Cationic domain 141-150 of apoE covalently linked to a class A amphipathic helix enhances atherogenic lipoprotein metabolism in vitro and in vivo

Geeta Datta,* David W. Garber,* Byung Hong Chung,* Manjula Chaddha,* Nassrin Dashti,* William A. Bradley,* Sandra H. Gianturco,* and G. M. Anantharamaiah[1,*,†]

Departments of Medicine* and Biochemistry and Molecular Genetics,† the Atherosclerosis Research Unit, University of Alabama at Birmingham Medical Center, Birmingham, AL 35294

Abstract **We previously showed (1) that a peptide, Ac-hE18A-NH_2, in which the arginine-rich heparin-binding domain of apolipoprotein E (apoE) [residues 141–150] (LRKLRKRLLR), covalently linked to 18A (DWLKAFYDKVAEKLKEAF; a class A amphipathic helix with high lipid affinity), enhanced LDL uptake and clearance. Because VLDL and remnants contain more cholesterol per particle than LDL, enhanced hepatic clearance of VLDL could lead to an effective lowering of plasma cholesterol. Therefore, in the present article we compared the ability of this peptide to mediate/facilitate the uptake and degradation of LDL and VLDL in HepG2 cells. The peptide Ac-hE18A-NH_2, but not Ac-18A-NH_2, enhanced the uptake of LDL by HepG2 cells 5-fold and its degradation 2-fold. The association of the peptides with VLDL resulted in the displacement of native apoE; however, only Ac-hE18A-NH_2 but not Ac-18A-NH_2 caused markedly enhanced uptake (6-fold) and degradation (3-fold) of VLDL. Ac-hE18A-NH_2 also enhanced the uptake (15-fold) and degradation (2-fold) of trypsinized VLDL Sf 100–400 (containing no immunodetectable apoE), indicating that the peptide restored the cellular interaction of VLDL in the absence of its essential native ligand (apoE). Pretreatment of HepG2s with heparinase and heparitinase abrogated all peptide-mediated enhanced cellular activity, implicating a role for cell-surface heparan sulfate proteoglycans (HSPG). Intravenous administration of Ac-hE18A-NH_2 into apoE gene knockout mice reduced plasma cholesterol by 88% at 6 h and 30% at 24 h after injection. We conclude that this dual-domain peptide associates with LDL and VLDL and results in rapid hepatic uptake via a HSPG-facilitated pathway.**—Datta, G., D. W. Garber, B. H. Chung, M. Chaddha, N. Dashti, W. A. Bradley, S. H. Gianturco, and G. M. Anantharamaiah. **Cationic domain 141-150 of apoE covalently linked to a class A amphipathic helix enhances atherogenic lipoprotein metabolism in vitro and in vivo.** ***J. Lipid Res.*** **2001.** 42: **959–966.**

Supplementary key words cationic peptides • enhanced uptake • degradation

Receptor interaction of atherogenic apolipoprotein B (apoB)-containing lipoproteins is essential for hepatic clearance (2–4), the major site of cholesterol catabolism to bile for elimination. Although LDL is cleared primarily by its interaction with the LDL-receptor (LDLR), VLDL and lipase-produced VLDL remnants are cleared, in large part, via the interaction of an appropriate conformation of apoE with several cell surface receptors. These include LDLR, the LDL-receptor related protein (LRP), and direct binding of apoE-containing VLDL and remnants to HSPG in the space of Disse (2–4). ApoE plays a critical role in lipoprotein metabolism. Its importance in inhibiting atherosclerosis in rabbits and mice has been established by numerous studies (5–10). For example, overexpression of human apoE in New Zealand White rabbits enhances the clearance of diet-induced lipoprotein remnants when compared with non-transgenic animals (5); intravenous injection of human apoE into cholesterol-fed Watanabe rabbits protects these animals from atherosclerosis (6). Mice overexpressing rat apoE have lower plasma cholesterol (7). Furthermore, loss of the apoE gene elicits spontaneous atherosclerosis (8, 9) that is ameliorated when macrophage-specific apoE expression is restored in apoE-deficient mice (10, 11). Finally, direct addition of apoE to the VLDL surface enhances the binding and uptake of VLDL (12–14), which carries more cholesterol per particle compared with LDL.

ApoE is a 299 amino acid residue protein that has been shown, initially by proteolysis studies (15, 16), to have two distinct domains: a receptor binding domain in the N-terminal region [residues 1–191] and a lipid binding domain at the C terminus [residues 192–299]. Subsequent studies with synthetic peptides and monoclonal antibodies (17–21) support this observation.

Abbreviations: Ac-, acetyl; apoB, apolipoprotein B; HSPG, heparan sulfate proteoglycan; LPDS, lipoprotein deficient serum; Mtr, 4-methoxy-2,3,6-trimethylbenzenesulfonyl; TFA, trifluoroacetic acid.

[1] To whom correspondence should be addressed at the Department of Medicine, UAB Medical Center, 1530 3rd Ave. S., Birmingham, AL 35294.

e-mail: ananth@uab.edu

Wilson et al. (22) crystallized the amino terminal domain of apoE [residues 1–191] containing the LDL receptor-binding domain, which demonstrated its globular structure. Using 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine complexes of the N-terminal 22-kDa thrombin fragment mutants of apoE, it has been shown (23) that conserved lysines and arginines within the 140–150 region of apoE are crucial for the interaction between apoE and the LDLR. The positive charge cluster appears to be involved in ionic interactions with negative charges of the LDLR and/or proteoglycans. We recently showed (1) that the dual-domain peptide Ac-hE18A-NH_2 with the sequence Ac-LRKLRKRLLRDWLKAFYDKVAEK LKEAF-NH_2, containing the arginine-rich cationic domain of apoE (LRKLRKRLLR), covalently linked to a class A amphipathic peptide 18A (DWLKAFYDKVAEKLKEAF), binds to LDL, and enhances its uptake and degradation in fibroblasts via the HSPG pathway. Previous studies from our laboratory (24) showed that class A amphipathic peptides interact with VLDL and displace apoE. We reasoned that an efficient removal of VLDL might result in a significant reduction of plasma cholesterol. Therefore we studied, and report here, the peptide-mediated uptake and degradation of LDL and VLDL by hepatocytes and the effect of acute intravenous administration of Ac-hE18A-NH_2 on plasma cholesterol levels in the apoE gene knockout mouse model.

