Enhancement by LDL of transfer of L-4F and oxidized lipids to HDL in C57BL/6J mice and human plasma

David Meriwether,⁎ Satoshi Imaiizumi,† Victor Grijalva,† Greg Hough,‡ Ladan Vakili,† G. M. Anantharamaiah,§ Robin Farias-Eisner,† Mohamad Navab,† Alan M. Fogelman,† Srinivasa T. Reddy,⁎† and Ishaiahu Shechter 1,†

Department of Obstetrics and Gynecology,⁎ and Department of Medicine, Division of Cardiology,¹ David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA; and Department of Medicine,§ Atherosclerosis Research Unit, University of Alabama at Birmingham, Birmingham, AL.

Abstract The apoA-I mimic peptide L-4F [(Ac-D-W-F-K-A-F-Y-D-K-V-A-E-K-F-K-E-A-F-NH2) synthesized from all L-amino acids] has shown potential for the treatment of a variety of diseases. Here, we demonstrate that LDL promotes association between L-4F and HDL. A 2- to 3-fold greater association of L-4F with human HDL was observed in the presence of human LDL as compared with HDL by itself. This association further increased when LDL was supplemented with the oxidized lipid 15S-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (15HETE). Additionally, L-4F significantly (P = 0.02) promoted the transfer of 15HETE from LDL to HDL. The transfer of L-4F from LDL to HDL was demonstrated both in vitro and in C57BL/6J mice. L-4F, injected into C57BL/6J mice, associated rapidly with HDL and was then cleared quickly from the circulation. Similarly, L-4F loaded onto human HDL and injected into C57BL/6J mice was cleared quickly with T1/2 = 23.6 min. This was accompanied by a decline in human apoA-I with little or no effect on the mouse apoA-I. Based on these results, we propose that (i) LDL promotes the association of L-4F with HDL and (ii) in the presence of L-4F, oxidized lipids in LDL are rapidly transferred to HDL allowing these oxidized lipids to be acted upon by HDL-associated enzymes and/or cleared from the circulation.—Meriwether, D., S. Imaiizumi, V. Grijalva, G. Hough, L. Vakili, G. M. Anantharamaiah, R. Farias-Eisner, M. Navab, A. M. Fogelman, S. T. Reddy, and I. Shechter. Enhancement by LDL of transfer of L-4F and oxidized lipids to HDL in C57BL/6J mice and human plasma. J. Lipid Res. 2011. 52: 1795–1809.

Supplementary key words atherosclerosis • apolipoprotein A-I • apolipoprotein A-I mimic peptides • lipoproteins • high density lipoprotein • low density lipoprotein

One class of apoA-I mimic peptides is 18 amino acids in length. The progenitor was originally designed as a tool for investigating the functional properties of apoA-I (1, 2). The 243 amino acids of apoA-I form a class A amphipathic helix, an α-helix with opposing polar and nonpolar faces, with the positively charged amino acids of the polar face being found at the polar/non-polar interface and with the negatively charged amino acids of this same face being found in its center (3, 4). ApoA-I mimetics do not share sequence homology with apoA-I, but each possesses the same class A amphipathic helical structure (5). The mimetics differ with respect to the number of phenylalanines present on the nonpolar face of the amphipathic helix, ranging from the two phenylalanines of the original 18A (since named 2F) up to seven phenylalanines (so-called 7F) (6). Because of their common class A helical structure, all six mimetics in this group reproduce the basic lipid-associated properties of apoA-I (1, 6).

ApoA-I has been shown to possess both anti-atherogenic (7–9) and anti-inflammatory properties (10–12). The apoA-I mimetics, however, differ with respect to their biological activity. 2F, for example, was not able to inhibit atherosclerosis when administered to atherosclerosis-sensitive mice (6). By contrast, 5F significantly reduced diet-induced atherosclerosis in C57BL/6J mice fed an atherogenic diet (13). The peptides were thus screened for their ability to inhibit LDL-induced monocyte chemotaxis in a coculture of human aortic wall cells (6). The performance

Abbreviations: ¹1⁴C-L-4F, carbon-14 labeled L-4F; 4F, (Ac-D-W-F-K-A-F-Y-D-K-V-A-E-K-F-K-E-A-F-NH2); FPLC, fast protein liquid chromatography; 5HETE, 5S-hydroxy-6E, 8Z, 11Z, 14Z-eicosatetraenoic acid; 12HETE, 12S-hydroxy-5Z, 8Z, 10E, 14Z-eicosatetraenoic acid; 12HETE-d₆, 12S-hydroxy-5Z, 8Z, 10E, 14Z-eicosatetraenoic-5, 6, 8, 9, 11, 12, 14, 15-d₆ acid; 15HETE, 15S-hydroxy-5Z, 8Z, 11Z, 13E-eicosatetraenoic acid; 15HETE-d₆, 15S-hydroxy-5Z, 8Z, 11Z, 13E-eicosatetraenoic-5, 6, 8, 9, 11, 12, 14, 15-d₆ acid; 15HETE, 15S-hydroxy-5Z, 8Z, 11Z, 13E-eicosatetraenoic acid; 15HETE-d₆, 15S-hydroxy-5Z, 8Z, 11Z, 13E-eicosatetraenoic-5, 6, 8, 9, 11, 12, 14, 15-d₆ acid; 15HETE, 15S-hydroxy-5Z, 8Z, 11Z, 13E-eicosatetraenoic acid; 15HETE-d₆, 15S-hydroxy-5Z, 8Z, 11Z, 13E-eicosatetraenoic-5, 6, 8, 9, 11, 12, 14, 15-d₆ acid; 15HETE, 15S-hydroxy-5Z, 8Z, 11Z, 13E-eicosatetraenoic acid; 15HETE-d₆, 15S-hydroxy-5Z, 8Z, 11Z, 13E-eicosatetraenoic-5, 6, 8, 9, 11, 12, 14, 15-d₆ acid; hHDL, mouse LDL; hHDL, human HDL, hHDL, human LDL; 13HODE, 13S-hydroxy-9Z,11E-octadecadienoic acid; L-4F, 4F synthesized from all L-amino acids; mHDL, mouse HDL; SPR, surface plasmon resonance; T1/2, half-life.

