Probing structure and function of VLDL by synthetic amphipathic helical peptides

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Abstract Class A amphipathic helical peptides have been shown to mimic many properties of exchangeable apolipoproteins. The three analogs of the class A amphipathic peptides were used to probe the structure and function of human very low density lipoproteins (VLDL): *1)* 18 residue peptide possessing a single helical domain (18A) with the sequence Asp-Trp-Leu-Lys-Ala-Phe-Tyr-Asp-Lys-Val-Ala-Glu-Lys-Leu-Lys-Glu-Ala-Phe; *2)* two domains of 18A separated by a Pro (37pA); and *3)* an 18A analog with the end groups protected to increase helicity (Ac-18A-NH_2). Upon incubation of the peptides with VLDL at a peptide to VLDL (protein) ratio of 1:1, the 37pA and Ac-18A-NH_2 were able to displace most of apolipoprotein (apo) Cs and E from VLDL without alteration in its lipid composition and morphology while 18A had minimal effect. The extent of displacement was a function of the peptide to VLDL ratio. The rank order of displaceability of apolipoproteins on VLDL was apoE > C-III > C-II. The displacement of apoE and/or Cs from VLDL by peptides variably affected the ability of VLDL to interact with purified bovine milk lipoprotein lipase (LpL) and cultured macrophages. Treatment of VLDL with Ac-18A-NH_2 markedly lowered its reactivity to LpL and its ability to induce lipid accumulation in cultured macrophages; however, treatment of VLDL with 37pA or 18A only minimally lowered their abilities. Ac-18A-NH_2 treatment of VLDL resulted in the increase of apparent K_m and a decrease of V_{max} for lipoprotein lipase (LpL)-catalyzed hydrolysis of VLDL triglycerides. When an artificial triglyceride emulsion was used as a substrate of LpL, 37pA, but not Ac-18A-NH_2, activated LpL. The above data indicate that *1)* amphipathic helical peptides can alter the metabolic and functional properties of VLDL by dissociating the functionally important exchangeable apolipoproteins from VLDL as well as by acting as a functional element of VLDL after their incorporation; and *2)* the class A amphipathic peptides having different lipid-associating properties exert significantly different effect on VLDL function.—**Chung, B. H., M. N. Palgunachari, V. K. Mishra, C. H. Chang, J. P. Segrest, and G. M. Anantharamaiah.** Probing structure and function of VLDL by synthetic amphipathic helical peptides. *J. Lipid Res.* 1996. **37:** 1099–1112.

Supplementary key words apolipoproteins • lipolysis • macrophages

Circulating VLDL in human blood is known to be heterogeneous in size, composition, and metabolic properties (1). Human VLDL contains several exchangeable apolipoproteins (apoC-I, C-II, C-III, and E) and nonexchangeable apoB (2). The important features of the heterogeneity of VLDL may be associated with variation in the levels and/or conformation of exchangeable apolipoproteins on VLDL as these exchangeable apolipoproteins play an exceedingly important role in regulating the metabolism of VLDL (3–11). It is well established that apoC-II on VLDL surface acts as a specific protein cofactor necessary for the hydrolysis of triglycerides by LpL (3), while apoE on the VLDL surface acts as a specific ligand for the binding of VLDL and their remnants to the lipoprotein receptors of hepatic and nonhepatic tissues (4–8). There is evidence that apoC-III is an inhibitor of LpL (9, 10) and/or inhibits the apoE-mediated binding of VLDL and their remnants to lipoprotein receptors (5–7, 11). Although the specific roles of individual exchangeable apolipoproteins on VLDL function have been identified by studying the functional properties of purified individual apolipoproteins, little information is currently available regarding how these exchangeable apolipoproteins on native VLDL modulate the metabolic and functional properties of VLDL.

Exchangeable apolipoproteins contain tandem repeating units of the amphipathic α-helixes, the structural and functional units of these proteins (12). A number of investigators have previously synthesized the fragments of several exchangeable apolipoproteins and the peptide analogs of the amphipathic helix that acti-

Abbreviations: apo, apolipoproteins; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; LpL, lipoprotein lipase; LCAT, lecithin:cholesterol acyltransferase; FFA, free fatty acids; BSA, bovine serum albumin; DMPC, dimyristol phosphatidylcholine; SDS, sodium dodecyl sulfate; HPLC, high pressure liquid chromatography; EDTA, ethylenediamine tetraacetic acid.

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vate two key lipolytic enzymes in plasma, lecithin:cholesterol acyltransferase (LCAT) and LpL, to study the domains of apolipoproteins responsible for their binding to lipids and binding of lipoproteins to lipoprotein receptors on cells (13–23). We have previously synthesized an 18 residue amphipathic model peptide, 18A, that can bind to lipids, and subsequently made a number of modifications on this peptide to increase its lipid binding potency (15, 21). The peptide 18A is a mimic of class A amphipathic helix in which both C- and N-terminal ends are free (15). We found that a peptide formed by connecting two domains of 18A by a Pro residue (37pA) had an increased helical content and possessed enhanced lipid binding and biological properties compared to 18A; this modification was made to investigate the effect of possible co-operativity between the two 18A helical domains (15). Another modification of 18A involving blockage of both C- and N-terminal ends of 18A by amidation and acetylation, respectively, produced a peptide (Ac-18A-NH_2) with enhanced lipid binding and biological properties compared to 18A (21). The blockage of the end groups of 18A stabilizes the α-helix because it prevents unfavorable electrostatic repulsion between the helix macrodipole and the terminal charges in the helix (21). The helical content of Ac-18A-NH_2 in the presence of lipid is much higher than that of 18A (72% vs. 54%) but is similar to that of 37pA (73%) (21, 24). We have shown previously that 37pA can displace up to 80% of apoA-I from native high density lipoproteins without a detectable change in the level of apoA-II, while 18A can displace up to 40% of apoA-I (25). The displacement of apoA-I from HDL by 37pA was found to have no apparent effect on the ability of HDL to act as a substrate for LCAT (25). Although Ac-18A-NH_2 has the lipid-associating property similar to that of 37pA, whether the Ac-18A-NH_2 with amino acid residues half the size of 37pA can also displace exchangeable apolipoproteins from native lipoproteins and alter the biological properties of lipoproteins has not been examined.

In the present study, we have examined the ability of synthetic amphipathic helical peptides derived from a prototype amphipathic helical parent peptide (18A) to displace exchangeable apolipoproteins on VLDL and the effect of the displacement of native apolipoproteins by these peptides on the metabolic and functional properties of VLDL.

MATERIALS AND METHODS

Preparation of lipoproteins

Fresh type IV hyperlipidemic sera were obtained from the Alabama Regional Blood Center at Birmingham, AL. VLDL was isolated from hypertriglyceridemic plasma by ultracentrifuging plasma in a 50.2 Ti fixed-angle rotor at 45,000 rpm for 16 h. The isolated VLDL was washed once by a modified method of short single vertical spin density gradient ultracentrifugation (26). The modified density gradient ultracentrifugation involves formation of a two-step density gradient in Beckman SW 41 swingout rotor tubes, layering 4 ml density-adjusted VLDL (d 1.30 g/ml) under 7 ml normal saline, and subsequent ultracentrifugation of density gradient tubes in a SW 41 swingout rotor for 4 h at 40,000 rpm. The VLDL that floated to the top of the tubes was recovered and then dialyzed against Tris-buffered saline (0.01 M Tris–0.15 M NaCl, pH 7.4) for 16 h. The concentration of VLDL was determined by measuring the levels of proteins by the method of Lowry et al. (27) or the level of total cholesterol by using an enzymatic cholesterol assay kit from Boehringer Mannheim Co. (Kit No. 12408), Indianapolis, IN. In order to clarify the turbidity of the protein assay reagent containing VLDL, 20 μl of 10% SDS was added per ml of assay reagent.

