Influence of phospholipid depletion on the size, structure, and remodeling of reconstituted high density lipoproteins

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Abstract This study shows that phospholipid depletion has a major impact on the size and structure of spherical, reconstituted high density lipoproteins (rHDL) and their remodeling by cholesteryl ester transfer protein (CETP). Spherical rHDL, 9.2 nm in diameter with a phospholipid/cholesteryl ester/unesterified cholesterol/apolipoprotein A-I (apoA-I) (PL/CE/UC/A-I) molar ratio of 37.3/24.5/4.1/1.0, were depleted progressively of phospholipids by incubation with phospholipase A_2. After 30 min of incubation the PL/CE/UC/A-I molar ratio of the rHDL was 8.0/31.2/4.4/1.0 and their diameter had decreased to 8.0 nm. Comparable changes in rHDL size and composition were also apparent when the incubations were carried out in the presence of other lipoprotein classes and lipoprotein-deficient plasma. The changes in size and composition were not accompanied by the dissociation of apoA-I from the rHDL. Phospholipid depletion did not affect rHDL surface charge or the structure and stability of apoA-I. The remodeling of unmodified and phospholipid-depleted rHDL by CETP was also investigated. When the rHDL were incubated for 3 h with CETP and Intralipid, transfers of core lipids between the phospholipid-depleted rHDL and Intralipid were decreased relative to unmodified rHDL. This difference was no longer apparent when the incubations were extended beyond 3 h. In these incubations apoA-I dissociated from the phospholipid-depleted and unmodified rHDL at 3 and 12 h, respectively. At 24 h the respective diameters of the unmodified rHDL and phospholipid-depleted rHDL were 8.0 and 7.8 nm. In conclusion, phospholipid depletion has a major impact on rHDL size and their remodeling by CETP.—Rye, K-A., and M. N. Duong. **Influence of phospholipid depletion on the size, structure, and remodeling of reconstituted high density lipoproteins. *J. Lipid Res.* 2000.** 41: **1640–1650.**

Supplementary key words phospholipase A_2 • cholesteryl ester transfer protein • apolipoprotein A-I • high density lipoprotein remodeling

Phospholipids and apolipoproteins are the two main surface constituents of high density lipoproteins (HDL). While the role of apolipoproteins in regulating the structure, function, and remodeling of HDL by plasma factors has been studied extensively, little is known about the influence of phospholipids on these processes. The present study is concerned with how phospholipid depletion affects the size and structure of HDL and their remodeling by plasma factors. This issue is of considerable physiological importance as HDL are the preferred substrates for phospholipase A_2, the plasma concentration of which increases several hundredfold under conditions of acute and chronic inflammation (1, 2). Evidence that the activity of phospholipase A_2 influences HDL levels in vivo comes from studies of mice transgenic for phospholipase A_2, which have low levels of small, phospholipid-depleted HDL (1).

Previous work in this area has focused on how phospholipid depletion affects the size and density of HDL isolated from human plasma. The results of those studies have been conflicting. For example, Perret et al. (3) found that the hydrated density of HDL increases and their size decreases after incubation with phospholipase A_2. Pattnaik, Kézdy, and Scanu (4), in contrast, reported that phospholipid depletion increases the hydrated density of HDL, but does not affect their size. Other studies have suggested that phospholipid depletion does not affect the association of apolipoprotein A-I (apoA-I) with HDL (3, 5), but does cause minor alterations in the immunoreactivity of some epitopes in the central region of the molecule (5).

The present study was carried out to determine precisely how phospholipid depletion affects the size and structure of HDL and their remodeling by cholesteryl ester transfer protein (CETP). As plasma HDL contain several populations of particles, which differ in size, as well as phospholipid composition and concentration, they were not appropriate for use in this study. Instead, the study was carried out with well-characterized preparations of spherical reconstituted HDL (rHDL) consisting of a single, homogeneous population of particles with apoA-I as the

Abbreviations: apo, apolipoprotein; BSA, bovine serum albumin; CD, circular dichroism; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; GdnHCl, guanidine hydrochloride; HDL, high density lipoprotein(s); rHDL, reconstituted high density lipoprotein(s); HL, hepatic lipase; LCAT, lecithin:cholesteryl acyltransferase; LDL, low density lipoprotein(s); LPDP, lipoprotein-deficient plasma; PL, phospholipid; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; TBS, Tris-buffered saline; TG, triglyceride; UC, unesterified cholesterol; VLDL, very low density lipoprotein(s).

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sole apolipoprotein constituent and cholesteryl esters as the only core lipids. Earlier work from this laboratory has shown that these rHDL are indistinguishable from plasma HDL in their interactions with plasma factors (6–8).

To ensure that changes in rHDL size, structure, and remodeling could be attributed unequivocally to phospholipid depletion, it was essential that the factor(s) used to hydrolyze the rHDL phospholipids did not affect the concentrations of any other rHDL constituents. The plasma factors lecithin:cholesterol acyltransferase (LCAT) and hepatic lipase (HL) both have phospholipase activities that hydrolyze HDL phospholipids and decrease their concentration at the particle surface (9, 10). However, in addition to their phospholipase activities, HL and LCAT also alter the concentrations of HDL core lipids and change the size of the particles. For example, the phospholipid hydrolysis mediated by LCAT is accompanied by cholesteryl ester formation, which increases HDL size (11). HL, on the other hand, hydrolyzes HDL triglycerides, as well as phospholipids, and decreases their size (12). As a result, neither of these plasma factors was suitable for use in the current project. Instead, bee venom phospholipase A_2, a type III secretory phospholipase, which specifically hydrolyzes phospholipid *sn*-2 acyl chains, was used for the study.

The results show that phospholipase A_2 decreases rHDL size and regulates their remodeling by CETP. It was also important to establish whether these changes in rHDL size and remodeling were apparent when other lipoproteins and plasma factors were present. This was achieved by incubating rHDL with phospholipase A_2, very low density lipoprotein (VLDL), low density lipoprotein (LDL), and lipoprotein-deficient plasma. The results of those studies show that the rapid in vitro remodeling of rHDL by phospholipase A_2 is affected minimally by other lipoproteins and is unaffected by the plasma factors phospholipid transfer protein (PLTP), CETP, and LCAT that are present in lipoprotein-deficient plasma.

MATERIALS AND METHODS

Purification of apoA-I

HDL were isolated from human plasma by sequential ultracentrifugation in the $1.07 < d < 1.21$ g/ml density range. ApoA-I was obtained by delipidation of ultracentrifugally isolated HDL as described (13). The resulting apoHDL was chromatographed on a Q Sepharose Fast Flow column (Pharmacia Biotech AB, Uppsala, Sweden) (14, 15).

Isolation of VLDL, LDL, and lipoprotein-deficient plasma

Samples of human blood were collected into EDTA-Na_2 tubes (final concentration, 1 mg/ml) and placed immediately on ice. Plasma was isolated at 4°C by centrifugation at 3,000 rpm for 15 min. VLDL and LDL were isolated ultracentrifugally as the fraction of $d < 1.063$ g/ml. The $d > 1.063$ g/ml fraction was adjusted to a density of 1.25 g/ml and lipoprotein-deficient plasma was isolated by ultracentrifugation as the fraction of $d > 1.25$ g/ml. All the ultracentrifugal procedures were carried out at 4°C at a speed of 100,000 rpm for 16 h, using a Beckman (Fullerton, CA) TLA-100.4 rotor in a Beckman TL-100 tabletop ultracentrifuge.

Purification of LCAT and CETP

LCAT and CETP were purified from pooled human plasma (Transfusion Service, Royal Adelaide Hospital, Adelaide, Australia) exactly as reported elsewhere (7, 16). The LCAT used in this study esterified 560 nmol of cholesterol/ml LCAT/h. The CETP had 35 units of activity/ml, where 1 unit is the transfer activity of 1 ml of pooled human lipoprotein-deficient plasma.