⁎ To whom correspondence should be addressed. e-mail: ishechter@mednet.ucla.edu
4F has the structure Ac-D-W-F-K-A-F-Y-D-K-V-A-E-K-F-K-E-A-F-NH₂. There are four phenylalanines on the hydrophobic face of the amphipathic helix. The peptide is capped at the N- and C-terminals with acetyl and NH₂ groups, respectively. These caps stabilize the α-helix of 4F and thereby increase its lipid binding capacity (15). 4F synthesized from L amino acids (L-4F) and from D amino acids (D-4F) have both been used in biological studies. D-4F and L-4F were shown to have the same in vitro and in vivo properties, indicating that stereo-specificity is not essential to the activity of the peptide (16, 17).

In mouse models of atherosclerosis, 4F significantly reduced atherosclerotic lesions while improving the anti-inflammatory properties of HDL (18). 4F has also been reported to show promise in the treatment of other inflammatory disorders in preclinical studies (14, 19–21) including diabetes (22, 23), arthritis (22), hyperlipidemia-induced renal inflammation (23), influenza A pneumonia (24), vascular dementia (25), Alzheimer’s disease (26), and ovarian cancer (27). As discussed in detail (28), two recent studies in humans gave conflicting results regarding the ability of the peptide to alter HDL inflammatory properties. Based on new mouse studies, the most likely explanation for the discrepancy is that the negative study employed doses that were significantly lower than the positive study (28).

At least two mechanisms of action have been proposed to explain the effects of 4F, particularly on atherosclerosis (16). First, it has been shown that 4F can cause the remodeling of HDL and the formation of preβ HDL enriched in both apoA-I and paraoxonase activity (29, 30). This transformation lowers lipoprotein hydroperoxide levels; HDL becomes anti-inflammatory; and HDL-mediated cholesterol efflux and reverse cholesterol transport from macrophages are stimulated (29). Reverse cholesterol transport through preβ HDL is considered atheroprotective (31). It has thus been hypothesized that it is through the remodeling of HDL and the stimulation of reverse cholesterol transport that 4F was able to reduce lesions in mouse models of atherosclerosis (29). In objection to this mechanism, it has been pointed out that the physiological concentration of 4F necessary for HDL remodeling is higher than the minimum that has been shown necessary for atheroprotection (16).

In a second hypothesis, 4F was proposed to be involved in the sequestration and removal of oxidized lipids. It has been shown that 4F binds oxidized phospholipids and oxidized fatty acids with a high affinity, which is several orders of magnitude greater than apoA-I (32). Because oxidized lipids play central roles in both atherosclerosis (33) and other inflammatory disorders (34–36) including cancer (37, 38), the sequestration and removal of oxidized lipids by 4F has been put forward as the basis of a general mechanism of action regarding its various positive effects (20).

Consistent with this second hypothesis, administration of 4F to diabetic apoE null mice has been shown to significantly reduce atherosclerotic lesions while also decreasing hepatic concentrations of 13S-hydroxy-5Z, 8Z, 11Z, 13E-eicosatetraenoic acid (15HETE), 12S-hydroxy-5Z, 8Z, 10E, 14Z-eicosatetraenoic acid (12HETE), and 13S-hydroxy-9Z,11E-octadecadienoic acid (13HODE) (39). It has also been shown that L-4F injected into apoE null mice resulted in a significant reduction in plasma levels of oxidized fatty acids, including 15HETE and 13HODE, while lowering the HDL inflammatory index (40). However, the mechanism by which 4F causes the clearance of oxidized lipids remains unknown.

Both proposed mechanisms may involve the interaction between 4F and HDL. Thus, one purpose of our investigation was to use 14C-labeled L-4F to investigate the in vivo interaction between 4F and HDL. This aspect of our study received added importance due to a recent report questioning this relationship (41). We report here that L-4F associates immediately and preferentially with HDL upon its introduction into the circulation of C57BL/6j mice, and that this complex of HDL and L-4F is cleared quickly.

In the course of this and other lipoprotein association studies, we observed for the first time that LDL plays an important role in the relationship between L-4F and HDL. Not only does LDL appear to direct L-4F to HDL, it also amplifies the association between HDL and L-4F by several-fold. In the process, L-4F transfers oxidized lipids from LDL onto HDL. Thus, our results suggest a possible model by which oxidized lipids are cleared from the system by L-4F. L-4F transfers oxidized lipids into HDL, at least in part from LDL, and this complex of HDL, L-4F, and oxidized lipids is then cleared from the circulation.

