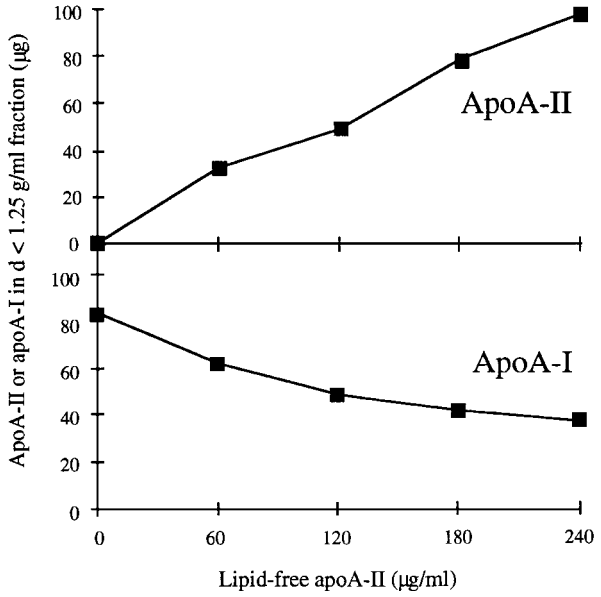

Figure 4 Effect of apoA-II concentration on the apoA-II and apoA-I recovered in the HDL density fraction after incubation of lipid-free apoA-II, lipid-free apoA-I, sodium oleate and LDL

Lipid-free apoA-I (1.2 mg/ml incubation mixture) was incubated with increasing concentrations of lipid-free apoA-II in the presence of 1.2 mM sodium oleate and LDL (313 μg of apoB/ml incubation mixture) for 3 h at 37 °C. During the last hour of the incubation, fatty-acid-free BSA (3%, w/v) was added to the mixture to bind the sodium oleate. After the incubation, lipid-associated apoA-II and apoA-I were separated from lipid-free apolipoproteins by ultra-centrifugation at a density of 1.25 g/ml. Concentrations of apoA-II (upper panel) and apoA-I (lower panel) in the $d < 1.25$ g/ml fraction were determined as described in the Experimental section.

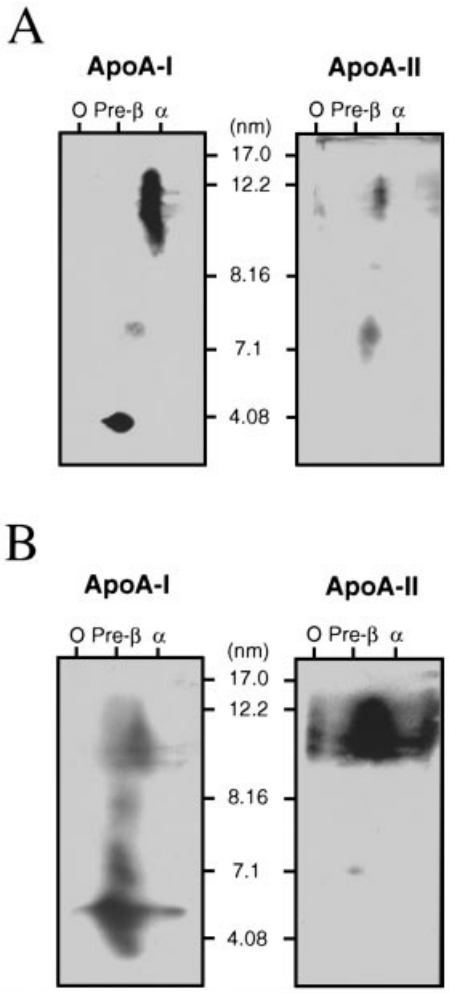

Figure 5 Distribution of apoA-I and apoA-II in the new HDL particles formed during incubation of lipid-free apoA-I, lipid-free apoA-II, sodium oleate and LDL, determined by two-dimensional gel electrophoresis

Lipid-free apoA-I (1.2 mg/ml incubation mixture) was incubated with lipid-free apoA-II (0.12 mg/ml incubation mixture) in the presence of sodium oleate and LDL, as described in the legend to Figure 4. After the incubation, the HDL density fraction of 1.1–1.25 g/ml was isolated and subjected to two-dimensional agarose–polyacrylamide gradient gel electrophoresis (**A**). A portion of the HDL density fraction was subjected to immunoaffinity chromatography on an anti-(apoA-I)–Sepharose column. An aliquot of the particles which had bound to and were eluted from the column were subjected to two-dimensional agarose–polyacrylamide gradient gel electrophoresis (**B**). Immunoblotting to detect apoA-I or apoA-II was carried out as described in the Experimental section. Numbers refer to the Stokes diameter of high-molecular-mass protein standards. O, the origin of the agarose gel.