Preparation of spherical rHDL

Discoidal rHDL containing 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) (Sigma, St. Louis, MO), unesterified cholesterol (UC) (Sigma), and apoA-I were prepared by the cholate dialysis method (17). LDL were isolated from pooled human plasma by sequential ultracentrifugation in the $1.017 < d < 1.055$ g/ml density range and dialyzed against 0.01 M Tris-buffered saline (TBS, pH 7.4) containing 0.15 M NaCl, 0.005% (w/v) EDTA-Na_2, and 0.006% (w/v) NaN_3. Spherical rHDL containing cholesteryl esters as the sole core lipids and apoA-I as the only apolipoprotein constituent were prepared by incubating the discoidal rHDL with LDL and LCAT at 37°C for 24 h as described (18). The spherical rHDL were isolated from the LDL and LCAT by ultracentrifugation in the $1.07 < d < 1.21$ g/ml density range with two spins at the upper density and a single spin at the lower density (6). The rHDL were dialyzed three times against 1 liter of TBS before use.

Electrophoresis

The surface charge of the spherical rHDL was assessed by agarose gel electrophoresis as described by Sparks and Phillips (19). Electrophoresis on nondenaturing 3/40% polyacrylamide gradient gels (Gradipore, Sydney, Australia) was used to determine rHDL size (20).

Incubations

All incubations were carried out in stoppered plastic tubes in a shaking water bath maintained at 37°C. Nonincubated control samples were stored at 4°C. Fatty acid-free bovine serum albumin (BSA) (Sigma) at a final concentration of 32 mg/ml was added to the incubations that did not contain lipoprotein-deficient plasma. All the incubations contained 10 mM $CaCl_2$. Incubations of rHDL with CETP and Intralipid (20% triglyceride; KabiVitrum AB, Stockholm, Sweden) were carried out as described elsewhere (6). When the incubations were complete the rHDL were isolated by ultracentrifugation in the $1.07 < d < 1.25$ g/ml density range with two spins at the upper density and a single spin at the lower density. These procedures were carried out at 4°C for 16 h at a speed of 100,000 rpm in a TLA-100.1 or a TLA-100.2 rotor, using a Beckman TL-100 tabletop ultracentrifuge. Details of the individual incubations are described in the figure legends.

Spectroscopic studies

A Perkin-Elmer (Norwalk, CT) LS-50 luminescence spectrometer fitted with polarizers and a thermostatted cell holder maintained at 25°C was used for these studies. Fluorescence emission spectra were recorded from 300 to 380 nm, using an excitation wavelength of 295 nm and excitation and emission band passes of 5 and 6 nm, respectively. The same excitation wavelength was used to measure intrinsic fluorescence polarization.

ApoA-I stability was assessed by incubating the rHDL at 25°C for 0–24 h with 0–8.0 M guanidine hydrochloride as described (16).

The circular dichroism (CD) studies were carried out by W. Sawyer (Department of Biochemistry, University of Melbourne, Australia) as described (6).

For the fluorescence quenching studies the areas under the curves of 300- to 380-nm uncorrected emission spectra were determined after incubation in the presence of increasing concen-

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trations of KI (0–0.33 M) (6). The data were analyzed by a modified Stern-Volmer equation (21):

$$\frac{I_0}{I_0 - I} = \frac{1}{f_a} + \frac{1}{f_a K_Q [KI]}$$

where I_0 and I are the emission intensities in the absence and presence of quencher, respectively, f_a is the fraction of Trp residues exposed to quencher, and K_Q is the Stern-Volmer quenching constant. For these studies the excitation wavelength was 295 nm and the excitation and emission band passes were 5.0 and 7.0 nm, respectively.

Immunoblotting

Aliquots of unprocessed incubation mixtures were electrophoresed on 3/40% nondenaturing poylacrylamide gradient gels, transferred electrophoretically to nitrocellulose membranes, and immunoblotted for apoA-I using a sheep anti-human apoA-I polyclonal antibody. ApoA-I was detected by enhanced chemiluminescence (Amersham Pharmacia, Piscataway, NJ).

Other techniques

A Cobas Fara centrifugal analyzer (Roche Diagnostics, Zurich, Switzerland) was used for all chemical analyses (16). Cross-linking was carried out according to the method of Staros (22). To determine if the rHDL that had been incubated with phospholipase A_2 contained lysophosphatidylcholine and nonesterified fatty acids the rHDL lipids were isolated according to the method of Folch, Lees, and Stanley (23) and resolved by thin-layer chromatography using chloroform–methanol–water 65:25:1 (v/v/v).

Statistical analysis

The Student's *t*-test for paired samples was used to determine whether there were significant differences between values.

RESULTS

Influence of phospholipid depletion on rHDL size and composition

To determine how phospholipid depletion affects the size of spherical rHDL, incubations were carried out at 37°C for 30 min with a constant amount of rHDL (0.3 mg/ml phospholipid final concentration), increasing amounts of phospholipase A_2 (0.015–0.124 μg/ml incubation mixture), and fatty acid-free BSA. The BSA was added to the incubations to remove from the rHDL the lysophosphatidylcholine and nonesterified fatty acids generated during the phospholipolysis reaction.

Changes in rHDL particle size were determined by nondenaturing gradient gel electrophoresis (**Fig. 1**). Most of the original rHDL were 9.2 nm in diameter. Their size was not affected by incubation for 30 min in the absence of phospholipase A_2. When the rHDL were incubated for 30 min with increasing amounts of phospholipase A_2 they were converted progressively to small particles 8.0 nm in diameter. At the higher concentrations of phospholipase A_2 a minor population of 9.2-nm rHDL was also present. The rHDL lipids were isolated by Folch extraction (23) and resolved by thin-layer chromatography. Lysophosphatidylcholine and nonesterified fatty acids were not detected in the rHDL lipids (result not shown).

The time course of the phospholipid hydrolysis was also

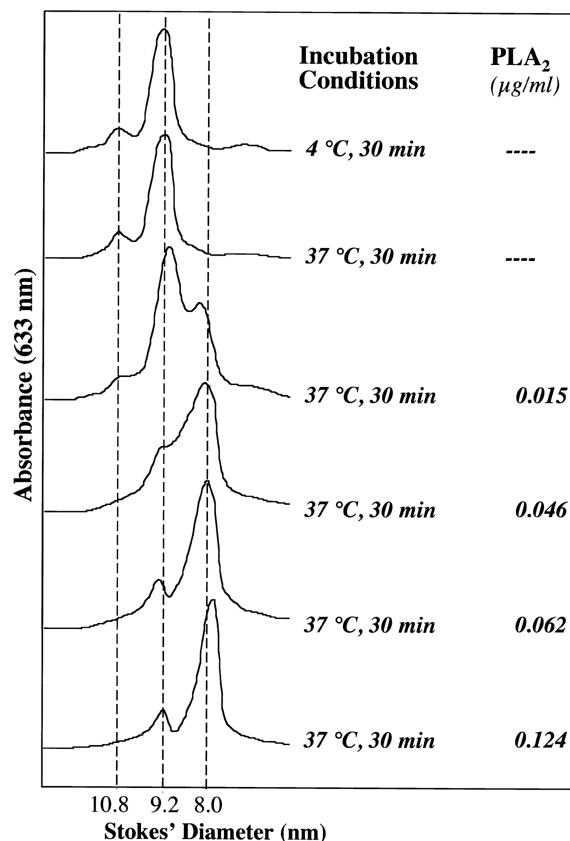

Fig. 1. Changes in the size of rHDL during incubation with increasing amounts of phospholipase A_2. Aliquots of rHDL (final phospholipid concentration, 0.3 mg/ml) were mixed with BSA, $CaCl_2$, and TBS. The mixtures were either maintained at 4°C or incubated at 37°C for 30 min in the presence or absence of increasing concentrations of phospholipase A_2 (final concentration, 0.015–0.124 μg/ml). The final volume of the incubation mixtures was 0.154 ml. Phospholipid hydrolysis was terminated by addition of EDTA-Na_2 (final concentration, 100 mM). The rHDL were isolated by ultracentrifugation as the fraction of d < 1.25 g/ml as described under Materials and Methods and 15 μg of apoA-I was loaded onto a 3/40% nondenaturing gradient gel. The gel was electrophoresed at 180 V for 18 h and stained with Coomassie Blue G-250. Laser densitometric scans of the stained gels are shown.