### MATERIALS AND METHODS

**ApoA-I mimetic peptide L-4F**

The amino acid sequence of 4F is Ac-DWFKAAYDK-VAEKFKEAF-NH₂. L-4F was synthesized from L-αmino acids by the solid phase peptide synthesis method previously described (42, 43) with the modification that rink amide resin instead of Wang resin was used. During the peptide chain elongation, the ε-amino groups of the lysines were protected by t-butyloxycarbonyl. The final step for the cleavage of the peptide from the resin along with the cleavage of t-butyloxycarbonyl protecting groups was accomplished using trifluoroacetic acid with suitable scavengers. Under these conditions, the N-terminal acetyl group is stable whereas all of the other t-butanol protecting groups are cleaved. For synthesizing 14C-peptide, 14C-acetic anhydride was used instead of acetic anhydride as previously described (44). Peptide purity was ascertained by mass spectral analysis and analytical HPLC (44). Both L-4F and 14C-L-4F were synthesized in the laboratory of G. M. Anantharamaiah of the University of Alabama at Birmingham.

**Human plasma**

Healthy human donor subjects were recruited after written consent was approved by the University of California at
Los Angeles (UCLA) Institutional Review Board. Fasting blood was collected in heparinized tubes (Becton Dickinson) and the plasma was separated by centrifugation.

**Mice and mouse plasma**

Both wild-type C57BL/6J mice and apoE null mice on a C57BL/6J background were obtained from the breeding colony of the Department of Laboratory and Animal Medicine at the David Geffen School of Medicine at UCLA after having been originally purchased from Jackson Laboratories. The mice were maintained on a chow diet (Ralston Purina). To obtain blood for assays, the mice were subjected after overnight fast to either local or terminal bleeds in which either 75 or 250 µl of blood was removed from the retro-orbital sinus. The mice were under mild isoflurane anesthesia, and the blood was collected into heparinized capillary tubes and chilled plasma separator tubes (Becton-Dickson). Plasma was preserved in 10% sucrose. All experiments were performed using protocols approved by the Animal Research Committee at UCLA and in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

**Isolation of lipoproteins**

Human LDL (hLDL) and HDL (hHDL) were isolated by density centrifugation and were obtained from the Atherosclerosis Research Unit Core facility. LDL was isolated between densities of 1.019–1.063 g/ml and HDL between densities of 1.063 and 1.21 g/ml. After isolation, the lipoproteins were obtained from the Atherosclerosis Research Unit Core facility. LDL was isolated by density centrifugation and were obtained from the Atherosclerosis Research Unit Core facility. LDL was isolated between densities of 1.019–1.063 g/ml and HDL between densities of 1.063 and 1.21 g/ml.

**FPLC separation and purification of plasma and lipoprotein mixtures**

Plasma and isolated lipoprotein mixtures were fractionated by gel permeation fast protein liquid chromatography (FPLC). The system consisted of dual Superose 6 columns in series (Amersham Biosciences) driven by a Beckman Coulter SystemGold 126NM Solvent Module through a 168NM Detector, controlled by Beckman’s 32 Karat 7.0 software, and collected by a Foxy 200 (Teledyne Isco). The column was eluted with a solution of 0.9% (w/v) NaCl, 0.03% (w/v) NaNO₃, pH 8.2 at a rate of 0.500 ml/min and fractions were collected. The column was flushed between runs with 0.5 ml of 1% Triton X-100 in PBS.

**Determination of cholesterol, radioactivity, and protein**

Cholesterol was determined using a commercially available kit (Thermo Scientific, 2011). Radioactivity was determined as disintegrations per minute (dpm) by scintillation counting either 0.250 or 0.500 ml of each fraction in 10.0 ml BioSafe II scintillation fluid (RPI Corporation) on a Packard Tri-Carb Model A900TR using QuantaSmart software. Protein concentration of isolated lipoproteins was determined by the Lowry method with Peterson’s modification using a commercially available kit (Sigma-Aldrich, TP0300).

**Incubation of ¹⁴C-L-4F with human and mouse plasma**

Human plasma, C57BL/6j plasma, and apoE⁻/⁻ plasma samples were incubated separately with ¹⁴C-L-4F (200 µl plasma; 50 µg ¹⁴C-L-4F with specific activity of 0.25 to 1.0 µCi) for 1 h at 37°C after light vortexing. Subsequently, the mixes were separated using FPLC and cholesterol and radioactivity were determined for the fractions.

**Lipid supplementation of LDL: 5HETE, 15HETE**

hLDL was supplemented separately with 5S-hydroxy-6E, 8Z, 11Z, 14Z-eicosatetraenoic acid (5HETE) and 15HETE. Both lipids were from Cayman Chemical (34230, 34720) and were supplied as 0.1 µg/µl solutions in ethanol. Lipid (0.5 µg/1.0 mg protein of LDL) was injected directly into the LDL, which had been brought up to 100 µl in PBS. The lipid was injected all at once from above the surface of the LDL and the resultant solution was pipetted up and down followed by gentle vortexing. The mixes were washed using a 100 kDa cut-off filter (Millipore, UFC810024) to remove any unassociated lipids.

**Isolated human lipoproteins**

Human lipoproteins (1.0 mg protein each of hHDL, hLDL, hLDL-5HETE, and hLDL-15HETE) were isolated by density centrifugation and were obtained from the Atherosclerosis Research Unit Core facility. LDL was isolated between densities of 1.019–1.063 g/ml and HDL between densities of 1.063 and 1.21 g/ml. After isolation, the lipoproteins were dialyzed to remove the added salts.

**FPLC separation and purification of plasma and lipoprotein mixtures**

Plasma and isolated lipoprotein mixtures were fractionated by gel permeation fast protein liquid chromatography (FPLC). The system consisted of dual Superose 6 columns in series (Amersham Biosciences) driven by a Beckman Coulter SystemGold 126NM Solvent Module through a 168NM Detector, controlled by Beckman’s 32 Karat 7.0 software, and collected by a Foxy 200 (Teledyne Isco). The column was eluted with a solution of 0.9% (w/v) NaCl, 0.03% (w/v) NaNO₃, pH 8.2 at a rate of 0.500 ml/min and fractions were collected. The column was flushed between runs with 0.5 ml of 1% Triton X-100 in PBS.