MATERIALS AND METHODS

Synthesis of the peptides

Peptides Ac-18A-NH_2 and Ac-hE18A-NH_2 were synthesized by Fmoc chemistry, using an automatic peptide synthesizer (PE Biosystems, Foster City, CA) according to the procedure described by us earlier (1). Purity of peptides was determined by C-18 analytical reversed-phase HPLC and confirmed by mass spectral analysis.

Preparation of VLDL, LDL, and lipoprotein-deficient serum

Plasma VLDL and LDL were isolated from human plasma obtained from the American Red Cross by sequential centrifugation as previously described (25, 26). The VLDL ($d < 1.006$ g/ml) was isolated by centrifugation of plasma at 50,000 rpm at 4°C for 24 h and washed once by reisolation under identical conditions. The LDL ($1.006 < d < 1.063$g/ml) was also isolated by centrifugation at 50,000 rpm at 4°C for 24 h and washed by overlayering with 150 mM NaCl and recentrifuged and dialyzed extensively against 150 mM NaCl containing 0.24 mM EDTA. The purity of the isolated lipoproteins was determined by Superose 6 (Pharmacia) column chromatography and by agarose electrophoresis. For preparation of lipoprotein-deficient serum (LPDS), the density of plasma was adjusted to 1.21 g/ml with potassium bromide and centrifuged at 50,000 rpm at 4°C. The top fraction consisting of lipoproteins was removed. The lower fraction, which is devoid of lipoproteins, was dialyzed extensively against 150 mM NaCl at 4°C for 60 h. The lipoprotein-deficient plasma was converted to LPDS by incubation with thrombin (10 U/ml) at 4°C for 24 h and removing the resulting clot by centrifugation.

Manual cholesterol analysis

Cholesterol was determined by adding 10-μl standard or plasma to 1-ml cholesterol reagent (4-g "Cholesterol 1000," Sigma Chemical Co., St. Louis, MO) dissolved in 100-ml deionized H_2O) and mixing. Absorbance at 500 nm was read in a spectrophotometer after 15 min of incubation at room temperature. Standards were Sigma Cholesterol Calibrator at 100, 200, and 400 mg/dl.

Trypsinolysis of VLDL

Trypsinolysis was carried out on VLDL S_f 100–400, essentially as described by Gianturco et al. (27). Briefly, the VLDL S_f 100–400 was treated with trypsin (1% by weight) in Tris buffer (pH 7.4) for 2 h at 37°C and passed over a benzamidine-sepharose column to remove active trypsin, and the protease-treated VLDL was reisolated by cumulative ultracentrifugation.

^{125}I labeling of LDL, VLDL, and peptides

LDL, VLDL, and peptides were labeled with ^{125}I using the method of Bilheimer, Eisenberg, and Levy (28). The ^{125}I-labeled material was separated from free ^{125}I by a desalting column (Biorad, Econopak 10DG). Fractions containing labeled material were pooled and specific activity determined. In the case of peptides, 90% of the peptide was labeled, whereas in LDL and VLDL, 80% and 70%, respectively, of the protein was labeled as determined by delipidation. The labeled LDL/VLDL was dialyzed extensively against PBS and was analyzed for protein and cholesterol content. ^{125}I-specific activities ranged from 250 to 400 dpm/ng of protein.

Interaction of the peptide with VLDL

The peptides Ac-hE18A-NH_2 and Ac-18A-NH_2 were added to VLDL at various protein/peptide (w/w) ratios and incubated at room temperature for 1 h. VLDL-peptide mixtures were subjected to ultracentrifugation to separate VLDL-peptide complexes from unbound peptides as previously described (24). The protein content of the isolated VLDL fraction was determined by the procedure of Lowry et al. (29). SDS-PAGE was carried out using the method of Laemmli (30). Pre-made Novex gels were used and the gel was stained with Coomassie blue to identify the protein bands. The bands were quantitated by densitometry using the gel documentation system from UVP (Upland, CA).

Binding of the peptides to VLDL was also examined by using ^{125}I-labeled peptides. Both Ac-hE18A-NH_2 and Ac-18A-NH_2 have a single Tyr residue that can be iodinated. VLDL (1-mg apoprotein) was incubated with 1 mg of labeled peptide, the VLDL-peptide complex was reisolated, and the bound peptide was separated from free peptide using density-gradient ultracentrifugation. The amount of peptide bound to VLDL was calculated using the specific activity of the peptide, which ranged from 250 to 300 dpm/ng of peptide.

Binding, internalization, and degradation of LDL and VLDL

The binding of lipoprotein (LDL or VLDL) to cells and its internalization and degradation were measured as described by Goldstein, Basu, and Brown (26). HepG2 cells were grown in DMEM containing 10% fetal calf serum (FCS) and penicillin-streptomycin-amphotericin in 6-well plates and used after reaching 75–90% confluency (2–3 days). The seeding density of cells used was 1.5×10^5 to 3.0×10^5 cells/ml medium. Cells were incubated with medium containing LPDS for 24 h prior to use. For binding experiments, the cells were incubated with peptide-treated and control ^{125}I-LDL or ^{125}I-VLDL at 4°C for 2 h and, for uptake (or internalization), they were incubated at 37°C for 2 h. After washing four times with ice cold PBS containing 2-mg/ml BSA to remove nonspecifically bound lipoprotein, the cells were incubated with dextran sulfate (4 mg/ml, Pharmacia, M_r 500,000) or heparin (10 mg/ml, Sigma) for 1 h to release specifically bound ^{125}I-LDL/ ^{125}I-VLDL. The counts

in the dextran sulfate/ heparin wash reflected the amount of LDL/VLDL specifically bound to cells. The cells were washed with cold PBS, dissolved in 1-ml 0.1N NaOH, and a 0.5-ml aliquot was counted. These counts reflect the amount of LDL/VLDL internalized. Protein was estimated by the method of Lowry et al. (29).