Immunoaffinity chromatography of HDL particles formed during incubations of lipid-free apoA-I and lipid-free apoA-II with LDL and sodium oleate

The data presented above raised the possibility that a proportion of the HDL particles formed in incubations containing lipid-free apoA-I and lipid-free apoA-II might have been A-I/A-II HDL. To investigate this possibility, the newly formed HDL particles were subjected to immunoaffinity chromatography on an anti-(apoA-I)–Sepharose column. In preliminary experiments involving this column, we established that A-I HDL, prepared by incubating lipid-free apoA-I alone with LDL and sodium oleate [19], bound quantitatively to the column, whereas no A-II HDL (prepared as described above) bound (result not shown). When the HDLs formed during incubations of both apoA-I and apoA-II with LDL and sodium oleate were applied to the anti-(apoA-I)–Sepharose column, again, virtually all of the apoA-I bound. In this case, however, 10–20% of the apoA-II also bound, indicating that at least this proportion of the apoA-II was associated with apoA-I-containing particles.

The mixture of A-I HDL and A-I/A-II HDL [which bound to and were subsequently eluted from the anti-(apoA-I)–Sepharose column], was subjected to two-dimensional gel electrophoresis to determine the distribution of apoA-I and apoA-II between the new HDL subpopulations. Figure 5(B) shows apoA-I and apoA-II immunoblot analysis of the two-dimensional gels. ApoA-I and apoA-II coincide in a major population of pre-β2 particles with a diameter of 10.8 nm and a minor population of pre-β2 particles of diameter 7.4 nm. There was also a substantial population of smaller pre-β2 particles of diameter 4.1 nm, in which only apoA-I was present.

When the HDL particles that had bound to the anti-(apoA-I)–Sepharose column were eluted, 79% of the apoA-I and 55% of the apoA-II was recovered. The composition of the eluted HDLs is shown in Table 2; lipid and protein constituents are expressed per mol of apoA-II. It should be noted that these bound particles probably include both A-I HDL and A-I/A-II HDL. The lipids present in this mixture of particles were predominantly phospholipids and unesterified cholesterol, with only trace amounts of cholesteryl esters and no measurable triacylglycerols or NEFA. Attempts were made to quantify the proportions of A-I HDL and A-I/A-II HDL in this mixture by

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Table 2 Composition of HDL particles eluted from an anti-(apoA-I)–Sepharose column after incubation of lipid-free apoA-II with lipid-free apoA-I, sodium oleate and LDL

Lipid-free apoA-II (0.12 mg/ml incubation mixture) was incubated with lipid-free apoA-I (1.2 mg/ml of incubation mixture) at 37 °C for 3 h in the presence of 1.2 mM sodium oleate and LDL (313 μg of apoB/ml incubation mixture). During the last hour of the incubation, fatty-acid-free BSA (3%, w/v) was added to the mixture to bind the sodium oleate. After the incubation, lipid-associated apoA-II and apoA-I were isolated by ultracentrifugation as the HDL density fraction of 1.1–1.25 g/ml and subjected to immunoaffinity chromatography on an anti-(apoA-I)–Sepharose column. The fractions were assayed as described in the Experimental section. The composition of the fraction which bound to and was eluted from the column is shown. PL, phospholipids; UC, unesterified cholesterol; CE, cholesteryl esters. Each value represents the mean of triplicate determinations.

	Stoichiometry (mol/mol)			
Experiment	ApoA-I/ApoA-II	PL/ApoA-II	UC/ApoA-II	CE/ApoA-II
I	1.8	19.6	13.0	3.5
II	2.9	28.2	25.6	0

immunoaffinity chromatography on an anti-(apoA-II)–Sepharose column. However, the recoveries from the column were too low to provide meaningful results.