**Determination of cholesterol, radioactivity, and protein**

Cholesterol was determined using a commercially available kit (Thermo Scientific, 2011). Radioactivity was determined as disintegrations per minute (dpm) by scintillation counting either 0.250 or 0.500 ml of each fraction in 10.0 ml BioSafe II scintillation fluid (RPI Corporation) on a Packard Tri-Carb Model A900TR using QuantaSmart software. Protein concentration of isolated lipoproteins was determined by the Lowry method with Peterson’s modification using a commercially available kit (Sigma-Aldrich, TP0300).

**Incubation of ¹⁴C-L-4F with isolated human lipoproteins**

Isolated human lipoproteins (1.0 mg protein each of hHDL, hLDL, hLDL-5HETE, and hLDL-15HETE) were separately mixed with ¹⁴C-L-4F (50 µg and 0.5 µCi) and brought up to 300 µl in PBS. Each mix was lightly vortexed and incubated at 37°C for 1 h before being fractionated using FPLC. Radioactivity and cholesterol were determined for the fractions. In additional experiments, ¹⁴C-L-4F (50 µg and 0.5 µCi) was combined with both hHDL and hLDL or LDL-15HETE (1.0 mg protein each). After light vortexing, the mixes were incubated for either 5 min or 1 h at 37°C before being fractionated using FPLC and assayed as described above.

**Loading of hLDL and hHDL with ¹⁴C-L-4F**

hLDL (2.0 mg protein) was mixed with 100 µg and 1.5 µCi ¹⁴C-L-4F and incubated at 37°C for 1 h. In order to remove unassociated ¹⁴C-L-4F, the mix was fractionated using FPLC after spin filtering. Cholesterol and radioactivity were determined in order to ensure that ¹⁴C-L-4F had associated with hLDL. The LDL fractions were pooled and subsequently concentrated using a 100 kDa cut-off filter (Millipore, UFC810024). Radioactivity per cholesterol was determined before and after concentration to ensure the integrity and adequate recovery of hLDL-¹⁴C-L-4F. Alternatively, hHDL (1.0 mg protein/sample) was loaded with ¹⁴C-L-4F (ranging from 5.0 to 200 µg) by mixing and a brief incubation at 37°C.

**Incubation of hLDL-¹⁴C-L-4F and mouse and human plasma**

hLDL was loaded with ¹⁴C-L-4F as described above. Human plasma (200 µl) or C57BL/6j plasma (120 µl) were mixed with concentrated hLDL-¹⁴C-L-4F (125 µl of a 250 µl concentrate, see above). The mixes were incubated at 37°C for 1 h before being fractionated using FPLC. Cholesterol and radioactivity were determined for the fractions.

**Lipid supplementation of hLDL with 15HETE-d₈**

hLDL was supplemented with 10.0 µg 15S-hydroxy-5Z, 8Z, 11Z, 13E-eicosatetraenoic-5, 6, 8, 9, 11, 12, 14, 15-d₈
acid (15HETE-d8) (Cayman, 334720). The solvent was evaporated under argon in a 10 ml glass test tube, and the lipid was brought up directly in hLDL (4.0 mg protein, 290 µl) by 5 min of gentle swirling and vortexing. The hLDL was then purified by washing three times using a 100 kDa cut-off filter. Cholesterol and protein were then determined for the concentrate.

Transfer of 15HETE-d8 from hLDL to hHDL

The experiment was carried out as follows: first, hLDL was supplemented with purified hHDL in the absence or the presence of L-4F. For this, 0.5 mg hLDL protein, 0.5 mg hLDL protein and 0.5 mg hHDL protein, and the latter together with 25 µg L-4F were brought up to a total volume of 150 µl in PBS. The three preparations were then incubated at 37°C for 1 h. An amount of 125 µl of each sample was then separated using FPLC as described. The isolated lipoprotein fractions from the various experimental groups were pooled, the lipids were extracted, and the extracts were analyzed by LC/MS/MS.

Lipid extraction and sample preparation for LC-ESI-MS/MS analysis

Lipoproteins were separated using FPLC; fractions for each individual lipoprotein were pooled in accord with the distributions of cholesterol, and the volumes of the pooled samples were recorded. 12HETE-d8 (Cayman, 334570) was added to each pooled sample as an internal standard (100 µL of 100 ng 12HETE-d8/ml methanol). The pH of the samples was adjusted to ~3.0 using formic acid. Oasis HLB 3 cc (60 mg) solid-phase extraction cartridges (Waters, 186001880) on a vacuum manifold were equilibrated with 5% methanol in water. The pH of the samples was adjusted to ~3.0 using formic acid. Oasis HLB 3 cc (60 mg) solid-phase extraction cartridges (Waters, 186001880) on a vacuum manifold were equilibrated with 2 ml methanol followed by 2 ml water. The samples were slowly loaded on the cartridges under vacuum. After the samples had completely flowed through at ~0.5 ml/min, the cartridges were washed with 2ml 5% methanol in water. Lipids were then eluted from the cartridges with 2 ml methanol. The eluates were evaporated to dryness under argon at 37°C. Each dried lipid extract was then resuspended in 100 µl of methanol using gentle vortexing. The samples were transferred to autosampler vials (Fisher scientific, 03-396-74) for LC/MS/MS analysis.