Degradation of LDL/VLDL was measured using the protocol described above for binding, except that the cells were incubated at 37°C for 5 h. Degradation was determined by precipitating ^{125}I-LDL or ^{125}I-VLDL from the medium by incubation at 4°C for 30 min with 16.7% TCA (0.5 ml of 50% TCA was added to 1 ml of medium) (25). The resulting precipitate was removed by centrifugation. The supernatant was treated with 10 μl of 40% potassium iodide and 40 μl of 30% hydrogen peroxide. The free ^{125}I liberated was extracted with 2 ml of chloroform. The upper aqueous layer (0.5 ml) was then counted. This represents the degradation of apolipoprotein in LDL/VLDL.

Cell-surface HSPG was removed by incubating the cells with 3 U/ml heparinase/heparitinase (Sigma) at 37°C for 2 h as previously reported (1). In all the cell experiments, mean values from replicate measurements of three independent experiments were used.

Effect of peptide in intravenous administration in apoE(−/−) mice

Mice were purchased from Jackson Laboratories (Bar Harbor, ME). Following an overnight fast, female mice were injected intravenously with 100 μg of Ac-hE18A-NH_2 or Ac-18A-NH_2 in 100 μl PBS through the tail vein; control mice were injected with 100 μl PBS. Blood was collected from the retro-orbital sinus under anesthesia immediately before injection and at 6 and 24 h following injection, and cholesterol was measured manually on all samples. Food was provided to the mice following the 6-h sample. Lipoprotein cholesterol profiles were determined using the CLiP method (31).

RESULTS

We have shown (1) that the dual-domain peptide, Ac-hE18A-NH_2, binds to LDL and enhances its uptake in fibroblasts. Given the importance of the liver in the clearance of LDL and VLDL, we determined the effect of this peptide on the uptake and degradation of LDL and VLDL in model hepatocytes. Ac-hE18A-NH_2 enhanced the uptake of LDL in HepG2 cells 5-fold and degradation 2-fold as seen in **Fig. 1**. Heparinase and heparitinase pretreatment of cells completely eliminated this effect (Fig. 1), suggesting that the LDL-Ac-hE18A-NH_2 is taken up via the HSPG pathway in hepatocytes as in fibroblasts (1). The level of enhanced uptake of LDL was dependent on the amount of Ac-hE18A-NH_2 incorporated into the LDL. Importantly, the control peptide Ac-18A-NH_2 not containing the cationic domain did not enhance the uptake of LDL in HepG2 cells at any concentration (data not shown).

Peptides associate with VLDL surface and displace apo E

Peptide binding to VLDL was studied by incubating VLDL with increasing amounts of Ac-hE18A-NH_2 at the weight ratios of 1:0.15, 1:0.3, and 1: 0.6 as indicated in the legend of **Fig. 2A**. The peptide-VLDL mixtures were subjected to density gradient ultracentrifugation to separate

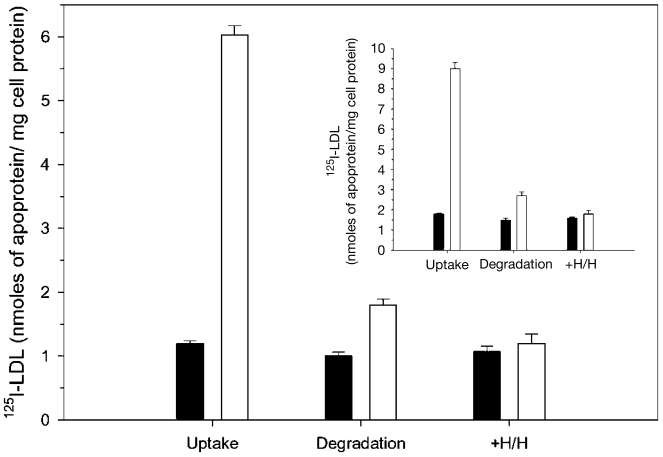
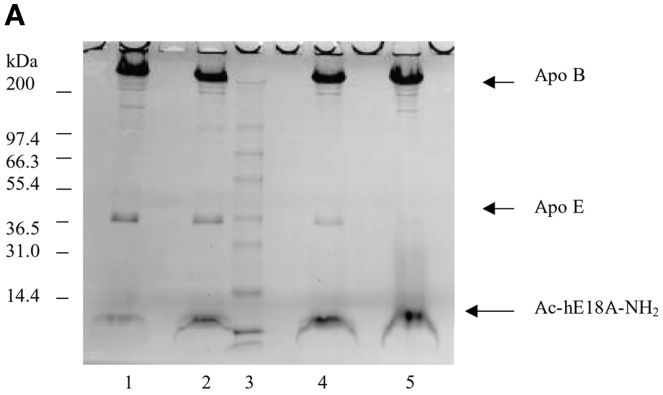
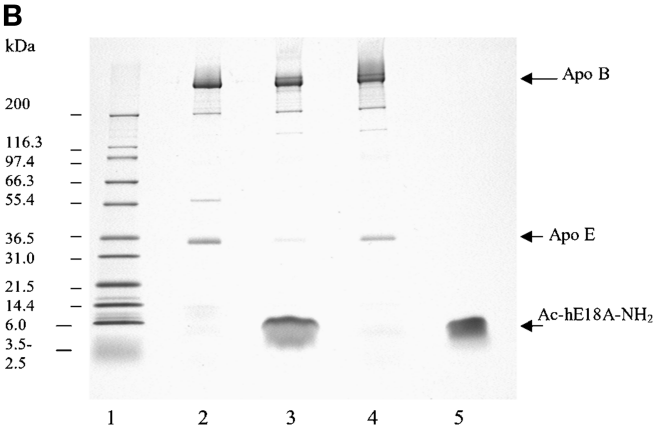