DISCUSSION

There are several mechanisms by which A-I HDL may be formed. They may be secreted into plasma as apoA-I–lipid complexes originating in the liver or intestine [12,13]. They may also be assembled in plasma from lipid-free (or lipid-poor) apoA-I. Lipid-free apoA-I has been shown to account for 5–15% of the apoA-I in plasma [29,30], and is constantly being generated as a product of the remodelling of HDLs by a variety of plasma factors [25,31,32]. Previous studies have shown that it can recruit lipids from cell membranes to form new A-I HDL [16–18]. More recently, we have shown that lipid-free apoA-I might also form new HDLs by recruiting lipids from other plasma lipoproteins in a process dependent on the presence of NEFA [19].

Much less is known about the formation of HDLs that contain apoA-II, whether it be the major subpopulation of A-I/A-II HDL or the minor subpopulation of A-II HDL. ApoA-II is synthesized in the liver, from where it is secreted into plasma as a component of preformed lipid complexes [33,34]. It is not known whether apoA-II is also secreted from the liver in a lipid-free form or whether lipid-free apoA-II, like apoA-I, is generated within the plasma as a product of the remodelling of HDLs by plasma factors. Lipid-free apoA-II has not been identified in human plasma, although this might reflect no more than its high affinity for lipids [27,28], which results in its rapid incorporation into protein–lipid complexes. There are several mechanisms by which lipid-free apoA-II could become a component of HDL. ApoA-II has been shown *in vitro* to displace apoA-I from pre-existing HDLs [27,28]. It is also known to recruit lipid from cell membranes to form HDL-like complexes [17]. The present study demonstrates that lipid-free apoA-II has the ability to recruit lipids from LDL in a NEFA-dependent process comparable with that observed with apoA-I [19].

The apoA-II–lipid complexes formed by this last mechanism are recovered in the HDL density fraction, and are separated by ultracentrifugation from any lipid-free proteins remaining in the $d > 1.25$ g/ml fraction. The complexes include two main subpopulations of particles with diameters of 7.4 and 7.8 nm. They have a pre-β2 migration that contrasts with the pre-β1 migration of lipid-free apoA-II. The major constituents of these complexes are apoA-II, phospholipids and unesterified cholesterol. The molar ratio of phospholipid (and unesterified cholesterol) to apolipoprotein in these A-II HDL is remarkably low, whether in comparison with the A-II HDL formed during incubations of lipid-free apoA-II and cells [17], or with the A-I HDL formed by the same technique as that used in the present study [19]. The fact that small, 'lipid-poor' A-II HDL have not been described in human plasma indicates that if such particles are formed *in vivo*, they must either be rapidly cleared from the plasma or rapidly incorporated into pre-existing A-I HDL to form A-I/A-II HDL.

The negligible amount of non-polar core lipids in the A-II HDL formed in the present study is consistent with them being discoidal particles. This contrasts with the spherical particles that comprise the minor subpopulation of A-II HDL found in some subjects [11]. It is most unlikely that the discoidal A-II HDL are converted into spherical A-II HDL, because lecithin:cholesterol acyltransferase, the enzyme which converts discoidal HDLs into spherical HDLs, is non-reactive with A-II HDL [17]. It is possible, however, that discoidal A-II HDL interacts with either discoidal or spherical A-I HDL to form discoidal or spherical A-I/A-II HDL. These interactions might involve plasma factors, such as phospholipid-transfer protein or cholesteryl-ester-transfer protein, which have been shown previously to promote fusion of HDL particles [35,36].