determined (**Table 1**). In this experiment the rHDL (0.3-mg/ml phospholipid final concentration) were incubated with phospholipase A_2 (final concentration, 0.062 μg/ml incubation mixture) for 0, 5, 10, 15, and 30 min. This concentration of phospholipase A_2 is comparable to what is observed under chronic inflammatory conditions (1). The phospholipid/cholesteryl ester/unesterified cholesterol/apoA-I molar ratio of the nonincubated rHDL was 37.3/24.5/4.1/1.0. These ratios were not affected by incubation for 30 min in the absence of phospholipase A_2. The rHDL phospholipids were hydrolyzed progressively by incubation in the presence of phospholipase A_2. At 30 min the phospholipid/apoA-I molar ratio of the rHDL was 8.0/1.0, corresponding to hydrolysis of 78.0% of the rHDL phospholipids.

Nondenaturing gradient gel electrophoresis was used to monitor changes in rHDL size (**Fig. 2**). The original rHDL

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TABLE 1. Composition of rHDL after incubation with phospholipase A_2

Incubation Conditions			Stoichiometry (PL/CE/UC/A-I)	Phospholipid Hydrolysis
Time	Temp.	PLA_2		
min	*°C*		*mol/mol*	*%*
30	4	−	37.3/24.5/4.1/1.0	
30	37	−	36.4/24.5/4.1/1.0	
5	37	+	29.5/28.6/4.7/1.0	19.0
10	37	+	20.5/31.1/4.9/1.0	43.7
15	37	+	14.8/31.9/4.3/1.0	59.3
30	37	+	8.0/31.2/4.4/1.0	78.0

Spherical rHDL were either maintained at 4°C, incubated for 30 min in the absence of phospholipase A_2 or incubated for 5, 10, 15, and 30 min in the presence of phospholipase A_2, then isolated by ultracentrifugation as described in the legend to Fig. 2. The compositional data represent the mean of triplicate determinations, which varied by less than 10%. Abbrevations: PLA_2, phospholipase A_2; PL, phospholipid; CE, cholesteryl ester; UC, unesterified cholesterol; A-I, apolipoprotein A-I.

were 9.2 nm in diameter. Their size was not affected by incubation for 30 min at 37°C in the absence of phospholipase A_2. Incubation in the presence of phospholipase A_2 was accompanied by a progressive reduction in rHDL size. By 30 min most of the rHDL were 8.0 nm in diameter.

Additional experiments were carried out to determine whether other lipoproteins and plasma factors affected the remodeling of rHDL by phospholipase A_2. The rHDL were incubated for 0, 5, 10, 15, and 30 min with phospholipase A_2 and a mixture of VLDL and LDL. The final concentrations of VLDL and LDL in the incubation mixtures were comparable to that of the plasma samples from which they were isolated. Under these conditions the decrease in rHDL size was indistinguishable from what is shown in Fig. 2 for incubations of rHDL with phospholipase A_2 alone (result not shown) and most of the rHDL were 8.0 nm in diameter after 30 min of incubation. The rHDL phospholipid hydrolysis was decreased slightly in the presence of VLDL and LDL compared with incubation with phospholipase A_2 alone (**Table 2**). After 30 min of incubation in the presence of phospholipase A_2 as well as VLDL and LDL, 54.5% of the rHDL phospholipids were hydrolyzed, compared with 78% for incubation with phospholipase A_2 alone.

The rHDL were also incubated with phospholipase A_2 and lipoprotein-deficient plasma, which contained PLTP, CETP, and LCAT. The rHDL phospholipid hydrolysis in these incubations was indistinguishable from what was found for incubations of rHDL with phospholipase A_2 alone (Table 2). The reduction in rHDL size was also comparable to what is shown in Fig. 2 for incubations of rHDL with phospholipase A_2 alone (result not shown).

When the rHDL were incubated with phospholipase A_2, VLDL, and LDL as well as lipoprotein-deficient plasma the phospholipid hydrolysis (Table 2) and changes in particle size (not shown) were comparable to what was found when the rHDL were incubated with phospholipase A_2 as well as VLDL and LDL.

The conversion of 9.2-nm rHDL to small, 8.0-nm particles has been reported in earlier work from this laboratory when rHDL were incubated with CETP and Intralipid (6). In those

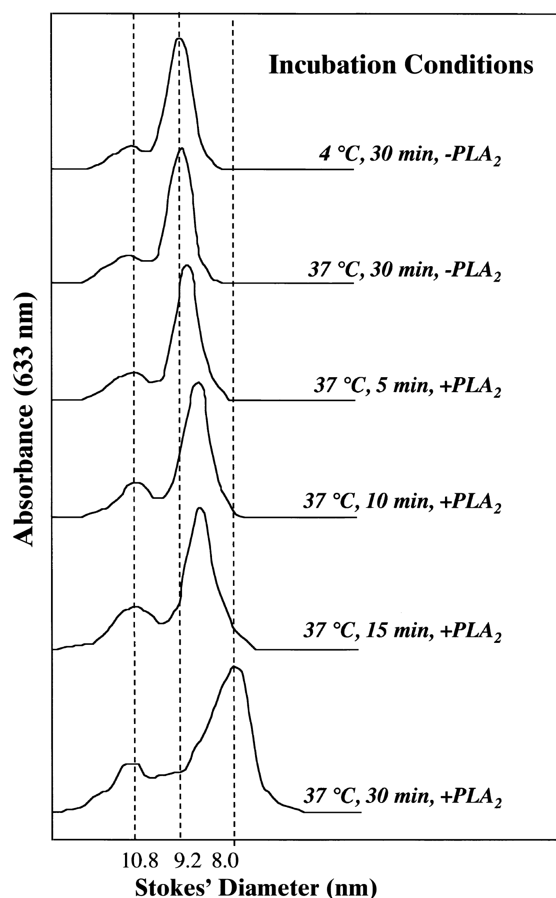

Fig. 2. Time course of rHDL size changes during incubation with phospholipase A_2. Spherical rHDL (final phospholipid concentration, 0.3 mg/ml) were mixed with BSA and $CaCl_2$ and either maintained for 30 min at 4°C, incubated at 37°C for 30 min in the absence of phospholipase A_2, or incubated for 5, 10, 15, or 30 min in the presence of phospholipase A_2 (final concentration, 0.062 μg/ml). The final volume of the incubation mixtures was 0.64 ml. When the incubations were complete the hydrolysis reaction was terminated as described in the legend to Fig. 1. The rHDL were then isolated by ultracentrifugation as the fraction of d $<$ 1.25 g/ml and subjected to nondenaturing gradient gel electrophoresis as described in the legend to Fig. 1. Laser densitometric scans of the stained gels are shown.