Fig. 1. Uptake and degradation of ^{125}I-LDL alone and ^{125}I-LDL-Ac-hE18A-NH_2 complex by Hep G2 cells. ^{125}I-LDL was incubated with Ac-hE18A-NH_2 (1:1 w/w) and the complex reisolated by density-gradient ultracentrifugation. The ^{125}I-LDL-peptide complex was used to determine the effect of the bound peptide on the uptake and degradation of ^{125}I-LDL. In each case, particles containing 50 μg of cholesterol were added and incubated with cells at 37°C for 2 h for uptake and for 5 h for degradation. Cell-surface bound LDL was removed in two different ways; in one experiment heparin was used (main figure) and in another, dextran sulfate (inset). The data represent a mean of triplicate values. The solid bars represent native ^{125}I-LDL, and the open bars represent ^{125}I-LDL-Ac-hE18A-NH_2. +H/H represents the uptake of LDL in cells pretreated with heparinase/heparitinase (3 U/ml).

VLDL-peptide complexes from unassociated peptide. The apolipoprotein distribution of the VLDL-peptide complexes re-isolated by density gradient ultracentrifugation was examined by SDS-PAGE and is shown in Fig. 2A. Since addition of peptide to VLDL could interfere with the quantitation of proteins on the lipoprotein, VLDL particle concentration was determined by cholesterol content. At equal VLDL particle concentration (based on cholesterol content), the ratio of apoE to apoB was determined at each peptide concentration by quantitating the intensity of the bands and is shown in **Table 1**. The apoE/apoB ratio (relative intensities) of each complex (lanes 2, 4, and 5 in Fig. 2A) was compared with that of VLDL alone (lane 1, Fig. 2A). The apoE/apoB ratio was lower in Ac-hE18A-NH_2-treated VLDL (Fig. 2A, lanes 2, 4, and 5) than in VLDL alone (Fig. 2A, lane 1). The apoE/apoB ratio decreases from 0.2 to 0.06 with increasing concentration of Ac-hE18A-NH_2 (Table 1). Negligible amounts of apoE were detectable in VLDL at the VLDL: peptide ratio of 1.0:0.6 (Fig. 2A, lane 5). The decrease in apoE content with increasing amount of peptide on VLDL indicated that apoE was displaced from the VLDL surface by Ac-hE18A-NH_2 in a concentration-dependent manner, whereas the increase in intensity of the band corresponding to the peptide demonstrated a concentration-dependent association of peptide with VLDL.

To compare the interaction of VLDL and Ac-hE18A-NH_2 with that of VLDL and the control peptide, Ac-18A-NH_2, complexes (1:1 w/w) of the two peptides were made with VLDL, isolated, and compared on the same SDS gel, Fig.





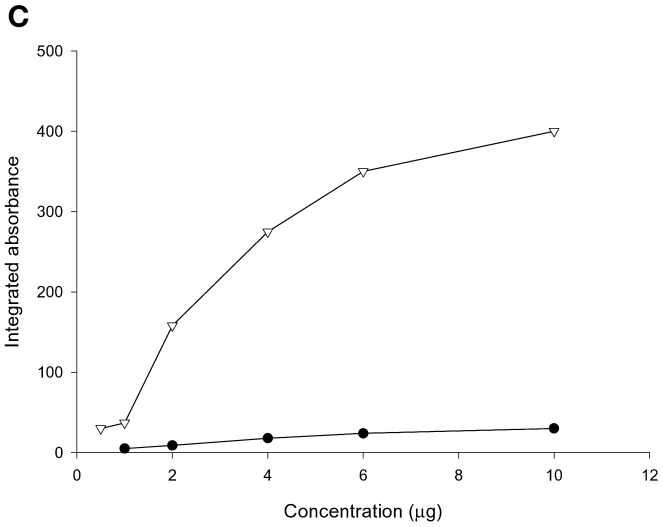


Fig. 2. A: Effect of increasing amount Ac-hE18A-NH_2 on its binding to VLDL. Apolipoproteins in VLDL and VLDL-Ac-hE18A-NH_2 complexes reisolated by density-gradient ultracentrifugation were separated on SDS-PAGE (10–20%). The gel is stained with Coomassie Brilliant blue. Lanes 1, VLDL alone; 2, VLDL-Ac-hE18A-NH_2 (1:0.15 w/w); 3, molecular weight standards; 4, VLDL-Ac-hE18A-NH_2 (1:0.3 w/w); and 5, VLDL-Ac-hE18A-NH_2 (1:0.6 w/w). The positions of the apolipoproteins and the peptide are indicated. The band at <14.4 kDa in lane 1 represents apoCs present in VLDL. Each of the lanes contains equivalent VLDL based on cholesterol content (50 μg). The decrease in the content of apoE per 50 μg of VLDL cholesterol with increasing addition of Ac-hE18A-NH_2 is evident in lanes 4 and 5. Increasing amounts of Ac-hE18A-NH_2 bound to the VLDL can be seen in lanes 2,4, and 5 indicated by its marker on the right side of the gel. B: Comparison of the effect of Ac-hE18A-NH_2 and Ac-18A-NH_2 on VLDL. SDS-PAGE (4–20%) of apolipoproteins in VLDL and VLDL-Ac-hE18A-NH_2 (1:1) and VLDL-Ac-18A-NH_2 (1:1) complexes reisolated by density-gradient centrifugation. Each of the lanes contains the same amount of VLDL based on cholesterol content (50 μg). Lanes 1, molecular weight standard; 2, control VLDL; 3, VLDL-Ac-hE18A-NH_2 (1:1 w/w); 4, VLDL-Ac-18A-NH_2 (1:1 w/w); and 5, Ac-hE18A-NH_2 alone (6 μg). C: Demonstration of the difference in the chromogenicity of Ac-hE18A-NH_2 and Ac-18A-NH_2. Different concentrations (1–10 μg) of Ac-hE18A-NH_2 and Ac-18A-NH_2 were loaded on a 10–20% SDS gel and electrophoresed. These gels were stained with colloidal blue, and the integrated band intensity was measured using the gel documentation system. The integrated band intensity was plotted against the concentration of the peptide. Ac-hE18A-NH_2 (open triangle) shows greater chromogenicity than Ac-18A-NH_2 (solid circle).