Preparation of peptides

Peptides were synthesized by the solid phase method using an automated solid phase peptide synthesizer (Advanced ChemTech., Louisville, KY). Peptides 18A, 37pA, and Ac-18A-NH_2 were synthesized by using the *p*-alkoxylbenzyl alcohol resin or Rink amide resin support (Peptide International, Louisville, KY) (28). For the attachment of the first amino acid to a *p*-alkoxybenzyl alcohol resin support, 9-fluorenylmethoxycarbonyl amino acids were coupled to the resin in the presence of dicyclohexyl carbodiimide and 4-dimethylaminopyridine (29). For the synthesis of Ac-18A-NH_2, 9-fluorenylmethyloxycarbonyl protection from the rink amide resin was removed with piperidine in dimethyl formamide, 10 ml per gram of the resin for two cycles of 5 min and 15 min, and the first 9-fluorenylmethyloxycarbonyl amino acid was added using dicylohexyl carbodiimide and 1-hydroxybenzotriazole. The other protecting groups used for the side-chain functional amino acids were *t*-butyl ether for Tyr, *t*-butylester for Asp and Glu, and *t*-butyloxycarbonyl for Lys. After the removal of 9-fluorenylmethyloxycarbonyl group from the first amino acid-resin by using piperidine in dimethylformamide, three equivalents of the appropriate 9-fluorenylmethyloxycarbonyl amino acids were coupled in the presence of dicyclohexyl carbodiimide and 1-hydroxybenzotriazole. The coupling and deprotection steps during the synthesis were monitored by the Kaiser's test (30). To obtain Ac-18A-NH_2, the N-terminal amino acid was acetylated using dicyclohexyl carbodiimide–acetic acid in dichloromethane for 30 min. The peptide was cleaved from the resin using 80% tri-

fluoroacetic acid containing 5% phenol, 5% water, 5% thioanisole, and 5% 1,2-ethanedithiol. The cleavage mixtures were filtered, and the filtrate was concentrated by rotary evaporation. After precipitation of peptides by using cold ether, the crude peptides were dissolved in 6 M guanidine hydrochloride, dialyzed against distilled water using dialysis membranes of 1000 MW cut off (Spectrapor, Fisher Scientific), and subsequently lyophilized. The peptides were purified using a preparative C-4 reverse-phase HPLC column (22 mm inner diameter × 25 cm, particle size 10 μm) on a Beckman HPLC system using a gradient of 25% to 58% acetonitrile in water containing 0.1% (v/v) trifluoroacetic acid. The purity of the peptides was checked on analytical HPLC (Beckman System Gold 166) using a C-18 reverse-phase column (4.6 mm inner diameter × 25 cm) using a gradient of 25% to 75% acetonitrile in water containing 0.1% trifluoroacetic acid. The purity and authenticity of each peptide were ascertained by subjecting them to amino acid and mass spectral analyses (31). The concentrations of peptides were determined by the method of Lowry et al. (27). The molar concentration of peptide solution was also determined by measuring the molar extinction coefficients of tryptophan and tyrosine in peptides (32).

Treatment of VLDL with synthetic peptides

Synthetic peptides dissolved in Tris buffer were mixed with VLDL to give the peptide to VLDL-protein ratios ranging from 0.25 to 1.0. Control VLDL and the VLDL–peptide mixtures were then incubated at room temperature for 16 h. In order to examine the extent of incorporation of peptides into VLDL, peptides were radiolabeled with ^{125}I by the procedure of McFarlane (33), and the ^{125}I-labeled peptides, and/or unlabeled peptides were incubated with VLDL. For the preparative isolation of the peptide-treated VLDL, the incubated samples of control VLDL (5 mg protein) and mixtures of VLDL (5 mg) and peptides (5 mg) were subjected to density gradient ultracentrifugation to separate VLDL from peptide or displaced apolipoproteins. VLDL that floated to the top of the tubes and the displaced free apolipoproteins and/or unbound free peptides remaining at the bottom of density gradient tubes were quantitatively recovered by collecting the top 3 ml and the bottom 4 ml fractions of the density gradient tube. In tubes containing radiolabeled peptides, 19 fractions were collected from each tube by puncturing the bottom using a density gradient fractionator (Hoeffer Scientific Instrument, San Francisco, CA). The levels of radioactivity and cholesterol in the density gradient fractions were then measured. The VLDL and free protein fractions collected from the density gradient tubes were dialyzed by using dialysis membranes (3500 mol wt cut off) against Tris buffer to remove excess KBr from the samples. The volumes of all dialyzed free protein fractions were then adjusted to 5 ml. After standardization of VLDL based on total cholesterol levels and free protein fraction based on volume, the compositions of apolipoproteins on VLDL and apolipoproteins displaced by peptides were examined by SDS gradient gel (34) or urea gel (2) electrophoresis. An aliquot of the VLDL density fraction containing 100 μg total cholesterol, or 250 μl free protein, was taken and concentrated to a total volume of approximately 50 μl by using a speed vacuum evaporator for their analysis by SDS and urea gel electrophoresis. Changes in the electrophoretic mobility of the VLDL after peptide treatment were examined by agarose gel electrophoresis (35). The morphology of VLDL was examined by negative staining with 2% potassium phosphotungstate and examining the grid on a Phillips 400 transmission electron microscope.

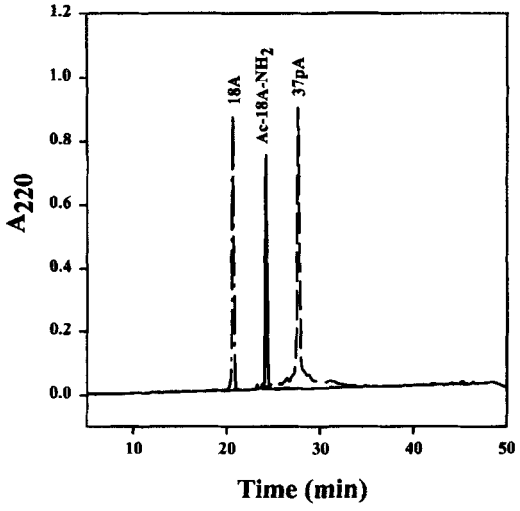

Fig. 1. Elution profiles of peptides from an analytical HPLC column.

Reactivity of control and peptide-treated VLDL to purified lipoprotein lipase

The reactivity of VLDL to the purified LpL was determined by measuring the rate and extent of hydrolysis of VLDL-triglycerides by purified milk LpL. The milk LpL was isolated and purified from bovine raw milk by the heparin–agarose affinity chromatographic method described by Iverius and Ostlund-Lindqvist (36). The specific activity of purified LpL was about 10 milliunits of lipolytic activity per μl, where 1 milliunit of enzyme activity is defined as the release of 1 nmol FFA per min at 37°C. Briefly, 5 μl of 1:10 diluted LpL was added to a 0.5 ml enzyme assay mixture containing control or peptide-treated VLDL (200 mg VLDL-triglycerides/dl)

TABLE 1. Mass spectral analysis of purified peptides

Peptides	Average Mass from 3 Estimates	Standard Deviation	Theoretical Mass
18A	2,200.69	± 0.73	2201.8
Ac-18A-NH_2	2,242.31	± 1.52	2242.8
37pA	4,480.76	± 1.10	4482.7

and fatty acid-poor bovine serum albumin (6%). The mixtures were incubated at 37°C for 120 min. Small aliquots (20 µl) of the samples in duplicate were taken at 0, 30, 60, 90, and 120 min of incubation and added to tubes containing 400 µl of chloroform–methanol 2:1 (v/v) to stop the enzyme reaction. After a vigorous vortexing followed by low speed centrifugation (1000 rpm for 10 min in a Sorvall low-speed centrifuge), a 200-µl aliquot of the clear solvent layer containing extracted lipids was taken from the bottom of the tubes into clean tubes. After evaporation of organic solvent, the levels of FFA in the tubes were assayed by an enzymatic method using the Boehringer Mannheim Reagent set #1082914. In order to determine the kinetics of LpL-catalyzed hydrolysis of triglycerides in control and peptide-treated VLDL, increasing concentrations of the control and peptide-treated VLDL (0.1–1.2 mM with respect to VLDL-triglycerides) were incubated with a constant amount of LpL (5 µl of 1:20 diluted LpL = 0.05 µg) at 37°C for 30 min. The enzyme reaction was terminated by extracting lipids using chloroform–methanol 2:1 (v/v). The assays were carried out in duplicate. Enzyme activity was calculated as µmol of free fatty acid released per min per mg enzyme proteins. Apparent K_m (Michaelis constant) and apparent V_{max} (maximum velocity) were determined by the Lineweaver-Burk plots of the data of enzyme velocities and substrate concentrations.