incubations the reduction in particle size was accompanied by the dissociation of apoA-I from the rHDL and a decrease in the number of apoA-I molecules from three to two per particle (6). To determine whether the reduction in rHDL particle size that occurs during incubation with phospholipase A_2 is also accompanied by a dissociation of apoA-I, the rHDL were incubated for 30 min in the presence and absence of phospholipase A_2. When the incubations were complete the control, unmodified rHDL and the phospholipid-depleted rHDL were cross-linked, delipidated, and electrophoresed on a sodium dodecyl sulfate (SDS) gradient gel. Cross-linked, lipid-free apoA-I was used as a standard (**Fig. 3**). The cross-linked, lipid-free apoA-I was a mixture of monomers, dimers, trimers, tetramers, and pentamers. The cross-linked apoA-I from both the unmodified and phospholipid-depleted rHDL migrated to the same position as the cross-linked, trimeric lipid-free apoA-I. This indicates that

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TABLE 2. Composition of rHDL after incubation with phospholipase A_2, VLDL, LDL, and LPDP

Incubation Conditions				
Time	Temp.	Additions	Stoichiometry (PL/CE/UC/A-I)	PL Hydrolysis
min	*°C*		*mol/mol*	%
30	4	$-PLA_2$	37.3/24.5/4.1/1.0	—
30	37	$-PLA_2$	36.4/24.5/4.1/1.0	—
5	37	$+PLA_2$	29.5/28.6/4.7/1.0	19.0
10	37	$+PLA_2$	20.5/31.1/4.9/1.0	43.7
15	37	$+PLA_2$	14.8/31.9/4.3/1.0	59.3
30	37	$+PLA_2$	8.0/31.2/4.4/1.0	78.0
30	4	$-PLA_2$ + VLDL + LDL	34.9/22.7/9.1/1.0	—
30	37	$-PLA_2$ + VLDL + LDL	33.6/22.3/8.8/1.0	—
5	37	$+PLA_2$ + VLDL + LDL	31.4/24.9/8.6/1.0	6.5
10	37	$+PLA_2$ + VLDL + LDL	25.4/27.0/8.6/1.0	24.4
15	37	$+PLA_2$ + VLDL + LDL	23.5/25.9/8.5/1.0	30.1
30	37	$+PLA_2$ + VLDL + LDL	15.3/25.1/8.2/1.0	54.5
30	4	$-PLA_2$ + LPDP	35.8/24.1/4.6/1.0	—
30	37	$-PLA_2$ + LPDP	34.1/24.0/4.2/1.0	—
5	37	$+PLA_2$ + LPDP	27.0/24.3/4.7/1.0	20.8
10	37	$+PLA_2$ + LPDP	20.4/25.8/4.8/1.0	40.2
15	37	$+PLA_2$ + LPDP	14.8/27.8/5.3/1.0	56.6
30	37	$+PLA_2$ + LPDP	10.5/30.8/3.7/1.0	69.2
30	4	$-PLA_2$ + VLDL + LDL + LPDP	34.6/17.2/12.1/1.0	—
30	37	$-PLA_2$ + VLDL + LDL + LPDP	32.8/19.6/11.6/1.0	—
5	37	$+PLA_2$ + VLDL + LDL + LPDP	29.5/23.4/11.3/1.0	10.1
10	37	$+PLA_2$ + VLDL + LDL + LPDP	25.9/26.3/13.4/1.0	21.0
15	37	$+PLA_2$ + VLDL + LDL + LPDP	20.3/24.3/12.1/1.0	38.1
30	37	$+PLA_2$ + VLDL + LDL + LPDP	12.7/25.0/11.3/1.0	61.3

Spherical rHDL were mixed with TBS, mixtures of VLDL and LDL or LPDP, and either maintained at 4°C, incubated for 30 min in the absence of phospholipase A_2 or incubated for 5, 10, 15, and 30 min in the presence of phospholipase A_2 as described in the legend to Fig. 2. The final volume of the incubation mixtures was 0.732 ml. The concentrations of VLDL TG and LDL CE in the incubations were 1.2 and 4.4 mM, respectively. When the incubations were complete the rHDL were isolated by ultracentrifugation as described in the legend to Fig. 2. The compositional data represent the mean of triplicate determinations, which varied by less than 10%. Abbreviations: PLA_2, phospholipase A_2; PL, phospholipid; CE, cholesteryl ester; UC, unesterified cholesterol; A-I, apolipoprotein A-I; LPDP, lipoprotein-deficient plasma.

the unmodified and phospholipid-depleted rHDL both contained three molecules of apoA-I per particle and that incubation with phospholipase A_2 is not accompanied by the dissociation of apoA-I from rHDL. This is in agreement with what has been shown indirectly for plasma HDL (3).

To confirm further that apoA-I does not dissociate from rHDL during the incubation with phospholipase A_2, aliquots of rHDL were either maintained at 4°C or incubated at 37°C for 30 min in the absence of phospholipase A_2 or incubated for 5, 10, 15, and 30 min in the presence of phospholipase A_2. The unprocessed incubation mixtures were electrophoresed on nondenaturing gradient gels, transferred to nitrocellulose membranes, and immunoblotted for apoA-I (**Fig. 4**). The rHDL that had either been maintained at 4°C or incubated at 37°C for 30 min in the absence of phospholipase A_2 are shown in tracks 1 and 2. Tracks 3–6 contain rHDL incubated with phospholipase A_2 for 5, 10, 15, and 30 min. Lipid-free apoA-I is shown in track 7. The absence of lipid-free apoA-I in tracks 3–6 confirms that apoA-I does not dissociate from rHDL during incubation with phospholipase A_2.

Influence of phospholipid depletion on the physical properties of rHDL

Previous studies from this laboratory have shown that rHDL that differ in size may also vary in their physical properties (6, 7). Spectroscopic experiments were carried out to determine if this is the case for the unmodified and phospholipid-depleted rHDL in the present study (**Table 3**).

Incubation with phospholipase A_2 did not affect the wavelength of maximum fluorescence (λ_{max}) of the rHDL. This indicates that phospholipid depletion does not change the environment of rHDL apoA-I Trp residues. In contrast, the steady state fluorescence polarization (P) of phospholipid-depleted rHDL was decreased relative to unmodified rHDL, indicating that the local rotational motions of rHDL apoA-I Trp residues are increased by phospholipid depletion.

The CD studies showed that lipid association increases the α-helical and β-turn content of apoA-I at the expense of the β-sheet and random coil content. This agrees with earlier work from our, and other, laboratories (7, 24). These results also show that phospholipid depletion does not alter the secondary structure of the apoA-I in rHDL. This is consistent with the quenching studies, where phospholipid depletion had no effect on the exposure of rHDL apoA-I Trp residues to the aqueous environment (f_a). Both the unmodified and phospholipid-depleted rHDL had comparable surface charges as judged by their electrophoretic mobilities after agarose gel electrophoresis. This is in agreement with the work of Pattnaik, Kézdy, and Scanu (4). When these results are considered together, it

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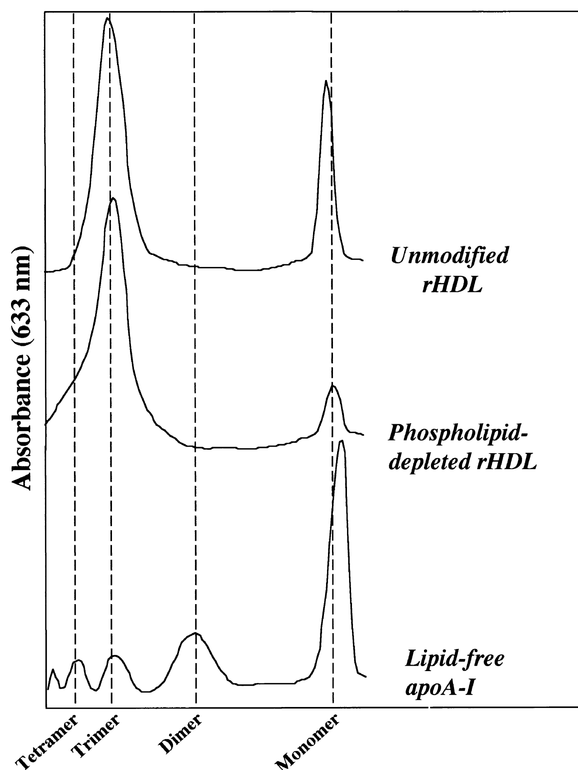