TABLE 1. Effect of increasing concentration of Ac-hE18A-NH_2 on apoE content of VLDL

VLDL + Ac-hE18A-NH_2[a]	ApoE/ApoB[b]
VLDL alone	0.20
1:0.15	0.15
1:0.30	0.10
1:0.60	0.06

[a] Complexes of VLDL and Ac-hE18A-NH_2 were prepared at different weight ratios as mentioned in Materials and Methods. The weight ratios used were 1:0, 1:0.15, 1:0.3, and 1:0.6 (lanes 1, 2, 4, and 5, respectively, from Fig. 2A).

[b] The apoE/apoB ratios were determined by quantitating the bands for apoB and apoE from Fig. 2A.

2B. Ac-18A-NH_2 also displaced apoE from VLDL (Fig. 2B, lane 4) as had been reported earlier (24), however, it displaced less apoE than Ac-hE18A-NH_2 (Fig. 2B, lane 3) at the same VLDL/peptide (w/w) ratio. Figure 2B, lane 5, shows the band due to the peptide Ac-hE18A-NH_2 alone. The apoE/apoB ratios (relative intensity) were determined as earlier and are found in **Table 2**. The apoE/apoB ratio decreased from 0.2 in VLDL alone (without peptide, Fig. 2B, lane 2) to 0.04 with Ac-hE18A-NH_2 (Fig. 2B, lane 3), and to 0.1 with Ac-18A-NH_2 (Fig. 2B, lane 4), respectively. A faint band corresponding to Ac-18A-NH_2 can also be seen in Fig. 2B, lane 4.

Using radiolabeled peptide (both Ac-hE18A-NH_2 and Ac-18A-NH_2), the binding of the peptide to VLDL was studied. It was determined that the amount of peptide bound to VLDL was the same in both cases (67 ± 5 nmol of Ac-hE18A-NH_2 and 73 ± 7 nmol of Ac-18A-NH_2 per mg of VLDL). Although the concentration of the two peptides in their respective complexes was the same, Ac-hE18A-NH_2 stained much more than Ac-18A-NH_2, possibly because the former contains a greater number of Arg and Lys residues. The difference in chromogenicity of the two peptides was also examined by staining SDS gels containing different amounts of peptides. Figure 2C shows the difference in chromogenicity, indicating increased chromogenicity of Ac-hE18A-NH_2 compared with that of Ac-18A-NH_2. The above results indicate that both peptides, Ac-hE18A-NH_2 and Ac-18A-NH_2, displaced apoE from the VLDL surface and that they bind to VLDL surface to produce an apoE-deficient, VLDL-peptide complex.

TABLE 2. Effect of Ac-hE18A-NH_2 and Ac-18A-NH_2 on apoE content of VLDL

Sample	ApoE/Apo B
VLDL	0.20
VLDL + Ac-hE18A-NH_2	0.04
VLDL + Ac-18A-NH_2	0.10

Complexes of VLDL and the two peptides, Ac-hE18A-NH_2 and Ac-18A-NH_2, were prepared at the weight ratio (1:1) as mentioned in Materials and Methods. The apoE/apoB ratios were determined by quantitating the bands for apoB and apoE from Fig. 2B.

The dual-domain peptide, but not the class A amphipathic helical peptide, enhances VLDL binding and degradation in HepG2 cells

To determine the effect of the dual-domain peptide on the binding (4°C, 2 h), uptake (37°C, 2 h), and degradation (37°C, 5 h) of ^{125}I-VLDL, HepG2 cells were incubated with ^{125}I-VLDL-Ac-hE18A-NH_2 and with ^{125}I-VLDL-Ac-18A-NH_2 complexes under the conditions described above and in Materials and Methods. Although apoE was displaced by both peptides, the presence of Ac-hE18A-NH_2 on VLDL markedly enhanced the binding, uptake, and degradation of ^{125}I-VLDL by HepG2, whereas that of Ac-18A-NH_2 did not. Binding of ^{125}I-VLDL at 4°C (3 μg of apoprotein/mg of cell protein ± 0.008) was enhanced in the presence of Ac-hE18A-NH_2 to 16.5 μg of apoprotein/mg of cell protein ± 0.14 (data not shown). These experiments were done in triplicate. Uptake (internalization) was measured by determining the amount of radioactivity in the cells after incubation at 37°C for 2 h, washing surface-bound VLDL with dextran sulfate. Compared with VLDL alone, the uptake and degradation of VLDL-Ac-hE18A-NH_2 were stimulated by 6-fold and 3-fold, respectively (**Fig. 3A**, inset). To confirm that the observed enhancement was real and not due to inefficient removal of surface-bound VLDL, separate experiments were carried out using heparin to remove surface-bound VLDL. As can be seen, both dextran sulfate (Fig. 3A, inset) and heparin (Fig. 3A) yielded similar results. In stark contrast, Ac-18A-NH_2 did not increase the uptake of VLDL; rather, the uptake was less than that of VLDL alone, in agreement with the observation that Ac-18A-NH_2 displaced apoE from VLDL and produced apoE-deficient VLDL. Both peptides associated with VLDL to the same extent as indicated above and both displaced the endogenous ligand apoE. However, only Ac-hE18A-NH_2 containing the cationic domain increased uptake and degradation of VLDL in HepG2 cells.