Effect of synthetic peptides on the hydrolysis of artificial lipid emulsion by LpL and pancreatic phospholipase

Effect of synthetic peptides on the hydrolytic activity of LpL and phospholipase were studied using artificial substrates. Briefly, an artificial substrate of LpL, glycerol-based emulsion of triglycerides, was prepared by mixing lecithin, glycerol, triolein, and fatty acid-poor albumin and sonicating the mixtures as described by Iverius and Ostlund-Lindqvist (36). The substrate of phospholipase, emulsions of lecithin–sodium deoxycholate–Triton X-100 containing bovine serum albumin, was purchased from Boehringer Mannheim Co., Indianapolis, IN. For the assay of LpL, 120-µl aliquots of lipid emulsion containing 36 µg lecithin, 300 µg triolein, and 10 mg BSA in 50 mM Tris and 150 mM NaCl, pH 8.5, were incubated with 8 µg synthetic peptides in the absence and presence of 0.6% normolipidemic serum. After 15 min preincubation, the enzyme reaction was started by the addition of 10 µl of 1:10 diluted LpL. For assay of phospholipase, 0.1 ml (5 mU) of purified porcine pancreatic phospholipase was added to a 0.5-ml aliquot of lecithin emulsion that had been preincubated with or without synthetic peptides for 15 min. A 20-µl aliquot (in duplicate) of the reaction mixture of LpL and phospholipase was then taken at 0, 15, 20, 30, 40, and 60 min of incubation, and the enzyme reaction was terminated by extracting lipids with chloroform–methanol 2:1 (v/v) or by adding 10 µl enzyme (phospholipase) stop reagent (12 mM EDTA, 50 mM phosphate buffer, pH 6.5). The amount of FFA released from triglyceride emulsion by LpL or from lecithin by phospholipase was then measured by using an enzymatic FFA assay kit (Boehringer Mannheim Co., kit No. 2082914).

Interaction of control and peptide-treated VLDL with cultured macrophages

To study the ability of control and peptide-treated VLDL to induce lipid accumulation in cultured cells, THP-1 monocyte-derived macrophages were interacted with control and peptide-treated VLDL. To prepare the THP-1 monocyte-derived macrophages, suspension cultures of THP-1 monocytes, kindly provided by Dr. Gianturco, University of Alabama at Birmingham, were grown in an RPMI 1640 medium containing 10% fetal bovine serum. Approximately 5–6 × 10^6 monocytes were then added into 6-well cell culture plates. Differentiation of monocytes into macrophages was induced by adding phorbol ester (phorbol-12-myristate-13 acetate, Sigma Chemical Co., St. Louis, MO) at 50 ng/ml culture medium and then incubating cells for 24 h. After removal of non-adherent cells by replacing the culture medium containing phorbol ester with fresh RPMI medium containing 10% fetal bovine serum, dishes were incubated until day 4 when the culture medium was replaced by RPMI 1640 containing 10% serum replacement CPSR-1 medium (Sigma Chemical Co.). After 24 h incubation, the culture medium was replaced by serum-free culture medium (RPMI 1640) containing 200 µg (cholesterol) of control and peptide-treated VLDL. The culture dishes were then incubated in a cell culture incubator for 24 h. The culture medium was then aspirated and the macrophages were washed 4 × with phosphate-buffered saline (0.05 M sodium phosphate, 0.15 M NaCl, pH 7.4). Cellular lipids were then extracted two times with 2 ml hexane–isopropanol 3:2 and once with hexane. The solvent extracts were pooled and aliquoted into two portions (1.5 ml and 4.5 ml) in order to assay cellular triglycerides and cholesterol. After the evapora-

tion of the solvent, the levels of triglycerides and cholesterol were measured by enzymatic assay kits (Boehringer Mannheim reagent sets #34292 and #12408). Cellular proteins were dissolved in 2 ml 0.1 N NaOH, and 0.1–0.2 ml aliquots were taken to assay the level of cellular proteins by the method of Lowry et al. (27).

RESULTS

Purification and purity of peptides

Figure 1 shows the elution profiles of 18A, Ac-18A-NH_2, and 37pA from an analytical reverse-phase HPLC column. The 18A, Ac-18A-NH_2, and 37pA were eluted from the HPLC column with retention times of 20.5 min, 24.2 min, and 27.7 min, respectively. The HPLC purification step removes all salts and other impurities associated with peptides. The mass spectral analysis of purified peptides showed that the masses of each of the purified peptides were identical to the theoretical masses of the corresponding peptides (**Table 1**).

Displacement of exchangeable apolipoproteins of VLDL by synthetic amphipathic helical peptides

In order to determine the ability of synthetic helical peptides having different lipid affinity (15, 21, 24) to displace exchangeable apolipoproteins from VLDL, incorporation of synthetic peptides into VLDL and dissociation of apolipoproteins from VLDL were compared after the incubation of VLDL and peptides. **Figure 2** shows the distribution of ^{125}I-labeled peptide (Ac-18A-NH_2) and VLDL cholesterol among density fractions obtained after density gradient ultracentrifugation of control VLDL, control peptide, and the mixture of VLDL and the peptide. The control VLDL, which was loaded at the bottom of the ultracentrifuge tube, floated to the top of the tube while the control peptide remained at the bottom of the tube (Fig. 2, top). Virtually all of the VLDL cholesterol and about 60% of radiolabeled peptide were recovered at the top of the tube after density gradient ultracentrifugation of the mixture containing VLDL and peptide with a ratio of 1:1; no detectable peak of cholesterol or radioactivity was found in the intermediate density regions of the density gradient tubes (Fig. 2, bottom). Further analysis on radiolabeled 18A and 37pA incubated with VLDL showed that 78% of 37pA and 38% of 18A were incorporated onto VLDL (**Table 2**). It should be noted that the amount of 37pA incorporated onto VLDL was about twofold more than that of 18A (78% vs. 38%). As 37pA consists of two domains of 18A, the moles of 18A and 37pA incorporated onto VLDL were about equal; moles of Ac-18A-NH_2 incorporated onto VLDL were greater than that of either 18A or 37pA (Table 2). The extent of incorporation of radiolabeled peptide (Ac-18A-NH_2) onto VLDL increased as the concentration of unlabeled peptide was lowered; when VLDL was incubated with only a trace amount of radiolabeled peptides, > 95% of peptides were incorporated onto VLDL (Table 2).