Fig. 3. Influence of phospholipid depletion on the number of apoA-I molecules in rHDL. Spherical rHDL (final phospholipid concentration, 0.3 mg/ml) were mixed with BSA and $CaCl_2$ and then incubated for 30 min in the presence or absence of phospholipase A_2 (final concentration, 0.062 μg/ml). When the incubations were complete the rHDL were isolated by ultracentrifugation as the fraction of d < 1.25 g/ml, dialyzed against 50 mM phosphate buffer, pH 7.4, and cross-linked as described under Materials and Methods. The cross-linked samples were delipidated and aliquots of the delipidated samples containing 8.0 μg of apoA-I were electrophoresed on a 3/40% SDS-polyacrylamide gradient gel, using cross-linked lipid-free apoA-I (8.0 μg) as a standard. Laser densitometric scans of the stained gels are shown.

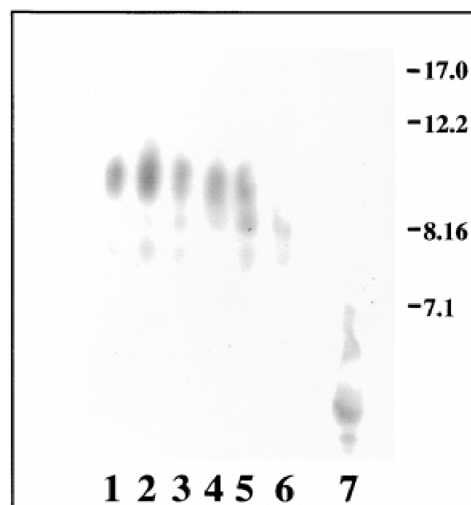

Fig. 4. Phospholipid depletion does not promote the dissociation of apoA-I from rHDL. Spherical rHDL were either maintained for 30 min at 4°C, incubated at 37°C in the absence of phospholipase A_2, or incubated in the presence of phospholipase A_2 for 5, 10, 15, or 30 min as described in the legend to Fig. 2. When the incubations were complete aliquots (0.5 μg of apoA-I) of the unprocessed incubation mixtures were electrophoresed on 3/40% nondenaturing polyacrylamide gradient gels. The rHDL were transferred electrophoretically to nitrocellulose membranes and immunoblotted with anti-apoA-I antibodies. A scan of the immunoblot is shown. Nonincubated rHDL is shown in lane 1. Lane 2 represents rHDL incubated for 30 min in the absence of phospholipase A_2. Lanes 3–6 show rHDL incubated with phospholipase A_2 for 5, 10, 15, and 30 min, respectively. Lipid-free apoA-I is shown in lane 7. The numbers on the right-hand side represent the diameters of high molecular weight standards.

appears that the reduction in rHDL particle size that occurs as a consequence of phospholipid depletion does not have a major effect on the conformation of apoA-I.

To determine if phospholipid depletion affects the stability of the apoA-I in rHDL, the unmodified and phospholipid-depleted rHDL were incubated with increasing concentrations of guanidine hydrochloride (**Table 4**). These results show that both rHDL preparations required comparable concentrations of guanidine hydrochloride to achieve 50% unfolding of apoA-I. The apoA-I in the unmodified and phospholipid-depleted rHDL also had similar free energies of unfolding (ΔG_{H_2O}) and there was no difference in the number of guanidine hydrochloride-binding sites (Δn) for the apoA-I in the folded and unfolded states. In addition, the apoA-I in the unmodified and phospholipid-depleted rHDL exhibited comparable kinetics of unfolding during incubation with guanidine hydrochloride (result not shown). When taken together these results indicate that phospholipid depletion does not affect the stability of apoA-I in rHDL.

Influence of phospholipid depletion on the remodeling of rHDL by CETP

Earlier work from this laboratory has shown that when rHDL are incubated with CETP and Intralipid, cholesteryl esters are transferred from the rHDL to Intralipid, and triglycerides are transferred in the reverse direction, from Intralipid to rHDL (6). Under circumstances in which the transfers of core lipids out of the rHDL are greater than the transfers of core lipids into the rHDL, the size of the particles decreases and apoA-I dissociates from the rHDL (6).

To determine whether phospholipid depletion affects the remodeling of rHDL by CETP, unmodified and phospholipid-depleted rHDL were mixed with Intralipid and either maintained at 4°C or incubated at 37°C for 24 h in the absence of CETP or incubated for 1, 3, 6, 12, or 24 h in the presence of CETP. **Figure 5** shows the cholesteryl ester/apoA-I (CE/apoA-I), the triglyceride/apoA-I (TG/apoA-I), and the phospholipid/apoA-I (PL/apoA-I) molar ratios for unmodified (closed symbols) and phospholipid-depleted rHDL (open symbols) at each time point after incubation with CETP. This experiment is representative of two separate experiments. The concentrations of the individual constituents were determined in triplicate for each experiment. After 24 h of incubation in the absence of CETP the respective CE/apoA-I molar ratios of the

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TABLE 3. Physical properties of rHDL after incubation in the presence and absence of phospholipase A_2

Sample	Incubation Conditions	λ_{max}[a]	Polarization[b]	f_a[c]	K_Q[d]	Electrophoretic Mobility[e]	ApoA-I Secondary Structure[f] α Helix	β Sheet	β Turn	Random Coil
		nm	*P*		M^{-1}	$\mu m\ sec^{-1}/V\ cm^{-1}$	%			
Unmodified rHDL	37°C, 30 min	334.3 ± 0.8	0.137 ± 0.001[g,h]	0.43 ± 0.06	3.55 ± 0.05	−0.72	65.6	0.1	14.5	19.8
Phospholipid-depleted rHDL	37°C, 30 min	333.7 ± 0.6	0.120 ± 0.002[i]	0.41 ± 0.18	2.03 ± 0.47	−0.75	66.1	0	14.7	19.2
Lipid-free A-I		335.7 ± 0.9	0.180 ± 0.003	0.58 ± 0.07	3.60 ± 0.16	−0.58	48.3	18.6	0	33.1

Spherical rHDL were incubated for 30 min at 37°C in the presence or absence of phospholipase A_2 and then isolated by ultracentrifugation as described in the legend to Fig. 2.

[a] Wavelength of maximum fluorescence determined at 25°C from a 300 to 380-nm emmission scan, using an excitation wavelength of 295 nm. Values represent means ± SD of three determinations.

[b] Intrinsic steady state fluorescence polarization determined at 25°C using an excitation wavelength of 295 nm. Values represents means ± SD of three determinations.

[c] Fraction of apoA-I Trp residues available for quenching. Determined from a modified Stern-Volmer plot as described under Experimental Procedures.

[d] Stern-Volmer quenching constant. Determined from a modified Stern-Volmer plot as described under Experimental Procedures.

[e] Determined by agarose gel electrophoresis as described under Experimental Procedures.

[f] Determined by circular dichroism.

[g] $P < 0.005$ compared with lipid-free apoA-I.

[h] $P < 0.005$ compared with phospholipid-depleted rHDL.