Heparinase and heparitinase remove HSPG from the surface of cells. We used this to determine whether HSPG had a role in Ac-hE18A-NH_2-mediated uptake of VLDL. Cells were pretreated with these enzymes as detailed in Materials and Methods. As can be seen in Fig. 3A, treatment with heparinase and heparitinase eliminated the enhancement of VLDL uptake by Ac-hE18A-NH_2, impli-

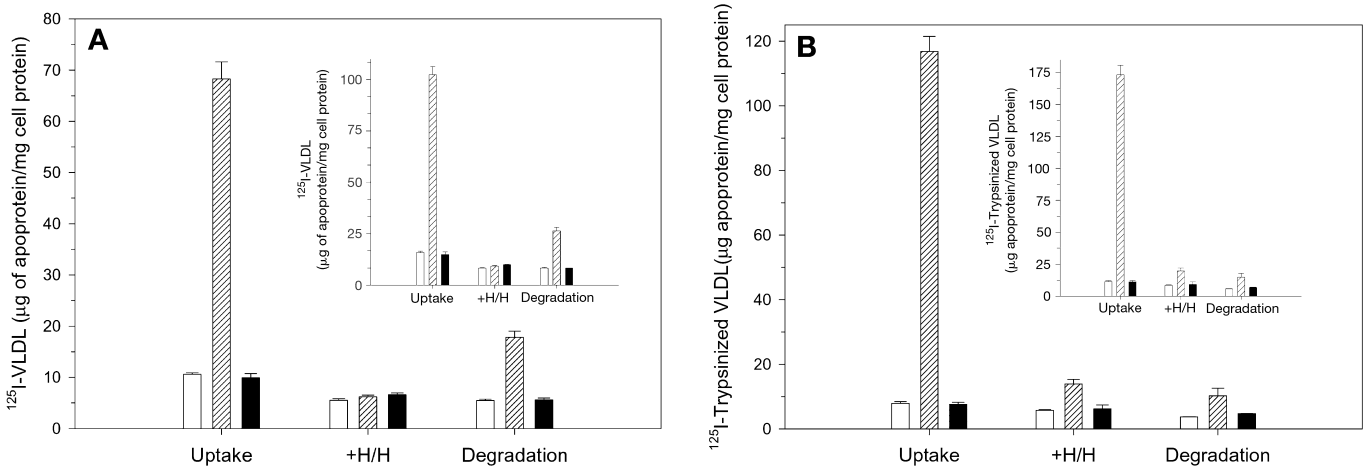


Fig. 3. A: Uptake and degradation of ^{125}I-VLDL alone and ^{125}I-VLDL-Ac-hE18A-NH_2 complex by Hep G2 cells. Ac-18A-NH_2 was used as a control. ^{125}I-VLDL was incubated with the peptide (1:1 w/w) and reisolated by density centrifugation. The peptide bound to ^{125}I-VLDL floated to the top and the unbound peptide was at the bottom. The ^{125}I-VLDL-peptide complex was used to determine the effect of the peptide on the uptake and degradation of ^{125}I-VLDL. In each case particles containing 50 μg of cholesterol were added and the cells incubated at 37°C for 2 h for uptake and 5 h for degradation. Cell-surface bound VLDL was removed in two different ways; in one experiment heparin was used (main figure) and in another, dextran sulfate (inset). ^{125}I-VLDL alone (open bar); ^{125}I-VLDL-Ac-hE18A-NH_2 (1:1 w/w) (hatched bar); and ^{125}I-VLDL-Ac-18A-NH_2 (1:1 w/w) (solid bar). +H/H indicates the uptake of ^{125}I-VLDL and ^{125}I-VLDL-Ac-hE18A-NH_2 in cells pretreated with heparinase/heparitinase (3 U/ml). The data represent a mean of triplicate values. B: Uptake and degradation of ^{125}I-trypsinized VLDL S_f 100–400 alone and ^{125}I-trypsinized VLDL-Ac-hE18A-NH_2 complex by Hep G2 cells. Ac-18A-NH_2 was used as a control. ^{125}I-trypsinized VLDL was incubated with the peptide (1:1 w/w) and reisolated by density centrifugation. The peptide bound to ^{125}I-trypsinized VLDL floated to the top and the unbound peptide was at the bottom. The ^{125}I-trypsinized VLDL-peptide complex was used to determine the effect of the peptide on the uptake and degradation of ^{125}I-trypsinized VLDL. In each case particles containing 50 μg of cholesterol were added. Conditions for uptake and degradation were as mentioned in Fig. 3A. Cell-surface bound VLDL was removed in two different ways; in one experiment heparin was used (main figure) and in another, dextran sulfate (inset). ^{125}I-trypsinized VLDL alone (open bars); ^{125}I-trypsinized VLDL-Ac-hE18A-NH_2 (1:1 w/w) (hatched bars); and ^{125}I-trypsinized VLDL-Ac-18A-NH_2 (1:1 w/w) (solid bars). +H/H indicates the uptake in presence of heparinase/heparitinase (3 U/ml), as in Fig. 3A. The data represent a mean of triplicate values.

cating HSPG in this peptide-mediated enhanced uptake of VLDL, similar to our previous observation of LDL-peptide complex uptake and degradation.

Ac-hE18A-NH_2 binds to trypsinized VLDL and enhances its uptake by HepG2 cells

Trypsinization of VLDL removes immuno-detectable apoE on VLDL S_f 100–400 (16). This VLDL subfraction is rendered LDL-receptor-inactive with the loss of its apoE receptor-binding domain on a particle in which the apoB is incompetent in binding to the LDLR (14, 15, 32). Both peptides studied here, Ac-hE18A-NH_2 and Ac-18A-NH_2, bound to trypsinized VLDL as determined by SDS electrophoresis (data not shown). It was therefore of interest to determine whether Ac-hE18A-NH_2 and/or Ac-18A-NH_2, when bound to trypsinized VLDL S_f 100-400, could facilitate its uptake by HepG2 cells. As with intact total VLDL, only Ac-hE18A-NH_2 enhanced the uptake of trypsinized VLDL S_f 100–400. The uptake increased by 15-fold over the control trypsinized VLDL, whereas Ac-18A-NH_2 had no effect (Fig. 3B). Degradation of trypsinized VLDL was also enhanced by Ac-hE18A-NH_2; again, Ac-18A-NH_2 had no effect. Although the Ac-hE18A-NH_2-mediated uptake was enhanced 15-fold, the degradation was only 2-fold greater than with trypsinized VLDL alone. To determine whether the HSPG pathway was involved in uptake and degradation in this case also, the cells were again pretreated with heparinase and heparitinase. The observed enhancement was nullified by pretreatment of the cells with heparinase and heparitinase. These data suggest that HSPG pathway plays a significant role in the enhanced uptake and degradation of Ac-hE18A-NH_2-mediated VLDL and trypsinized VLDL. The peptide mimics at least one of the functions of apoE; i.e., binding to HSPG.