When a mixture containing both lipid-free apoA-I and lipid-free apoA-II was incubated with LDL and sodium oleate, both apolipoproteins were incorporated into new HDL particles. Using immunoaffinity chromatography on an anti-(apoA-I)–Sepharose column, it was found that a proportion of the HDL-associated apoA-II bound to the column. This apoA-II was, by definition, a component of particles that also contained apoA-I. The new A-I/A-II HDL appeared as one major population of particles with a diameter of 10.8 nm and pre-β2 mobility. A proportion of the HDL-associated apoA-I that bound to the column coincided with that of apoA-II in both electrophoretic mobility and size, and was presumably also a component of the new A-I/A-II HDL. However, much of the apoA-I resided in smaller HDLs that did not contain apoA-II, indicating that the HDLs that bound to the anti-(apoA-I)–Sepharose column included a mixture of both A-I HDL and A-I/A-II HDL. As was the case with the A-I HDL described previously [19] and the A-II HDL formed in the present study, the mixture of A-I HDL and A-I/A-II HDL contained phospholipids and unesterified cholesterol, but only trace amounts of cholesteryl esters or triacylglycerol, and were therefore probably discoidal rather than spherical particles. Electron microscopy and composition analysis of the new A-I/A-II HDL could not be carried out, however, because attempts to separate them from the A-I HDL by immunoaffinity chromatography on an anti-(apoA-II)–Sepharose column resulted in yields that were insufficient for either characterization or quantification.

The A-I/A-II HDL formed in the present study might have been assembled directly from the lipid-free apolipoproteins via a NEFA-dependent mechanism similar to that which we proposed in our earlier study [19]. According to this mechanism, NEFA that were either generated by lipolysis [19] or provided exogenously accumulate at the surface of the LDL. Previous studies *in vitro* [37] have shown that the majority of the NEFA generated during lipolysis of hypertriacylglycerolaemic or post-prandial sera exceed the binding capacity of albumin, and consequently partition to the lipoprotein fraction of plasma. The NEFA would then mediate the binding of the lipid-free apolipoproteins to the LDL, as shown previously by Musliner et al. [38], with apoA-I and apoA-II dissociating as apolipoprotein–lipid

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complexes, a proportion of which include A-I/A-II HDL particles. An additional possibility is that the hybrid particles were the result of lipid-free apoA-II displacing apoA-I from preformed A-I HDL. Regardless of the sequence of events, it is apparent that when lipid-free apoA-I and lipid-free apoA-II are exposed to LDLs in the presence of sodium oleate, one of the products formed is a population of A-I/A-II HDL. It should be noted that the present studies were carried out in the absence of albumin to allow the NEFA to partition to the LDL; albumin was added subsequently to remove any NEFA which might have bound to the newly formed HDL particles. However, in experiments in which the NEFA were produced by lipolysis of VLDL, new HDL particles were formed from either lipid-free apoA-I [19] or lipid-free apoA-II, even when albumin was present for the entire incubation.

It remains to be determined whether lipid-free apoA-I and lipid-free apoA-II contribute to the formation of A-I/A-II HDL *in vivo*. Several lines of evidence support this proposal. First, lipid-free apoA-I is constantly being generated as a product of remodelling of HDLs by a variety of plasma factors [25,31,32]. Secondly, NEFA are also constantly generated by the actions of lipolytic enzymes, such as LPL. This is of particular relevance in situations such as post-prandial lipaemia or hypertriacylglycerolaemia, where lipolysis generates levels of NEFA which exceed the binding capacity of albumin [37]. Finally, lipoprotein fractions, such as LDLs or VLDLs, are readily available to provide a source of lipid for formation of the new particles. A major limiting factor to the production of the new A-I/A-II HDL particles appears to be the generation of lipid-free apoA-II. It is not known whether lipid-free apoA-II, like apoA-I, is freely generated as a product of HDL remodelling; this, however, is unlikely to be due to apoA-II's high affinity for lipids [27,28]. This suggests that formation of new A-I/A-II HDL *in vivo* by the processes described in the present study would be dependent on the rate of synthesis of apoA-II. Indeed, Ikewaki et al. [39] have shown that the production rate of apoA-II is the major determinant of A-I/A-II HDL levels in normolipidaemic humans.

In summary, we have shown that lipid-free apoA-II can recruit lipids from other lipoproteins in a NEFA-dependent process to form new 'lipid-poor' A-II HDL, in a similar manner to that previously described for apoA-I [19]. When both lipid-free apolipoproteins are present, a proportion of the new HDLs formed contain both apoA-I and apoA-II. This observation has potential importance in terms of understanding the formation of apolipoprotein-specific HDLs with human plasma.

This work was supported by a grant from the National Heart Foundation of Australia.

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Received 13 July 1998/12 October 1998; accepted 9 November 1998

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