[i] $P < 0.001$ compared with lipid-free apoA-I.

unmodified rHDL and phospholipid-depleted rHDL were 19.3/1 and 19.6/1. After 1 h of incubation in the presence of CETP the CE/apoA-I molar ratio of the unmodified rHDL decreased to 8.3/1, while that of the phospholipid-depleted rHDL was 14.4/1. At 3 h the difference between the unmodified and phospholipid-depleted rHDL was still apparent, with the CE/apoA-I molar ratio of the unmodified rHDL being 2.7/1, compared with 6.3/1 for the phospholipid-depleted rHDL. When the incubations were extended beyond 3 h this difference was no longer apparent.

In the initial stages of the incubation triglyceride transfers from Intralipid into phospholipid-depleted rHDL were also decreased relative to the unmodified rHDL. After 24 h of incubation in the absence of CETP the respective TG/apoA-I molar ratios of the unmodified and phospholipid-depleted rHDL were 0.5/1 and 0.6/1. These values probably reflect small amounts of Intralipid remaining with the rHDL after ultracentrifugation, rather than the spontaneous transfer of triglycerides from Intralipid into rHDL. After 1 h of incubation with CETP the TG/apoA-I molar ratio of the unmodified rHDL was 12.9/1, compared with 9.7/1 for the phospholipid-depleted rHDL. At 3 h the TG/apoA-I molar ratios of the unmodified and phospholipid-depleted rHDL were 17.9/1 and 16.6/1, respectively. When the incubations were extended beyond 3 h, the TG/apoA-I molar ratios of both rHDL preparations were comparable.

The decrease in the rHDL TG/apoA-I molar ratio that was apparent when the incubation with CETP and Intralipid was extended from 3 to 24 h is due to the transfers of core lipids out of the rHDL exceeding the transfers of core lipids into the rHDL. This observation is discussed in detail elsewhere (6).

Phospholipids were also transferred from Intralipid to the unmodified and phospholipid-depleted rHDL during the incubation with CETP. In both cases the rHDL PL/apoA-I molar ratio increased linearly during the first 6 h of incubation. There was no further change in the PL/apoA-I molar ratio when the incubations were extended from 6 to 24 h.

The changes in particle size that occurred during incu-

TABLE 4. Influence of phospholipid depletion on the conformational stability of apoA-I in spherical rHDL

Sample	Incubation Conditions	$[GdnHCl]_{1/2}$[a]	$[GdnHCl]_{1/2}$[b]	ΔG_{H_2O}[c]	Δn[d]
		M	*M*	*kcal/mol*	
Unmodified rHDL	37°C, 30 min (−PLA_2)	2.7 ± 0.2	2.7 ± 0.1	4.7 ± 0.2	15.4 ± 1.1
Phospholipid-depleted rHDL	37°C, 30 min (+PLA_2)	2.4 ± 0.3	2.3 ± 0.1	4.6 ± 0.6	16.5 ± 2.3
Lipid-free A-I		1.0 ± 0.1	1.0 ± 0.1	2.5 ± 0.5	26.4 ± 2.1

Spherical rHDL were incubated for 30 min at 37°C in the presence or absence of phospholipase A_2 and then isolated by ultracentrifugation as described in the legend to Fig. 2. The rHDL were then incubated at 25°C for 0–24 h in the presence of increasing concentrations of GdnHCl as described under Experimental Procedures.

[a] Concentration of GdnHCl required to achieve 50% unfolding of apoA-I. Determined directly from a plot of the concentration of GdnHCl versus wavelength of maximun fluorescence.

[b] Calculated concentration of GdnHCl required to achieve 50% unfolding of apoA-I. Values were obtained as described (16).

[c] Conformational stability of apoA-I in the absence of GdnHCl. Determined as described (16).

[d] Difference in the number of GdnHCl-binding sites of apoA-I in the folded and unfolded state calculated as described (16).

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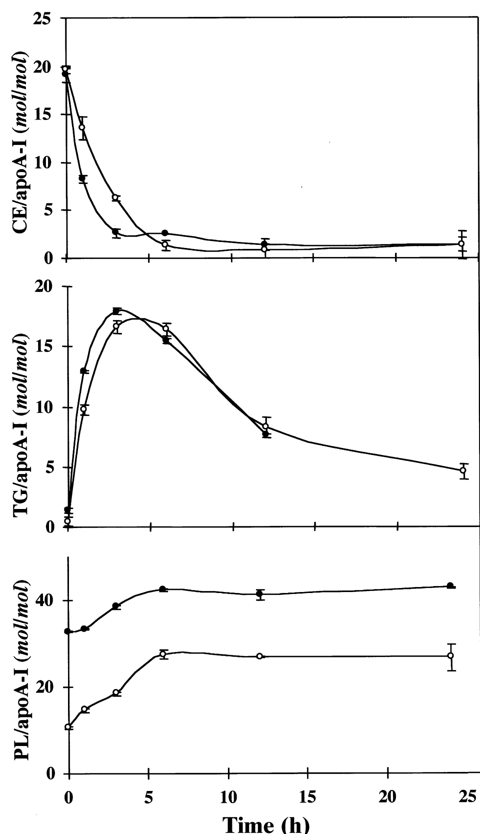

Fig. 5. Incubation of unmodified and phospholipid-depleted rHDL with CETP and Intralipid: effect on rHDL composition. Unmodified (closed symbols) and phospholipid-depleted rHDL (open symbols) were mixed with Intralipid alone or with Intralipid and CETP. The final rHDL cholesteryl ester and Intralipid triglyceride concentrations were 0.1 and 4.0 mmol/l, respectively. The samples that did not contain CETP were either maintained at 4°C or incubated at 37°C for 24 h. The samples containing CETP (final activity, 2.7 units/ml) were incubated at 37°C for 1, 3, 6, 12, or 24 h. The final volumes of the incubation mixtures were 2 ml. When the incubations were complete the rHDL were isolated by ultracentrifugation and their composition was determined as described under Materials and Methods. The rHDL CE/apoA-I, TG/apoA-I, and PL/apoA-I molar ratios are shown as a function of time. Means ± SD of triplicate determinations are shown.