Effect of acute administration of the peptide in apoE gene knockout mice

Intravenous administration of 100 μg Ac-hE18A-NH_2 in 100 μl PBS into fasting apoE gene knockout mice (n = 8) reduced plasma cholesterol from 394 ± 12 mg/dl (zero time) to 47.7 ± 10.3 mg/dl (88% reduction) 6 h after injection ($P < 0.001$ compared with zero time by one-way ANOVA; **Fig. 4A**). Food was provided after the 6-h blood sample. At 24 h, cholesterol levels had increased, but remained significantly less than the initial levels (282 ± 18 mg/dl; $P < 0.001$ compared with zero time by one-way ANOVA; Fig. 4A). Injection of PBS (n = 7) did result in significant reduction of plasma cholesterol at 6 h compared with initial levels ($P < 0.001$), but the reduction was significantly less than in the Ac-hE18A-NH_2 mice (Fig. 4A). The reduction in plasma cholesterol in Ac-hE18A-NH_2-injected mice was primarily in the VLDL and IDL/LDL regions of the profile; a representative profile from an individual animal is presented in Fig. 4B. Injection of Ac-18A-NH_2 into fasting apoE gene knockout mice did not result in significant reductions of plasma cholesterol at 4 h compared with PBS. Total cholesterol levels at 4 h in Ac-18A-NH_2-injected mice were reduced 22.7 ± 3.2% (n = 4) compared with initial levels, whereas those in PBS-injected mice were reduced 22.2 ± 2.7% (n = 4) compared with initial levels. These reductions were comparable to those seen in PBS-injected mice in Fig. 4A (29.1 ± 1.4%).

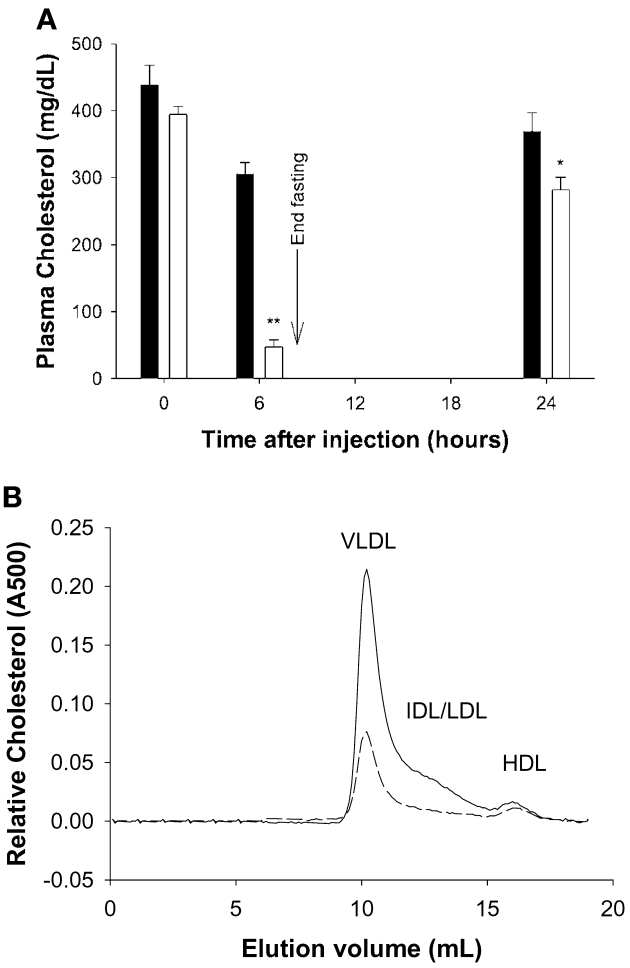


Fig. 4. Clearance of plasma cholesterol following intravenous injection of Ac-hE18A-NH_2 in apoE gene knockout mice. Fasting female apoE gene knockout mice were injected intravenously through the tail vein with either PBS (100 μl; n = 7) or Ac-hE18A-NH_2 (100 μg in 100 μl PBS; n = 8). Blood samples were taken under anesthesia immediately before injection (zero time) and at 6 and 24 h. Plasma was collected and analyzed for total cholesterol and for plasma cholesterol profiles by the CLiP method (31). A: Total cholesterol levels in PBS (solid bars) or peptide-injected (open bars) mice are shown as mean ± SEM. * $P < 0.05$ versus PBS at the same time point by two-tailed *t*-test; ** $P < 0.0001$ versus PBS at the same time point by two-tailed *t*-test. B: Representative plasma cholesterol profiles at zero time (solid line) and 6 h (dashed line) in a single peptide-injected mouse.