Figure 3A shows the SDS gradient gel electrophoregrams of top VLDL density and bottom lipid-free protein fractions obtained from the density gradient tubes containing control VLDL and incubation mixtures of VLDL and various peptides. The control VLDL, which was incubated without peptides, contained gel bands of apoB-100, albumin, apoE, and apoCs (Fig. 3A, right,

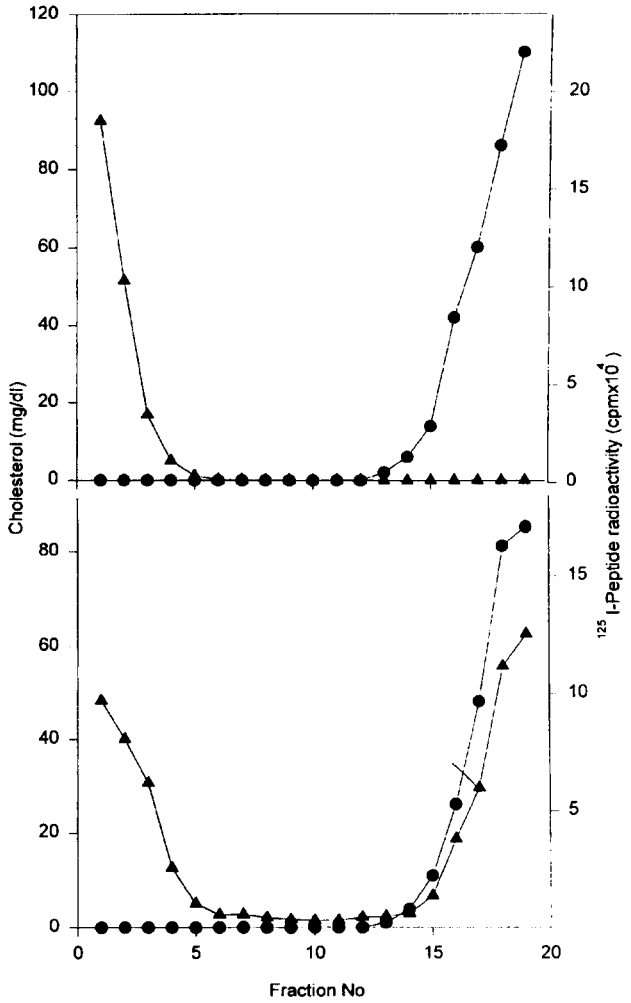

Fig. 2. Distribution of radioactivity of ^{125}I-labeled peptide (Ac-18A-NH_2) and VLDL cholesterol in the density gradient fractions obtained after density gradient ultracentrifugation of control VLDL and peptides (top) and mixtures of VLDL and peptides (bottom). Control VLDL (0.5 mg), control radiolabeled peptides (0.5 mg), and mixtures containing 0.5 mg VLDL and 0.5 mg radiolabeled peptides were subjected to the density gradient ultracentrifugation. After downward fractionation of the samples in the tubes, the levels of radioactivity (▲-▲) and cholesterol (●-●) in each gradient fraction were measured.

TABLE 2. Distribution of radioactivity of ^{125}I-labeled peptides incubated with or without VLDL (0.5 mg protein) among density gradient fractions

Peptides in the Incubation Mixtures			Distribution of ^{125}I-Radioactivity		
^{125}I-Labeled Peptides (trace amount)	Unlabled Peptides	Peptides Conc	Bottom Fraction (Tubes 1–5)	Middle Fraction (Tubes 11–14)	Top Fraction (Tubes 15–19)
		mg		*% of total*	
Ac-18A-NH_2	Ac-18A-NH_2	0.50	38.1	2.1	59.8
18A	18A	0.50	60.7	1.2	38.1
37pA	37pA	0.50	20.5	1.4	78.1
Ac-18A-NH_2	Ac-18A-NH2	0.25	11.9	2.6	85.5
Ac-18A-NH_2	Ac-18A-NH_2	0.125	7.6	1.8	90.6
Ac-18A-NH_2	Ac-18A-NH_2	0	3.2	1.4	95.4
Ac-18A-NH_2	18A	0.445[a]	14.6	3.2	82.2
Ac-18A-NH_2	37pA	0.51	21.1	2.8	76.1
Ac-18A-NH_2	37pA	1.02[a]	39.8	2.1	58.2

Values are a mean of two determinations.

[a]As molecular weights of 18A, Ac-18A-NH_2, and 37pA are 2,200, 2,243, and 4,580, respectively, 0.50 mg of Ac-18A, 0.445 mg of 18A, and 1.02 mg of 37pA are all 0.223 μmol of peptides.

lane 1). The VLDL incubated with Ac-18A-NH_2 contained no detectable apoE and apoCs (Fig. 3A, right, lane 4) while the VLDL incubated with 18A and 37pA contained a small amount of apoCs but no detectable apoE (Fig. 3A, right, lanes 2 and 3). ApoE and/or Cs were detected in the lipid-free protein fractions of all the peptide-treated VLDL samples but not in the control VLDL sample (Fig. 3A, left). It should be noticed that the albumin band is detectable in control VLDL but not in peptide-treated VLDL (Fig. 3A, right). These data suggest that amphipathic helical peptides enhanced the dissociation of albumin from VLDL. Although small molecular weight peptides entered into the gels are not readily fixable, the presence of peptides banded below apoCs in gels of both VLDL and free protein fractions is evident (Fig. 3A).

Further study showed that the extent of displacement of apoCs and E by peptides is a function of peptide to VLDL ratio in the incubation mixture (Fig. 3B). ApoE was completely displaced from VLDL by peptides at a peptide to VLDL ratio of 0.25:1 (Fig. 3B, lanes 1–2), while the complete displacement of apoCs occurred at a peptide to VLDL ratio of 1:1 (Fig. 3B, lane 4). The control VLDL (lane 1), incubated at room temperature for 16 h, contained numerous faint protein bands just below apoB-100 (Fig. 3B). The VLDL incubated with Ac-18A-NH_2 at peptide to VLDL ratio of 1:1 contained an increased number of intense protein bands below the apoB-100 and a less intense apoB-100 band than those on the control VLDL (Fig. 3B, lanes 1 and 4). It has been shown that VLDL isolated from hypertriglyceridemic serum contains variable amounts of serine protease, and that the proteolytic degradation of apoB occurs during storage of VLDL when proteinase inhibitor is not included in the sample (37). As we have not included proteinase inhibitors in the incubating sample, the numerous bands just below apoB may likely be the proteolytic degradation products of apoB-100.

The relative levels of individual C apolipoproteins in control and peptide-treated VLDL were further examined by urea gel electrophoresis and a subsequent densitometric scanning of the gels (Fig. 3C). Although the VLDL incubated with Ac-18A-NH_2 or 37pA at a VLDL to peptide ratio of 1:1 had little or no detectable apoCs on VLDL (Fig. 3C, scans B and C), the gel scan of VLDL incubated with 18A showed that the relative ratio of apoC-II to apoC-III in the peptide-treated VLDL was much higher than that in control VLDL (Fig. 3C, scans A and D), indicating that apoC-IIIs are more readily displaceable by peptides than apoC-II. The greater dissociation of apoC-IIIs than apoC-II from VLDL by peptides is also evident in the gels of another preparation of VLDL incubated with 37pA at a VLDL (proteins) to peptide ratio of 0.25:1. (Fig. 3C, scans E and F). The presence of 37pA incorporated onto VLDL at the left shoulder of apoC-III_1 peak is evident (scan F of Fig. 3C).

In order to further compare the relative potencies of 18A, 37pA, and Ac-18-NH_2 to displace exchangeable apolipoproteins from VLDL, the extent of association of radiolabeled Ac-18-NH_2 to VLDL in the presence of the excess various unlabeled peptides was examined (Table 2). As Table 2 shows, the association of radiolabeled Ac-18A-NH_2 to VLDL can be inhibited by the presence of an excess of all unlabeled peptides. The association of radiolabeled Ac-18A-NH_2 to VLDL was somewhat more effectively inhibited by unlabeled Ac-18A-NH_2 than by 37pA when VLDL was incubated with an equal mass of peptides (Table 2). When VLDL was

incubated with equal molar amounts of unlabeled peptides, the Ac-18A-NH_2 was less effective than 37pA (Table 2). The unlabeled 18A was least effective in inhibiting the association of radiolabeled Ac-18A-NH_2 to VLDL (Table 2).