LC/MS/MS analysis

LC/MS/MS was performed using a 4000 QTRAP quadrupole mass spectrometer (Applied Biosystems) equipped with electrospray ionization source. The HPLC system utilized an Agilent 1200 series LC pump equipped with a thermostatted autosampler (Agilent Technologies). Chromatography was performed using a Luna C-18(2) column (3 µm particle, 150 × 3.0mm; Phenomenex) with a security guard cartridge (C-18; Phenomenex) at 40°C. Mobile phase A consisted of 0.1% formic acid in water and mobile phase B consisted of 0.1% formic acid in acetonitrile. The autosampler was set at 4°C. The injection volume was 10 µl and the flow rate was controlled at 0.4ml/min. The gradient program was as follows: 0–2 min, 50% B; 2–3 min, linear gradient from 50% to 60% B; 3–15 min, linear gradient from 60 to 65% B; 15–17 min, 65% B; 17–19 min, linear gradient from 65 to 100% B; 19–21 min 100% B; 21–23 min, linear gradient from 100% to 50% B; 23–27 min, 50% B. The data acquisitions and instrument control were accomplished using Analyst 1.4.2 software (Applied Biosystems). Detection was accomplished by using the multiple reaction monitoring mode with negative ion detection; the parameter settings used were: ion spray voltage = −4500 V; curtain gas = 20 (nitrogen); ion source gas 1 = 50; ion source gas 2 = 30; ion source gas 2 temperature = 550°C. Collision energy, declustering potential, and collision cell exit potential were optimized for each compound to obtain optimum sensitivity. The transitions monitored were: m/z 327.1→226.1 for 15HETE-d8 and 327.1→184.0 for 12HETE-d8.

Binding studies

Binding studies were performed by surface plasmon resonance (SPR) on a BIAcore 3000 system (BiaCore AB) as previously described (32). In short, L-4F was immobilized on a BIAcore CM5 sensor chip activated per the manufacturer’s protocol. hLDL and hHDL that had been isolated by ultracentrifugation was further purified using FPLC. FPLC was also used to isolate HDL from the plasma of a male C57Bl/6j mouse. The FPLC fractions were pooled and the protein concentrations of the lipoproteins were first determined. Molarity was subsequently calculated by assuming an average molecular weight (MW) for HDL of 360 kDa and for LDL of 2500 kDa, also supposing that protein makes up 40% of the mass of HDL and 22.5% of the mass of LDL (46). Further solutions of these analytes were then prepared in buffer containing 10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) surfactant P20. Binding was measured by observing the change in the SPR angle as 30 µL of analyte in various concentrations flowed over the sample for 3 min at 10 µL/min. Equilibrium affinity constant (Kd) values were calculated from assays performed with five different analyte concentrations that gave binding responses of 50 to 300 resonance units. The calculations were done with BIAcore’s BIAevaluation software, version 4.1, assuming a molar ratio of 1:1 analyte:peptide binding.

Injection of mice and collection of plasma

C57Bl/6j mice were fasted overnight. Samples of 14C-L-4F alone, hHDL/hHDL-14C-L-4F, or hLDL-14C-L-4F were introduced directly into the circulation of these mice via their tail veins using a 29 gauge insulin needle and syringe (Becton Dickinson, 329424). In the case of 14C-L-4F alone (600 µg and 0.5 µCi brought up to 150 µl in 1× PBS), the peptide was introduced directly into each of five female 12-week-old mice. In the case of hHDL/hHDL-14C-L-4F, each of six female 12-week-old mice was injected with hHDL (1.0 mg protein) that had been loaded with 0 to 200 µg 14C-L-4F and brought up to 150 µl in 1× PBS in the manner described above. In the case of hLDL-14C-L-4F, three male 10-week-old mice were injected with hLDL-14C-L-4F concentrate (0.10 µCi, 1.2 mg protein LDL, 70 µl total volume). Blood was drawn at various times after injection (14C-L-4F, from 3 min
to 30 min; hHDL/hHDL-$^{14}$C-L-4F, from 0 min to 120 min; and hLDL-$^{14}$C-L-4F, from 3 min to 60 min). During each bleed, $\sim$100 µl of blood was drawn from the retro-orbital sinus under mild isoflurane anesthesia as above. The plasma samples were separated and preserved as above. The plasma samples were then either assayed directly or fractionated before further assays were performed.

**Western blotting and quantification**
Samples were first separated by gel electrophoresis under reduced and denaturing conditions on 10% NuPAGE Novex bis-tris gels (Invitrogen) using MOPS SDS running buffer (Invitrogen). Gels were transferred to polyvinylidene fluoride membrane (Millipore, IPVH00010) and some probed 1:100 with rabbit, human-and-mouse-specific anti-apoA-I antibody (Abcam, ab33470) and 1:5000 with HRP-conjugated anti-rabbit IgG (Amersham, NA931). Others were probed 1:10,000 with rabbit, anti human-specific anti-apoA-I (Abcam ab52945) or 1:20,000 with rabbit anti mouse-specific anti-apoA1 (Abcam ab20355). For loading control the blots were probed 1:1500 with mouse, mouse-and-human-specific anti-albumin (Santa Cruz, sc58588) followed 1:5000 with HRP-conjugated anti-rabbit IgG or 1:5000 with anti-mouse IgG (Amersham, NCA931). Blots were detected fluorescently on a Typhoon 9400 fluorescent scanner (GE Healthcare) using ECL Plus Western blotting detection reagent (Amersham, RPN2106). In order to quantify the amount of apoA-I present in each sample, the scan of each blot was subjected to analysis using ImageQuant 5.0 software (Molecular Dynamics).