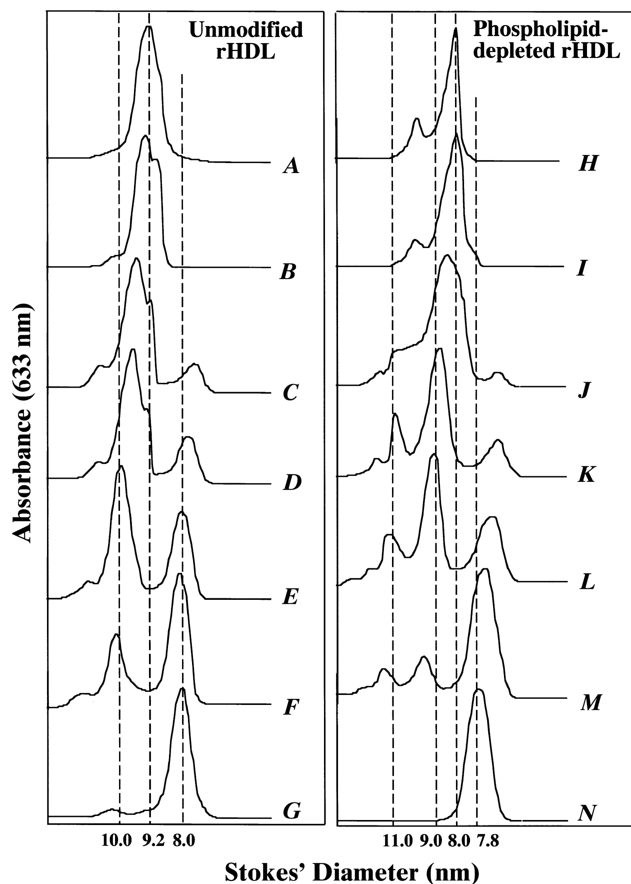

Fig. 6. Incubation of unmodified and phospholipid-depleted rHDL with CETP and Intralipid: effect on rHDL size. Unmodified and phospholipid-depleted rHDL were incubated with Intralipid alone or with Intralipid and CETP as described in the legend to Fig. 5. The rHDL were isolated by ultracentrifugation as the fraction of d < 1.25 g/ml and then subjected to nondenaturing gradient gel electrophoresis as described in the legend to Fig. 1. Laser densitometric scans of the stained gels are shown. Profiles A and H show unmodified and phospholipid-depleted rHDL, respectively, which were maintained at 4°C for 24 h. Profiles B and I show unmodified and phospholipid-depleted rHDL, respectively, after 24 h of incubation at 37°C in the presence of Intralipid. Profiles C–G show, respectively, unmodified rHDL after 1, 3, 6, 12, and 24 h of incubation in the presence of Intralipid and CETP. The size distribution of the phospholipid-depleted rHDL after 1, 3, 6, 12, and 24 h of incubation in the presence of Intralipid and CETP is shown in profiles J–N, respectively.

bations of unmodified and phospholipid-depleted rHDL with Intralipid and CETP are shown in **Fig. 6**. The unmodified rHDL were 9.2 nm in diameter (Fig. 6, profile A). Their size was not affected by incubation for 24 h with Intralipid alone (Fig. 6, profile B). When the unmodified rHDL were incubated with CETP for 1, 3, 6, 12, and 24 h (Fig. 6, profiles C–G) they were converted progressively to small particles 8.0 nm in diameter. Larger particles, 10 nm in diameter, were also apparent during the first 12 h of the incubation. These size changes have been described in detail elsewhere (6).

The nonincubated, phospholipid-depleted rHDL were 8.0 nm in diameter (Fig. 6, profile H). Their size was not affected by incubation for 24 h in the presence of Intralipid alone (Fig. 6, profile I) or for 1 h in the presence of Intralipid and CETP (profile J). At 3 h (Fig. 6, profile K) and 6 h (profile L) the diameter of the predominant population of particles was 9.0 nm. Larger and smaller particles were also apparent at these times. At 12 h most of the rHDL were 7.8 nm in diameter (Fig. 6, profile M). By 24 h the rHDL were all 7.8 nm in diameter (Fig. 6, profile N). The appearance of larger particles at 3 and 6 h is most likely due to enrichment of the phospholipid-depleted rHDL with triglyceride (see Fig. 5).

Earlier work from this laboratory has shown that the decrease in particle size that occurs when unmodified rHDL are incubated with Intralipid and CETP is accompanied by the dissociation of apoA-I from the rHDL and a decrease in the number of apoA-I molecules from three to two per particle (6). To determine whether apoA-I also dissociates from phospholipid-depleted rHDL, unmodi-

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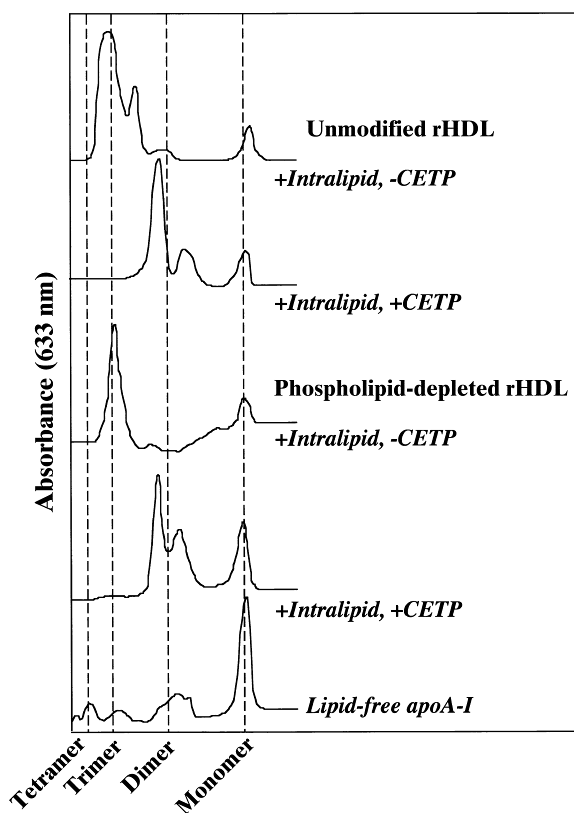

Fig. 7. Incubation of unmodified and phospholipid-depleted rHDL with Intralipid and CETP: effect on the number of apoA-I molecules per particle. Unmodified and phospholipid-depleted rHDL were incubated at 37°C for 24 h with Intralipid alone or with Intralipid and CETP at the concentrations described in the legend to Fig. 5. When the incubations were complete the rHDL were isolated by ultracentrifugation, dialyzed against 50 mM phosphate buffer, pH 7.4, and cross-linked. The cross-linked samples were delipidated and electrophoresed on a 3/40% SDS-polyacrylamide gradient gel as described in the legend to Fig. 3, using cross-linked lipid-free apoA-I as a standard. Scans of the stained gels are shown.

fied and phospholipid-depleted rHDL were mixed with Intralipid and incubated for 24 h in the presence and absence of CETP. When the incubations were complete the rHDL were cross-linked and delipidated, then electrophoresed on an SDS gradient gel using cross-linked, lipid-free apoA-I as a standard (**Fig. 7**). After incubation in the absence of CETP the unmodified and phospholipid-depleted rHDL both contained three molecules of apoA-I/particle. After 24 h of incubation in the presence of CETP both preparations of rHDL contained two molecules of apoA-I/particle.

The time course of the dissociation of apoA-I from the unmodified and phospholipid-depleted rHDL is shown in **Fig. 8**. In this experiment the rHDL were either maintained at 4°C or incubated at 37°C for 24 h with Intralipid alone or incubated for 1, 3, 6, 12, and 24 h with Intralipid and CETP. Aliquots of the unprocessed incubation mixtures were then subjected to nondenaturing gradient gel electrophoresis, transferred to nitrocellulose membranes, and immunoblotted for apoA-I. Lipid-free apoA-I was also applied to the gels (Fig. 8, track 1). Tracks 2 and 3 show, respectively, rHDL that had been mixed with Intralipid and either maintained at 4°C or incubated at 37°C for 24 h. Neither of these samples contained lipid-free apoA-I. Lipid-free apoA-I was not detected in the unmodified rHDL that had been incubated with Intralipid and CETP for 1 h (Fig. 8, track 4), 3 h (track 5), or 6 h (track 6). It was, however, present at 12 h (Fig. 8, track 7) and 24 h (track 8). In the case of the phospholipid-depleted rHDL, lipid-free apoA-I was evident at 3 h (Fig. 8, track 5), 6 h (track 6), 12 h (track 7) and 24 h (track 8). This result shows that apoA-I dissociates more readily from phospholipid-depleted rHDL than from unmodified rHDL.

DISCUSSION

In inflammatory states human plasma contains high levels of a secretory, nonpancreatic phospholipase A_2 that hydrolyzes the phospholipids in both acute phase and normal HDL (1, 2). Until now the effects of phospholipid depletion on the size, structure, and remodeling of HDL have been poorly understood. These issues have been addressed in the present study by incubating well-defined, homogeneous preparations of rHDL with bee venom phospholipase A_2, a secretory phospholipase A_2 that hydrolyzes HDL phospholipids without altering the concentrations of other HDL constituents. The results show that phospholipid depletion has a major effect on the size of rHDL, their remodeling by CETP, and the CETP-mediated dissociation of apoA-I.