Figure 4 left nm showed that the treatment of VLDL with peptides decreased the electrophoretic mobility of VLDL. The above changes are likely due to the displacement of anionic apoCs and E on VLDL by neutral synthetic peptides. Electron micrographic examination revealed that displacement of exchangeable apolipoproteins on VLDL by amphipathic peptides had no apparent effect on the morphology of VLDL (Fig. 4 right) although there were some changes in average sizes of VLDL. The VLDL treated with peptides was somewhat smaller in size than that of control VLDL; the average sizes of control VLDL, 18A-, 37pA-, and Ac-18A-NH_2-treated VLDL were 369 ± 100 Å, 345 ± 96 Å, 338 ± 74 Å, and 330 ± 91 Å, respectively (n = 100 particles).

Effect of the displacement of apoCs and/or E on VLDL by synthetic peptides on the reactivity of VLDL to purified LpL

To study whether the displacement of apoC-II, an activator of LpL, from VLDL by synthetic peptides affects the ability of VLDL to serve as a substrate for LpL, the reactivities of control VLDL and peptide-treated VLDL to purified bovine milk LpL were compared. Treatment of VLDL with Ac-18A-NH_2 markedly lowered the initial velocity of hydrolysis of triglycerides in VLDL by LpL, while the treatment of VLDL with 18A or 37pA peptides minimally affected its initial velocity (**Fig. 5A**). The kinetics of LpL activity on control and Ac-18A-NH_2-treated VLDL were further compared after incubation of increasing concentrations of control VLDL and Ac-18A-NH_2-treated VLDL with a constant amount of LpL for 30 min. The double reciprocal plots of enzyme activity (1/v) versus substrate (VLDL-triglyceride) concentration (1/s) resulted in straight lines indicating that the hydrolysis followed Michaelis-Menten kinetics (Fig. 5B). The kinetic parameters, apparent V_{max} and apparent K_m, were calculated from the axial intercepts of the Lineweaver-Burk plot. The displacement of exchangeable apolipoproteins on VLDL by Ac-18A-NH_2 caused a marked increase of apparent K_m (from 0.29 to 1.05 mM) and a decrease of apparent V_{max} (from 83 to 32 μM FFA/min/mg LpL) for LpL-catalyzed hydrolysis of VLDL-triglycerides. The LpL reactivity of Ac-18A-NH_2-treated VLDL, defined as the ratio of V_{max} to K_m is thus markedly (9 ×) lower than that of control VLDL (Fig. 5B).

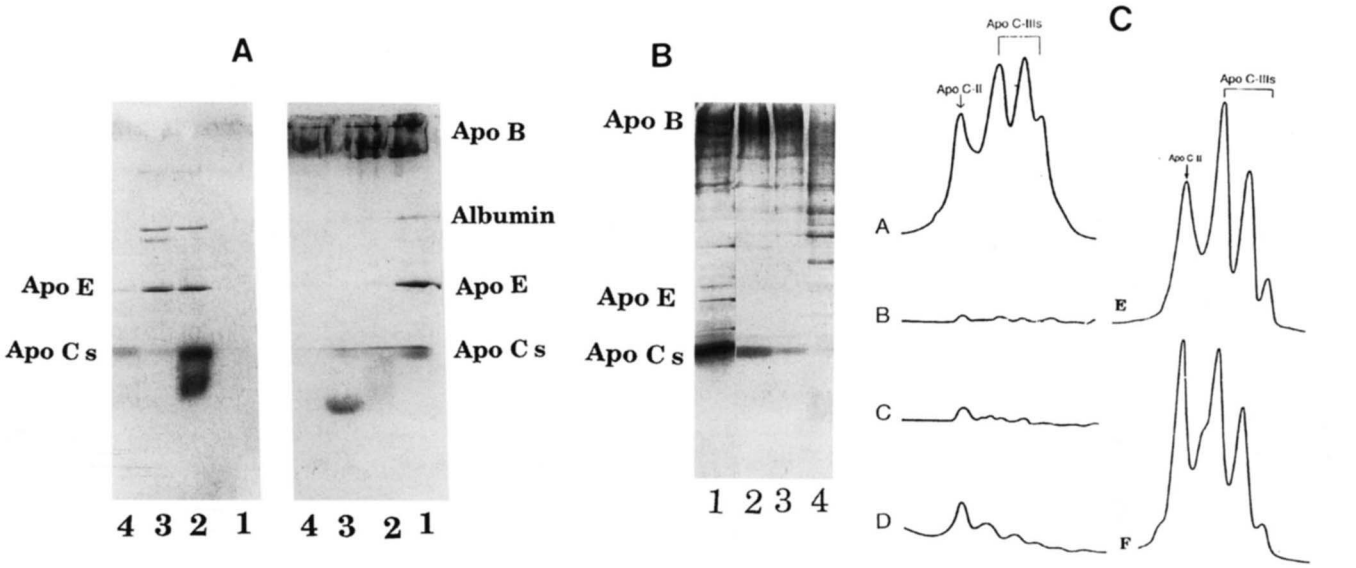

Fig. 3. A, Left: SDS gradient gel electrophoregrams of control and peptide-treated VLDL and apolipoproteins displaced (dissociated) from VLDL by synthetic peptides. Control VLDL (1) and mixtures of VLDL and 18A (2), 37pA (3) or Ac-18-NH_2 (4) were subjected to short spin density gradient ultracentrifugation to separate VLDL from free proteins. Apolipoprotein patterns of the VLDL fraction (right) and the free protein fraction (left) were then analyzed by SDS gradient gel electrophoresis after standardization of VLDL based on cholesterol level and of free protein fractions based on volume. B, Middle: SDS gradient gel electrophoregrams of control VLDL and VLDL modified by an increasing amount of Ac-18A-NH_2. One mg control VLDL (1) was treated with 0.25 mg (2), 0.5 mg (3), and 1 mg (4) of Ac-18A-NH_2. Standardization for gels of VLDL was based on VLDL cholesterol level. C, Right: Densitometric scans of urea gels of control VLDL and peptide-treated VLDL. Scans A–D are control VLDL (A) or VLDL incubated with an equal amount of Ac-18A-NH_2 (B), 37pA (C), or 18A (D). Scans E and F are another preparation of control VLDL (D) and VLDL incubated with 37pA at the VLDL to peptide ratio = 1:0.25 (F). Standardization of the VLDL for gel scans A–D was made based on the level of cholesterol content and of the VLDL and for gel scans E and F was made based on the content of tetramethylurea-soluble proteins.

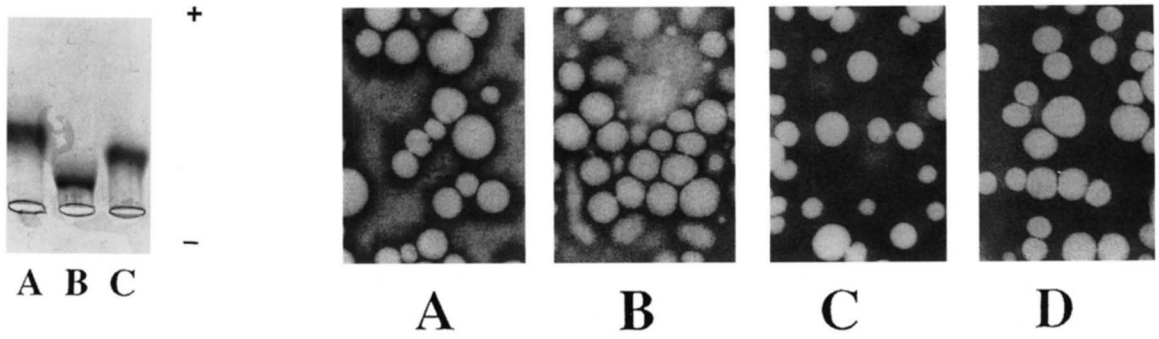

Fig. 4. Left: Electrophoretic mobility of VLDL modified by synthetic peptides. One mg control VLDL (A) was treated with 1 mg 37pA (B) or Ac-18A-NH_2 (C). Right: Electron micrographs of control VLDL (A) and VLDL treated with Ac-18A-NH_2 (B), 37pA (C), or 18A (D).