**Statistical analyses**
Statistical analyses were performed by unpaired two-tail $t$-test.

**RESULTS**
When $^{14}$C-L-4F was incubated with fasted human plasma it associated almost completely with the HDL fraction as determined by FPLC fractionation (Fig. 1A). On occasion, when a low level of VLDL was present in some of the donors’ plasma, $^{14}$C-L-4F was detected in this fraction as well (data not shown). However, repeatedly, the LDL fraction of the human plasma (fractions 18–26) contained little to no radioactivity under these conditions.

![Fig. 1. L-4F association with lipoproteins in human plasma and in C57BL/6J and apoE$^{-/-}$ mouse plasma.](image)

$^{14}$C-L-4F (50.0 µg with 0.25 µCi) was incubated with 200 µl human plasma for 1 h at 37°C. The mix was separated using FPLC, and radioactivity (○) and cholesterol (□) were determined for the fractions (A). $^{14}$C-L-4F (50 µg with 1.0 µCi) was also incubated with 200 µl of either wild-type C57BL/6J or apoE$^{-/-}$ plasma for 1 h at 37°C. The mixes were fractionated using FPLC, and radioactivity (■, apoE$^{-/-}$; △, BL6) and cholesterol (□, apoE$^{-/-}$; □, BL6) were determined for the fractions (B). The figure depicts almost complete association with HDL in human and C57BL/6J plasma and preferential association with apoB-containing lipoproteins in plasma of the apoE$^{-/-}$ mouse. (The figure is a representative of four different experiments.)
Similar results were obtained for the association of 
$^{14}$C-L-4F with C57BL/6j mouse plasma lipoproteins. Here, 
too, the L-4F radioactivity was associated almost completely 
with the HDL fraction of fasting C57BL/6j mice (Fig. 1B). 
In contrast, when $^{14}$C-L-4F was incubated with the plasma 
of fasting apoE-null mice, very little L-4F is associated with 
the HDL fraction. Rather, L-4F was detected in earlier 
fractions together with the distribution of cholesterol.

When incubated with ultracentrifuge-purified hHDL, 
$^{14}$C-L-4F was associated with the HDL fraction off the 
FPLC. However, unlike in full human plasma, when ultra-
centrifuge-purified hLDL alone was incubated with $^{14}$C-L-4F, 
in the absence of other lipoproteins, the radioactivity was 
associated with the LDL. (Fig 2B). This association of L-4F 
with isolated LDL can be enhanced when the LDL is pre-
loaded with the oxidized lipid 15HETE. This enhanced 
association is dose-dependent and a calculated 2.07-fold 
significant increase in radioactivity associated with LDL was 
observed when 5.0 µg 15HETE was preloaded into the pu-
rified hLDL ($P < 0.001, n = 3$) (Fig 2B). Similar results were 
observed for 5HETE-loaded hLDL (data not shown).

In an attempt to study partially reconstituted human 
plasma, the purified human HDL and LDL were incubated 
with $^{14}$C-L-4F both separately and together (Fig. 3A). As 
observed before, each of the isolated lipoproteins associ-
ated with $^{14}$C-L-4F, with the HDL efficiency for the associa-
tion exceeding by 8-fold that of the purified LDL alone on 
a per cholesterol basis. However, a highly synergistic effect 
in the binding of $^{14}$C-L-4F to the HDL was observed when 
LDL was present. Although there was a 60% decrease in 
the binding of $^{14}$C-L-4F to the LDL (a loss of 7,350 counts), 
a most pronounced 2.5-fold increase in its binding to HDL 
is shown (a gain of 36,307 counts). The association of $^{14}$C-
L-4F with the HDL component of the mix was virtually 
complete in 5 min (data not shown) as compared with 
60 min. A comparable low level of $^{14}$C-L-4F with the LDL 
was also observed for the two time periods. This result was 
not plasma donor-specific and was reproducible with 
three different donor pairs of HDL and LDL prepara-
tions. The ability of L-4F to be transferred from hLDL to 
mouse HDL (mHDL) in full C57BL/6j plasma is demon-
strated in Fig. 3B. When $^{14}$C-L-4F-preloaded hLDL isolated 
by FPLC (from Fig. 2B) was mixed with C57BL/6j mouse 
plasma, a distinct transfer of the L-4F to the HDL fraction 
was observed. This transfer was incomplete under the 
in vitro experimental conditions and when compared with

---

**Fig. 2.** L-4F associates with isolated human HDL and LDL. 15HETE supplementation of LDL increases 
the association between L-4F and LDL. hHDL and 
hLDL were isolated from plasma by ultracentrifuga-
tion. 1.0 mg of HDL protein (A) was incubated for 
1 h with $^{14}$C-L-4F (50 µg; 0.5 µCi), and radioactivity 
and cholesterol were determined for the iso-
FPLC fractions. 15HETE enhances the association 
between L-4F and LDL. In a parallel experiment, 
samples of 1.0 mg protein of hLDL were supple-
cemented with 0 to 5.0 µg 15HETE. The lipoprotein 
preparations were then incubated with $^{14}$C-L-4F (50 µg; 
0.25 µCi), isolated on FPLC and the FPLC fractions 
were assayed for both radioactivity and cholesterol. 
A dose-dependent increase in association be-
tween L-4F and LDL was observed, with calculated 
total counts in the various LDL fractions of 3,975, 
4,653, 5,661 and 8,258 dpm accordingly. A significant 
2.07-fold increase was observed between the associa-
tion of $^{14}$C-L-4F with 5.0 µg 15HETE supplemented 
and with 15HETE unsupplemented LDL ($P < 0.001, 
n = 3$) (B).
the transfer of $^{14}$C-L-4F from the same preloaded LDL to the mHDL upon introduction to the mouse circulation (see below). Similar transfer to the HDL fraction in isolated human plasma was observed (data not shown).