To ascertain the physiological relevance of these observations it was important to determine whether the phospholipase A_2-mediated remodeling of rHDL occurred in the presence of other lipoproteins and plasma factors. This was achieved by including VLDL, LDL, and/or lipoprotein-deficient plasma in the incubations. When the rHDL were incubated with phospholipase A_2 as well as VLDL and LDL, the phospholipid hydrolysis was reduced slightly at 30 min. However, the VLDL and LDL had no effect on the reduction in rHDL particle size, with the diameter of most of the rHDL having decreased to 8.0 nm by the end of the incubation. This indicates that phospholipase A_2 hydrolyzes VLDL and LDL phospholipids as well as rHDL phospholipids, but exhibits a preference for the phospholipids in rHDL. This is consistent with what has been reported by Pruzanski et al. (1).

When the rHDL were incubated with phospholipase A_2 and lipoprotein-deficient plasma the extent of rHDL phospholipid hydrolysis and the reduction in particle size were indistinguishable from what was observed for incubations of rHDL with phospholipase A_s alone. This highlights the fact that phospholipase A_2 hydrolyzes rHDL phospholipids rapidly (up to 80% of the rHDL phospholipids hydrolyzed in 30 min) whereas the remodeling of HDL by CETP, LCAT, and PLTP proceeds over a number of hours.

One of the most interesting findings to emerge from this study is that phospholipid depletion does not alter the number of apoA-I molecules associated with the

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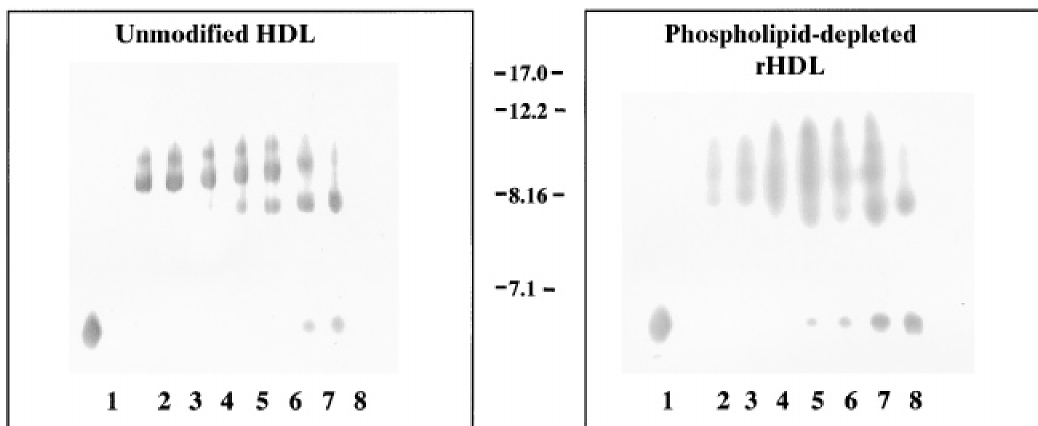

Fig. 8. Dissociation of apoA-I from unmodified and phospholipid-depleted rHDL during incubation with Intralipid and CETP. Unmodified and phospholipid-depleted rHDL were either maintained at 4°C with Intralipid or incubated with Intralipid alone or Intralipid and CETP as described in the legend to Fig. 5. Aliquots (0.5 μg of apoA-I) of the unprocessed incubations were electrophoresed on a 3/40% nondenaturing gradient gels and immunoblotted for apoA-I. Scans of the immunoblots are shown. Track 1 shows lipid-free apoA-I. Tracks 2 and 3 show, respectively, rHDL maintained either at 4°C or incubated at 37°C for 24 h with Intralipid. The rHDL that were incubated with Intralipid and CETP for 1, 3, 6, 12, and 24 h are shown, respectively, in tracks 4, 5, 6, 7, and 8. The numbers between the panels represent the diameters of high molecular weight standards.

rHDL. Both the unmodified 9.2-nm rHDL and phospholipid-depleted, 8.0-nm rHDL contain three apoA-I molecules per particle. This is in agreement with what has been reported in earlier studies in which plasma HDL were incubated with phospholipase A_2 (3, 4). It also suggests that the reduction in rHDL size is a direct consequence of phospholipid depletion. To determine if this is the case we used the Stokes' radii in Fig. 2 to calculate that the surface areas of the 9.2- and 8.0-nm rHDL are 266 and 201 nm^2, respectively. To ascertain whether phospholipid depletion alone could account for this 65-nm^2 reduction in rHDL surface area, the areas of the phospholipids in the 9.2- and 8.0-nm rHDL were calculated. Using the cross-linking data in Fig. 3, the stoichiometries in Table 1, and assuming that each phospholipid molecule occupies 0.65 nm^2 (25), it follows that the 9.2-nm rHDL contain 112 phospholipid molecules that occupy 73 nm^2, and that the 8.0-nm rHDL contain 24 phospholipids that occupy 16 nm^2. This indicates that phospholipid depletion alone can account for the reduction in rHDL size.

One of the main goals of the present study was to determine how phospholipid depletion affects the remodeling of rHDL by CETP. When unmodified rHDL are incubated with CETP and Intralipid, the rHDL donate cholesteryl esters to and accept triglycerides from Intralipid (6). The results in Fig. 5 show that phospholipid-depleted rHDL also donate cholesteryl esters to and accept triglycerides from Intralipid during incubation with CETP. However, during the initial stages of the incubation, the transfers of core lipids into and out of the phospholipid-depleted rHDL were decreased relative to those of unmodified rHDL. This observation can be explained in two ways.

As HDL-CETP interactions are mediated by the binding of CETP to phospholipid head groups (26), the reduced transfers of core lipids into and out of the phospholipid-depleted rHDL may simply reflect the smaller number of phospholipid head groups available for binding CETP. If this is the case the core lipid transfers should become indistinguishable from those of unmodified rHDL when the phospholipid-depleted rHDL acquire extra phospholipids. The data in Fig. 5 are consistent with this idea.

An alternative explanation for the reduced CETP-mediated transfers of core lipids between phospholipid-depleted rHDL and Intralipid is that a proportion of the remaining phospholipid head groups in the phospholipid-depleted rHDL are masked by non-lipid-associated domains of apoA-I and are unavailable for binding to CETP. If this were the case, core lipid transfers should increase if these domains reassociated with the rHDL surface. Reassociation of apoA-I domains may occur if a molecule of apoA-I dissociates from rHDL, thus increasing the surface area available for association of the remaining apoA-I with the rHDL surface. The results in Figs. 5 and 8, showing that the dissociation of apoA-I from the phospholipid-depleted rHDL coincides with the "normalization" of the transfers of core lipids between phospholipid-depleted rHDL and Intralipid, support this suggestion.

In conclusion, this is the first systematic investigation of the effects of phospholipid depletion on the size and structure of HDL and their remodeling by CETP. The results show that phospholipid depletion decreases rHDL size, decreases the CETP-mediated transfers of core lipids between rHDL and Intralipid, and enhances the CETP-mediated dissociation of lipid-free apoA-I from the particles. As earlier findings from this laboratory have demonstrated that the lipid-free apoA-I that dissociates from HDL can be recycled into new HDL particles (11), the

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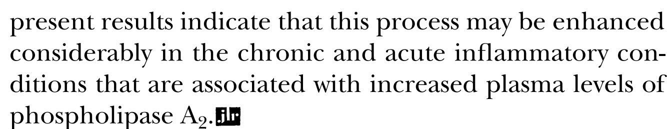

present results indicate that this process may be enhanced considerably in the chronic and acute inflammatory conditions that are associated with increased plasma levels of phospholipase A_2.

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