To determine whether lowering of reactivity of VLDL to LpL after treatment with Ac-18A-NH_2 is due to the lack of apoC-II on VLDL, we examined the effect of including the displaced apolipoproteins in the lipolysis assay mixtures on the extent of hydrolysis of triglycerides in peptide-treated VLDL by LpL. As Fig. 5C shows, the inclusion of the displaced apolipoproteins in the lipolysis mixture somewhat enhanced the hydrolysis of TG in peptide-treated VLDL, but the extent of hydrolysis of VLDL-TG in this lipolysis mixture was much less than that of control VLDL. These data suggest that *1)* Ac-18A-NH_2 incorporated onto VLDL surface inhibits interaction of apoC-II with LpL; *2)* the full activation of LpL by apoC-II may require the apoC-II bound to VLDL surface; or *3)* the presence of apoC-II unbound to VLDL may not be sufficient for a full activation of LpL.

As the displacement of most of apoC-II from VLDL by 37pA minimally affected the reactivity of VLDL to LpL, we further examined whether 37pA incorporated onto VLDL can serve as an activator of LpL. As **Fig. 6A** shows, 37pA added to an artificial LpL substrate (serum-free triolein emulsion) can stimulate the hydrolysis of emulsion triglycerides to the same extent as that activated by apoC-II in diluted serum (Fig. 6A, left), indicating that 37pA can act as an activator of LpL. The 37pA added to the artificial LpL substrate containing diluted serum further increased the extent of hydrolysis of emulsion TG (Fig. 6A, right), indicating that 37pA has an additive effect on the activation of LpL. The Ac-18A-NH_2 added to lipid emulsion failed to activate LpL; the hydrolysis of triglycerides in emulsion containing diluted serum was inhibited by the presence of Ac-18A-NH_2 (Fig. 6A, left and right).

To further examine whether the inhibition of LpL activity by Ac-18A-NH_2 was a result of dissociating an activator of LpL (apoC-II) from the substrate or due to the modification or inactivation of the enzyme by peptides, we studied the effect of adding synthetic peptides on the activity of another lipolytic enzyme (pancreatic phospholipase) which does not require a cofactor for its activity. The Ac-18A-NH_2 added to phosphatidylcholine emulsion had no apparent inhibitory effect on the hydrolysis of phosphatidylcholine by the pancreatic phospholipase; the 37pA added had some stimulatory effect on the activity of pancreatic phospholipase (Fig. 6B).

Effect of the displacement of apoCs and/or E from VLDL by synthetic peptides on the ability of VLDL to induce lipid accumulation in cultured macrophages

The ability of control VLDL and peptide-treated VLDL to induce cellular lipid accumulation was studied in THP-1 monocyte-derived macrophages. The control VLDL added to the culture medium caused about a 10-fold increase in the level of cellular triglycerides and 1.5-fold increase in the level of cellular cholesterol (**Fig. 7**). The addition of an equal amount of Ac-18A-NH_2-treated VLDL into the culture medium caused only a minimal (2-fold) increase in the cellular triglyceride level with virtually no change in the cellular cholesterol level (Fig. 7). The levels of TG and/or cholesterol in cells incubated with 37pA-treated VLDL were much less than those of cells incubated with control VLDL but were more than those of cells incubated with Ac-18A-NH_2 (Fig. 7). The 18A-treated VLDL had virtually the same ability to induce cellular triglyceride and cholesterol accumulation when compared with the control VLDL (Fig. 7).

DISCUSSION

The present study demonstrated that the abilities of amphipathic helical peptides to displace native exchangeable apolipoproteins from VLDL are directly related to their lipid-associating ability. The displacement of exchangeable apolipoproteins by amphipathic peptides is likely due to the fact that the lipid-associating properties of peptides are greater than that of native

apolipoproteins. Displacement of apoA-I from the HDL surface by apoA-II, which has higher lipid-associating properties than apoA-I, is well documented (38). We have reported previously that the rank order of the ability of several synthetic amphipathic peptide analogs, including 18A and 37pA, to displace apoA-I from HDL is related to the rank order of lipid affinity and percent α-helicity of each peptide (15, 24). The α-helicity of Ac-18A-NH_2 in the presence of DMPC is very similar to that of 37pA (24, 39). Thus, these two peptides have similar ability to displace apoCs and E from VLDL (Fig. 3). We have reported earlier that the lipid-associating abilities of peptides depend on the cooperative binding to arrange themselves in an antiparallel fashion (40, 41). This cooperative binding increases with the increased helicity. As the α-helicity and the lipid-associating property of Ac-18A-NH_2 are similar to those of 37pA (21, 24), it is not surprising that Ac-18A-NH_2 and 37pA are equally effective in displacing the apoCs and E from VLDL in spite of the fact that Ac-18A-NH_2 is only half the size of 37pA. We have reported that Ac-18A-NH_2 has higher helical content and lipid-associating properties than 18A (21). Thus, the Ac-18A-NH_2 is more effective than 18A in displacing exchangeable apolipoproteins from VLDL (Fig. 3). This study revealed that a simple change, such as protection of N- and C-terminus in peptide to increase the helicity, could have a profound effect on the properties of peptide to alter structure and function of VLDL.

The present study demonstrates that exchangeable apolipoproteins associate with VLDL with different binding affinity. The apolipoprotein compositional data of control and peptide-treated VLDL suggest that apoE is bound to VLDL with a lower affinity than apoCs, while apoC-II bound to VLDL with higher affinity than apoC-III. Weisgraber et al. (42) have shown recently that apoC-I and C-II, but not apoC-III, are capable of displacing apoE on β-VLDL. The greater displaceability of apoE than apoC-II from VLDL by synthetic peptides is compatible with their data that apoC-II can displace apoE.