15HETE increased the binding of $^{14}$C-L-4F to isolated hLDL as demonstrated in Fig. 2B. One might expect, therefore, that a preloading of LDL with 15HETE would result in a decrease in association between L-4F and HDL when all three are coincubated, as the 4F would adhere to the LDL. Instead, a 50% increase in the association of $^{14}$C-L-4F with hHDL was observed for a mixture of HDL with 15HETE-preloaded hLDL as compared with LDL by itself. Under the incubation conditions, there was no observed net transfer of free or esterified cholesterol between the two purified lipoproteins (Fig. 4B). However, we have obtained evidence that the presence of L-4F can initiate the transfer of oxidized lipids present in LDL to the HDL fraction. Figure 5 depicts the effect of L-4F on the transfer of deuterated 15HETE-d₄ from preloaded hLDL to hHDL as determined by LC/MS/MS analysis. Only a residual transfer of this oxidized lipid to the HDL was observed in the absence of L-4F. However a 35-fold increase was observed when L-4F was added to the mix (Fig. 5).

The association of $^{14}$C-L-4F with lipoproteins was also studied in the circulation by introducing the radioactive peptide or a lipoprotein preloaded with it by tail vein injection into C57BL/6J mice. Fractionation of plasma obtained immediately post tail vein injection with $^{14}$C-L-4F for 1 h before FPLC fractionation. Radioactivity (●, LDL only; ■, HDL only; ○, LDL+HDL 60 min) and cholesterol (○, LDL+HDL 60 min) were determined for the isolated fractions (A). Radioactivity and cholesterol for the 5 min sample were very similar to those of the 60 min sample (data not shown). In a separate experiment, hHDL was first preloaded with $^{14}$C-L-4F by incubating 2.0 mg hLDL protein with 100 µg (1.0 µCi) $^{14}$C-L-4F before processing by FPLC to remove unbound peptide. The LDL fractions were pooled and concentrated, and the hHDL-$^{14}$C-L-4F concentrate was incubated for 1 h with isolated C57BL/6J plasma. The reaction mixture was fractionated by FPLC, and radioactivity (●) and cholesterol (○) were determined for the individual fractions (B). This result was not plasma donor-specific and was reproducible with three different donor pairs of hHDL and hLDL preparations as well as using isolated plasma from a different mouse.

The association of $^{14}$C-L-4F with lipoproteins was also studied in the circulation by introducing the radioactive peptide or a lipoprotein preloaded with it by tail vein injection into C57BL/6J mice. Fractionation of plasma obtained immediately post tail vein injection with $^{14}$C-L-4F showed association with a fraction containing cholesterol and the protein apoA-I. This fraction is eluted off the FPLC column with retention expected for HDL (Fig. 6A). The amount of L-4F radioactivity associated with this fraction rapidly declined in time with each subsequent bleed from the same mouse (Fig. 6B). After 30 min, it accounted for only 30% of the amount observed 3 min after injection. The radioactive material associated with the HDL fraction was identified as intact L-4F by LC/MS/MS. There was a time-dependent increase of radioactivity in the later eluting fractions 41 to 44. However, no intact $^{14}$C-L-4F was present in these fractions as determined by LC/MS/MS. The identity of this material(s) is presently unknown, but it appears to be an aggregation of degraded/processed substance(s). The results of this independent experiment confirm and extend our previously published data (19).
(Fig. 6C). By 7 min, there was already a significant 15% reduction ($P < 0.01$) in $^{14}$C-L-4F associated with the HDL-containing fractions of the plasma. Further significant reductions of 28% ($P < 0.01$) and 50% ($P < 0.001$) were observed by 10 and 15 min, respectively. Finally, by 30 min, there was a decline in 80% of the $^{14}$C-L-4F radioactivity ($P < 0.001$).

Clearance of radioactivity and human apoA-I from $^{14}$C-L-4F-preloaded hHDL was measured by injecting four fasting C57BL/6J female mice with this substance and comparing to two mice injected with the same hHDL lacking L-4F (Fig. 7). In this study, samples of hHDL (1.0 mg protein) were preloaded with increasing amounts of $^{14}$C-L-4F, ranging from 0 µg up to 200 µg. Blood was drawn and plasma isolated immediately postinjection (time 0), and at 30, 60, and 120 min. Time-dependent clearance of radioactivity from the circulation was observed for each of the mice. The average half-life ($T_{1/2}$) clearance value for all concentrations of $^{14}$C-L-4F is calculated to be 23.6 min (SD = 5.0 min) (Fig. 7A). In addition, using monospecific antibodies, time-dependent decline in the amounts of human and mouse apoA-I were determined in these plasma samples by Western blot analyses. $^{14}$C-L-4F concentration-dependent rate of decline was observed for the human apoA-I at all amounts of $^{14}$C-L-4F (Fig. 7B) whereas lack of such decline was observed for the mouse apoA-I for 120 min in up to 100 µg $^{14}$C-L-4F (Fig. 7C). A 12% decline in mouse apoA-I was observed only for 200 µg $^{14}$C-L-4F after 2 h as compared with about 40% decline in human apoA-I in the same plasma for all amounts of $^{14}$C-L-4F. No decline in either human or mouse apoA-I was detected in the plasma of mice injected with hHDL lacking L-4F. At 120 min, a decrease of about 20% total HDL cholesterol is observed (Fig. 7D). This decrease in HDL cholesterol is dependent on the presence of L-4F and cannot be observed in its absence.