DISCUSSION

The present data show that Ac-hE18A-NH_2 and Ac-18A-NH_2 associate with both LDL and VLDL, but only the dual-domain peptide, Ac-hE18A-NH_2, enhanced the uptake and degradation of VLDL and LDL by HepG2 cells. ApoE is re-

quired for the LDLR-mediated uptake of VLDL S_f >60 (2, 14). Both Ac-hE18A-NH_2 and Ac-18A-NH_2 displaced apoE from VLDL. Therefore, these apoE-deficient VLDL would be expected to be internalized by apoE-mediated cellular pathways to a lesser degree than the control VLDL. This was observed for Ac-18A-NH_2-treated and trypsinized VLDL; however, Ac-hE18A-NH_2-treated VLDL associated with HepG2 cells more efficiently (6-fold) than the control VLDL. The degradation was also enhanced 3-fold over the control. Thus the increased cell association of apoE-deficient VLDL-Ac-hE18A-NH_2 complex implies that Ac-hE18A-NH_2 is able to mimic apoE functionally for the enhancement of uptake and degradation of VLDL. The uptake of the VLDL-dual-domain peptide complex may be greater than VLDL alone because the number of Ac-hE18A-NH_2 molecules (potential ligand domains) bound per VLDL is greater than the number of apoE molecules in the native control VLDL. The relative enhanced uptake observed with trypsinized VLDL, despite the fact that all immunochemically detectable apoE was lost due to trypsinization, may be due to the same reason just stated or, perhaps, to the lower uptake of the trypsinized VLDL relative to the complex. Although the enhancement of VLDL internalization was 6-fold in presence of the peptide, degradation was not enhanced to the same extent (only 3-fold). This suggests the possibility that the VLDL-peptide complex internalization may occur through both receptor-dependent and receptor-independent pathways and/or that the degradation is at its maximum. It is also possible that the degradation pathway is inhibited by the peptide. These issues will be the subject of further investigation. Although HSPG is implicated in the internalization process, other receptor-dependent pathways may also be contributing to the transfer of VLDL from HSPG to receptor for endocytic degradation. All these parameters might reflect a greater internalization relative to the measured endocytic degradation.

The effect of Ac-hE18A-NH_2 on the uptake of LDL and VLDL in HepG2 cells (Figs. 1 and 3A) was examined using ^{125}I-VLDL treated with peptide at a 1:1 ratio and reisolating the lipoprotein-peptide complex. When the peptide-mediated LDL and VLDL uptake and degradation were compared on the basis of the amount of cholesterol to which the cells were exposed, the following results were obtained. Assuming as a first approximation and based on the protein-to-total cholesterol ratio per lipoprotein, it can be seen from the apoprotein uptake in Fig. 1 that 1.8 μg of LDL cholesterol/mg of cell protein are taken up. However, in the presence of peptide, ~9.0 μg of cholesterol/mg of cell protein was taken up. The results with VLDL suggest that approximately 3.2–5 μg of VLDL cholesterol/mg of cell protein are internalized in the absence of Ac-hE18A-NH_2 and ~20–32 μg of VLDL cholesterol/mg of cell protein in the presence of the dual-domain peptide (Fig. 3A), based on the calculation. Because VLDL is a larger particle than LDL, it contains more total cholesterol per particle. Thus peptide-mediated enhanced uptake of VLDL could result in internalization of greater amounts of cholesterol per particle. In the range of the peptide concentration studied (1:0.15, 1:0.3, 1:0.6, and 1:1), the uptake of both lipoproteins was dependent on the concentration of the peptide. The greater the number of Ac-hE18A-NH_2 molecules bound to the lipoprotein, the greater the internalization of the lipoprotein. Thus it appears that due to the greater surface area of VLDL, less surface pressure on VLDL, or both, more peptide can bind per VLDL particle as compared with LDL. Furthermore, displacement of apoE from the VLDL surface by the peptide perhaps exposes more lipid surface for peptide to associate. A greater number of peptide molecules compared with apoE (increased ligand-binding domains relative to one apoE) could bind VLDL; thus more Ac-hE18A-NH_2-VLDL is taken up than VLDL itself. Although both Ac-hE18A-NH_2 and Ac-18A-NH_2 bind VLDL to the same extent on a molar basis, only Ac-hE18A-NH_2 facilitates an increased binding and uptake of VLDL, suggesting that the net positive charge plays an important role in the cell association to HepG2 cells and fibroblasts (1).

ApoE is involved normally in the clearance of remnant atherogenic lipoproteins via not only the LDLR and LDLR-related proteins but also the HSPG-mediated pathway (2). It is possible that Ac-hE18A-NH_2 on the basis of our in vitro results might also assist in the rapid clearance of atherogenic lipoproteins in vivo. In experiments presented here, intravenous administration of the Ac-hE18A-NH_2 in apoE knockout mice reduced plasma cholesterol by 88% within 6 h and reduced levels were maintained at 24 h even with only a single injection (Fig. 4A). However, administration of the control peptide Ac-18A-NH_2 reduced plasma cholesterol levels only by 22% as did PBS. These results were similar to those reported by us (33) with intraperitoneal administration of Ac-18A-NH_2 in C57BL/6J mice fed an atherogenic diet.

HSPG mediate the internalization of apoE-containing lipoproteins either via the receptor-facilitated mechanism or independently. The Arg-rich heparin-binding region [141–150] of apoE, LRK**LRKRLLR**, would be expected to have a high affinity for cell-surface proteoglycans (34); our data support this observation. The portion of the sequence in bold corresponds to the consensus sequence of heparin-binding proteins, which bind glycosaminoglycans (GAG) (34). It appears that the amphipathic lipid-binding domain of Ac-hE18A-NH_2 binds to the lipoprotein surface while the positively charged domain directs the lipoprotein-peptide complex to the negatively charged proteoglycan for enhanced clearance via several potential pathways.

In summary, Ac-hE18A-NH_2 significantly enhances the uptake of atherogenic lipoproteins LDL and VLDL by HepG2 cells, whereas the peptide Ac-18A-NH_2, which does not possess the Arg-rich sequence LRKLRKRLLR does not. Enhanced VLDL uptake and degradation was observed despite the loss of apoE from the VLDL surface. A rapid and enhanced clearance of VLDL/LDL cholesterol in apoE knockout mice suggests that the peptide has the potential to lessen lesion formation by lowering plasma levels of atherogenic apoB-containing lipoproteins. Studies are in progress to determine whether lesion formation is decreased and the mechanism and site of this enhanced uptake of the apoB-containing lipoproteins and their fate after internalization.

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