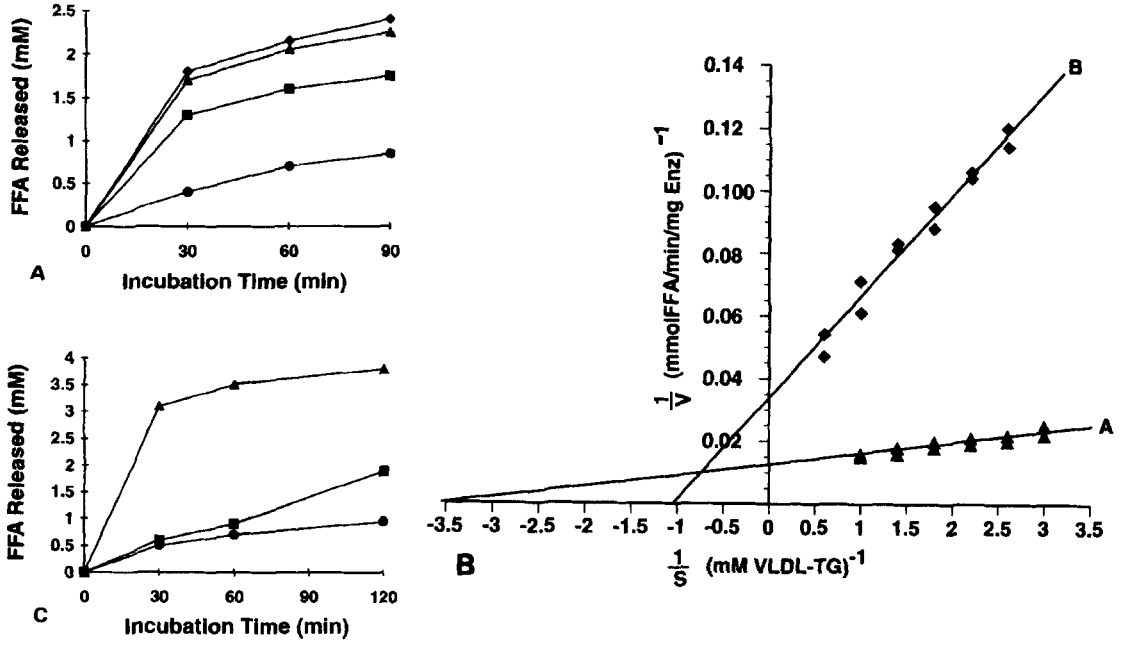

Fig. 5. A: Time-dependent release of free fatty acids from control VLDL and VLDL modified by various peptides during their incubation with purified bovine milk LpL. Control VLDL (◆-◆) and VLDL treated with 18A (▲-▲), 37pA (■-■), or Ac-18A-NH_2 (●-●) were incubated with purified bovine milk LpL and aliquots of sample (in duplicate) were taken at indicated times to measure the levels of FFA released. B: Kinetics of hydrolysis of control and Ac-18A-NH_2-treated VLDL-TG by lipoprotein lipase. The Lineweaver-Burk plots of control VLDL (A) and Ac-18A-NH_2-treated VLDL (B) were obtained after incubation of increasing amounts of control and Ac-18A-NH_2-treated VLDL-TG, in duplicate, with a constant amount (0.05 μg) of LpL. Values for V_{max} (μmol FFA/min per mg LpL) and K_m (mM) for control VLDL and Ac-18A-NH_2-treated VLDL were 83 and 0.29 (plot A) and 32 and 1.05 (plot B), respectively. C: Time-dependent release of free fatty acids during incubation of LpL with control VLDL and peptide-modified VLDL with or without removal of displaced apolipoproteins. One mg control VLDL was incubated with 1 mg Ac-18A-NH_2 and then the mixture was aliquoted into two portions. One portion of the sample was subjected to density gradient ultracentrifugation to separate VLDL from peptides and/or displaced apolipoproteins. Control VLDL (▲-▲), unfractionated VLDL and peptide mixtures (■-■), and VLDL separated from displaced apolipoprotein (●-●) were then incubated with purified LpL. Each value is a mean of duplicated determinations.

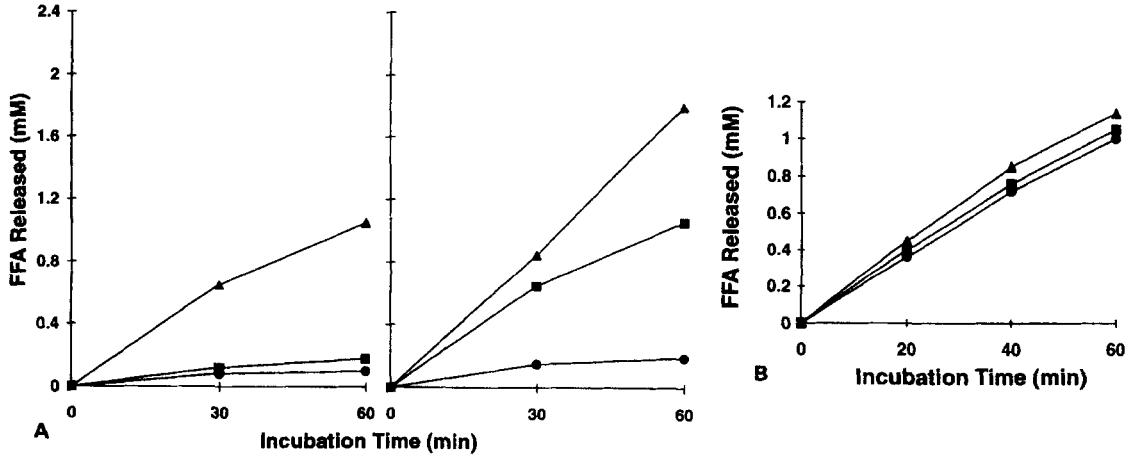

Fig. 6. A: Effect of amphipathic helical peptides on the hydrolysis of artificial lipid emulsion-triglycerides by LpL. An artificial LpL substrate (sonicated mixtures of triolein, glycerol, lecithin, and albumin) supplemented with (right) and without (left) diluted serum (0.6%) was incubated with bovine milk LpL in the absence of peptides (■-■) or in the presence of 37pA (▲-▲) or Ac-18A-NH_2 (●-●). Each value is a mean of duplicated determinations. B: Effect of amphipathic helical peptides on hydrolytic activity of pancreatic phospholipase. Phospholipid emulsion was incubated with pancreatic phospholipase in the absence of peptides (■-■) or in the presence of 37pA (▲-▲) or Ac-18A-NH_2 (●-●). Each value is a mean of duplicated determinations.

Although we have not determined the stoichiometry of displacing exchangeable apolipoproteins by amphipathic helical peptides, the peptides would be more efficient in associating with the VLDL compared to the exchangeable apolipoproteins of VLDL as exchangeable apolipoproteins of VLDL are known to contain several regions that are not associated with the phospholipid surface (12).

A number of studies have previously shown that the isolation of VLDL and LDL from certain serum accompanies the co-isolation of proteinases, where these proteinases can cause the degradation of apoB and/or apoE on these lipoprotein particles (35, 43). We observed that control VLDL, isolated from hypertriglyceridemic serum, contained some fragmented apoB after its incubation at room temperature for 16 h (Fig. 1B). The levels of fragmented apoB increased markedly when Ac-18A-NH_2 was included during the incubation of VLDL (Fig. 1B). These data indicate that displacement of exchangeable apolipoproteins by amphipathic helical peptides may increase the susceptibility of apoB to proteolysis by endogenous enzymes on VLDL. Yang et al. (44) have shown recently that phospholipid liposome-mediated removal of apoCs from VLDL affect the accessibility of apoB-100 to the cleavage by thrombin and epitope of monoclonal antibody for apoB. We have shown previously that lipolysis-induced alteration in the structure of VLDL increases the susceptibility of apoB to degradation by proteinases co-isolated with VLDL (45). Our present data support the hypothesis that dissociation of exchangeable apolipoproteins from VLDL alters the accessibility of VLDL apoB to proteolytic enzymes.

It is well known that under normal metabolic process, the loss of apoCs and apoE from VLDL requires extensive modification of VLDL core and/or surface lipids by lipolytic enzymes, and this structural modification alters the metabolic and functional properties of VLDL (1). The effect of dissociating apoCs and/or E without alterations in lipid composition of VLDL on the metabolic and functional properties of VLDL has not been examined previously. Our data show that the displacement of exchangeable apoCs and/or E by synthetic amphipathic peptides has variably affected the functions of VLDL to interact with LpL and cultured macrophages (Figs. 5 and 7). It is difficult to determine whether these functional changes are due to the removal of functionally important exchangeable apolipoproteins or the addition of peptides onto VLDL surface. The decrease in LpL reactivity of VLDL after its treatment with Ac-18A-NH_2 could be a result of the depletion of apoC-II, an activator of LpL, from VLDL and/or of inhibitory effect of the incorporated peptide. Kinetic analysis on the hydrolysis of VLDL-triglycerides by LpL showed that treatment of VLDL with Ac-18A-NH_2 caused a decrease in the V_{max} and an increase in the apparent K_m for LpL (Fig. 3B). Haberbosch et al. (46) have shown that triglyceride-rich lipoproteins (VLDL and chylomicrons) isolated from apoC-II-deficient patients have lower V_{max} and higher K_m for LpL-catalyzed hydrolysis of triglycerides in triglyceride-rich lipoproteins than those from normal subjects. Other studies (47, 48) using artificial lipid emulsion have shown that the deficiency of cofactor (apoC-II) resulted in an increase of apparent K_m and decrease of V_{max} of LpL. The change in the kinetics of

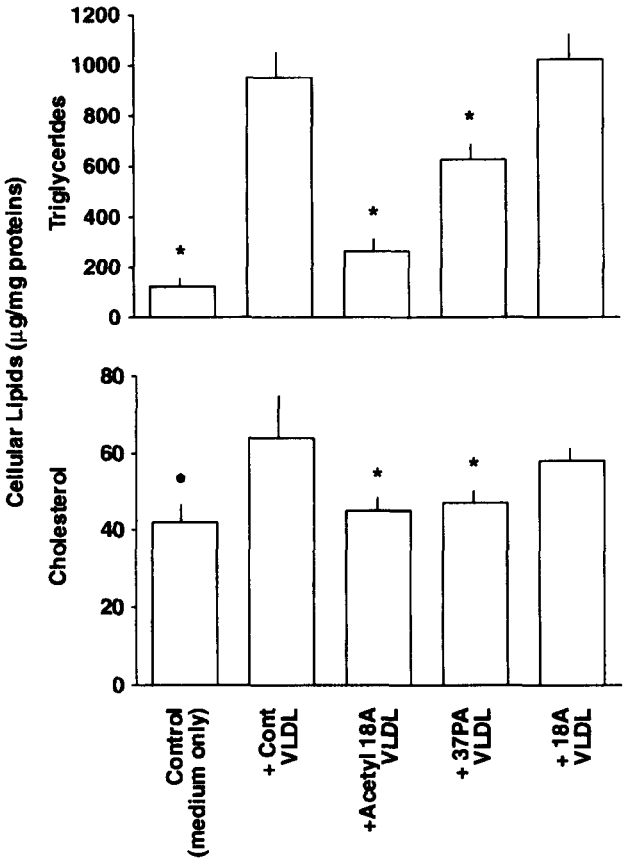

Fig. 7. Cellular lipid levels of THP-1 monocyte-derived macrophages incubated with control VLDL or VLDL treated with various peptides. Cellular triglyceride and cholesterol levels were determined after incubation of duplicated cells with medium only (control), medium plus control VLDL or VLDL treated with 18A, 37pA, or Ac-18A-NH_2 (n = 3); *different from cells incubated with control VLDL at the 95% confidence level.

VLDL to interact with LpL after the displacement of exchangeable apolipoproteins by Ac-18A-NH_2 (Fig. 3B) is similar to the kinetic changes that occurred during deficiency of apoC-II (46–48).

A number of in vitro studies have shown that native apoC-III and/or synthetic fragment of apoC-III added to VLDL and/or lipid emulsion can inhibit lipolysis by acting as an inhibitor of LpL (9, 10, 20). The kinetic analysis of this inhibition indicated that apoC-III acts as a noncompetitive inhibitor of LpL, and thus, the inhibitory effect of apoC-III cannot be reversed by apoC-II (9, 10). We observed that the lowering of LpL reactivity of Ac-18A-NH_2-treated VLDL can be partially restored by adding back the displaced apoC-II into the lipolysis mixture (Fig. 5C). Although the above enzyme kinetic data postulate that the decrease in LpL reactivity of VLDL after its treatment by Ac-18A-NH_2 is due to the depletion of apoC-II from VLDL particles, the possibility of Ac-18A-NH_2 acting as an enzyme inhibitor cannot be ruled out.

In contrast to Ac-18A-NH_2-treated VLDL, the treatment of VLDL with 37pA has only a minimal effect on the reactivity of the VLDL to LpL, although most of the apoCs and E on VLDL were displaced by peptides (Fig. 5A). The minimal change in LpL reactivity of 37pA-treated VLDL, which is deficient in apoC-II, is likely due to the effect of the incorporation of 37pA onto VLDL. As 37pA can activate LpL (Fig. 6A), the displacement of apoC-II by 37pA is not expected to affect the reactivity of VLDL to LpL. The mechanism whereby 37pA activates LpL is not known. ApoC-II has been postulated to contain three functional domains that are necessary to activate LpL (49). These are the lipid-binding domains, the LpL-activating domains, and LpL-binding domains. The lipid-binding domains (residues 13–22, 29–40, and 43–52) have been identified as amphipathic α-helical sequences, and the LpL activation domain consists of residues 56–67 (Ser-Thr-Ala-Ala-Met-Ser-Thr-Tyr-Thr-Gly-Ile-Phe) (49). The synthetic peptides containing both the LpL-activating domain and the lipid-binding domain have been shown to activate LpL as effectively as the whole apoC-II (50). The 37pA contains the sequences that can form amphipathic α-helix, but has no sequence homology to the LpL-activating domains of apoC-II. Further studies remain to be done to elucidate the structural features of 37pA that enable the activation of LpL.

It is well established that apoE is the major ligand responsible for the binding of hypertriglyceridemic VLDL to the lipoprotein receptors on macrophages and other cells (51, 52). In vitro studies have shown that enrichment of VLDL or LDL with apoE can increase cellular metabolism of these lipoproteins (11), but the effect of depleting apoE from VLDL on the metabolic properties of VLDL is not known. We found in this study that the displacement of apoE by various synthetic amphipathic helical peptides variably lowered the ability of VLDL to induce foam cell formation in cultured macrophages (Fig. 7). Displacement of apoE and/or apoCs by Ac-18A-NH_2 markedly suppressed the ability of VLDL to induce cellular triglyceride and cholesterol accumulation, while the displacement by 18A or 37pA had only a minimal effect (Fig. 7). As binding kinetics of VLDL to macrophages was not determined in the present study, it is not known whether the peptide-induced change in potencies of VLDL to induce foam cell formation in cultured macrophages is due to changes in the extent of apoE receptor-mediated uptake of VLDL. Our data show that the 18A-treated VLDL contained no detectable apoE but had an ability similar to that of control VLDL to induce the lipid accumulation in cultured macrophages (Figs. 3A and 7). The above data thus suggest that apoE may not be required for the VLDL to induce cellular lipid accumulation. The ability

of control or peptide-treated VLDL to induce cellular triglyceride and/or cholesterol accumulation related closely to the ability of VLDL to interact with LpL rather than the levels of exchangeable apoproteins displaced from VLDL by synthetic peptides (Figs. 3A, 5A and 7).

A number of studies have shown that FFA can induce TG accumulation in a variety of cultured cells (53) and can enhance the cellular uptake of cholesterol-rich lipoproteins (54). As macrophages are known to secrete LpL (55, 56), FFA should be generated during incubation of VLDL with macrophages. Lindqvist et al. (57) have shown that accelerated lipolysis of VLDL during its incubation with macrophages increases cellular lipid accumulation. The lower capacity of Ac-18A-NH_2-treated VLDL to induce cellular lipid accumulation than the control VLDL or 18A- and 37pA-treated VLDL would likely be associated with diminished production of FFA due to a low reactivity of Ac-18A-NH_2-treated VLDL to LpL secreted by macrophages. Rumsey et al. (58) have shown that LpL can enhance cellular uptake of lipoproteins in cultured THP-1 monocyte-derived macrophages by acting as a ligand for binding of lipoproteins to these cells. It is possible that treatment of VLDL with Ac-18A-NH_2 could suppress a potential interaction of VLDL with LpL secreted by macrophages and thus suppress the LpL-ligand-mediated uptake of VLDL.

In summary, using synthetic class A amphipathic helical peptides, we have demonstrated that different exchangeable apolipoproteins on VLDL possess different affinities for the VLDL surface. ApoC-II is bound with higher affinity than apoC-III to VLDL surface while apoC-III is bound more strongly than apoE. Amphipathic helical peptides can alter metabolic and functional properties of VLDL without altering their lipid composition and morphology by dissociating functionally important exchangeable apolipoproteins from VLDL and by acting as functional elements of VLDL after their incorporation into VLDL. An amphipathic helical peptide, as a proline-punctuated dimer, exerts a different effect on the function of VLDL compared to a single helix, although the secondary structures of these two peptides are similar. This study thus indicates that, in addition to the secondary structure of exchangeable apolipoproteins, the arrangement of exchangeable apolipoproteins on the VLDL surface is important in modulating the function of VLDL.

This work was supported in part by Public Health Service Grants HL 34343 and a grant from the National Livestock and Meat Board.

Manuscript received 25 July 1995 and in revised form 24 January 1996.

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