The transfer of $^{14}$C-L-4F from preloaded hLDL (from Fig. 2B) to plasma lipoproteins and its clearance from the circulation was studied by injecting it into fasting female C57BL/6J mice (Fig. 8). Unlike in the in vitro experiment (Fig. 3B), here by 3 min postinjection, the majority of radioactivity present in the plasma was associated with the HDL-containing fractions of the plasma and only 14% of the counts were detected on the LDL fraction. These counts were altogether cleared from the LDL fraction by 10 min. Time-dependent clearance of radioactivity was observed in the HDL fraction as well. At 60 min, only 15% of the radioactivity remained in the HDL compared with the 3 min time collection (Fig. 8A). Because no substantial parallel decrease in the LDL cholesterol was observed (Fig. 8B), it indicates that L-4F was transferred from hLDL to mHDL in the circulation both quickly and completely to the limits of detection.

The affinity of the individual isolated lipoproteins to L-4F was evaluated by a determination of their dissociation constants ($K_D$) to immobilized L-4F onto a sensor chip in a BIACore 3000 system. The binding was determined by an SPR using multiple concentrations of the lipoproteins. These results are depicted in Fig. 9 and show that L-4F binds to HDL with significantly greater affinity than to LDL. The affinities between L-4F and HDL, hHDL, and mHDL were not significantly different, with $K_D = 3.36 \times 10^{-8}$ and $1.03 \times 10^{-8}$ M, respectively. By contrast, the affinity between L-4F and LDL was significantly less than the affinity between L-4F and either HDL or mHDL, with $K_D = 1.17 \times 10^{-6}$ M ($**$, $*: P = 0.001, n = 5$).

**DISCUSSION**

Our in vitro studies indicate that L-4F associates with the HDL fraction of human and C57BL/6J plasma (Fig. 1A, B). Similarly, L-4F associated with isolated hHDL (Fig. 2A)
LDL enhances transfer of L4-F and oxidized lipids to HDL. However, this difference in relative affinity to the two isolated lipoproteins cannot by itself fully account for the lack of association between LDL and L-4F observed in plasma. Based on the differing affinities, it would be anticipated that a partition of radioactivity would be observed between LDL and HDL when 14C-L-4F was applied to full plasma. However, in most experiments, as the one depicted in Fig. 1A, the L-4F radioactivity was associated with HDL with no detectable radioactivity in the LDL fraction.

To further elucidate the binding of L-4F with HDL and LDL, we studied the interaction of L-4F with lipoproteins in partially reconstituted human plasma that consisted of only HDL and LDL. As noted above, L-4F will associate with purified HDL (see Fig. 2B). Likewise, if LDL is markedly decreased, as is the case in apoE−/− mouse plasma, the association profile of L-4F with the FPLC fractions of the plasma mirrors the profile of cholesterol across those same fractions (see Fig. 1B).

L-4F displays greater relative affinity for purified HDL than for purified LDL when the binding is compared on a per cholesterol basis of the lipoproteins. A 12-fold greater relative affinity of L-4F is shown for isolated hHDL than for isolated hLDL (Fig. 3A, B). Even when LDL is supplemented with oxidized lipids, which are reported to bind 4F with high affinity (32), L-4F still exhibits an almost 9-fold greater relative affinity for an unsupplemented hHDL than for 15HETE-supplemented hLDL. A more quantitative determination of the affinities of the various lipoproteins to L-4F is the determination of the individual K_Ds. (Fig. 9). In agreement with the data presented in Fig. 3, L-4F binds with about two orders of magnitude higher affinity (lower K_D) to both mHDL and hHDL as compared with the affinity to hLDL. However, this difference in relative affinity to the two isolated lipoproteins cannot by itself fully account for the lack of association between LDL and L-4F observed in plasma. Based on the differing affinities, it would be anticipated that a partition of radioactivity would be observed between LDL and HDL when 14C-L-4F was applied to full plasma. However, in most experiments, as the one depicted in Fig. 1A, the L-4F radioactivity was associated with HDL with no detectable radioactivity in the LDL fraction.

To further elucidate the binding of L-4F with HDL and LDL, we studied the interaction of L-4F with lipoproteins in partially reconstituted human plasma that consisted of only HDL and LDL. As noted above, L-4F will bind with isolated HDL or LDL. Interestingly, when L-4F was coincubated with both HDL and LDL, the association between L-4F and LDL decreased by at least 60% whereas the association between L-4F and HDL increased 2- to 3-fold (Fig. 3A). This cannot be the consequence of a competition for the L-4F between HDL over LDL because, in the absence of LDL, the binding of the L-4F to the HDL is several fold lower compared with the same binding in the presence of LDL under the same experimental conditions. Therefore, there was a true synergistic binding of L-4F to HDL in the presence of LDL.

as was reported by others (13, 29, 47). What is interesting to note is the variable association between LDL and L-4F under different conditions. When HDL was present, as in full human plasma, L-4F associated little to none with the LDL fraction of the plasma, even when LDL exceeded HDL by ~2-fold on a cholesterol basis (see Fig. 1A). However, if no other lipoprotein is present, L-4F will associate with purified hHDL (see Fig. 2B). Likewise, if LDL is markedly decreased, as is the case in apoE−/− mouse plasma, the association profile of L-4F with the FPLC fractions of the plasma mirrors the profile of cholesterol across those same fractions (see Fig. 1B).

L-4F enhances the transfer of 15HETE from hLDL onto hHDL. LDL was supplemented with 2.5 µg deuterated 15HETE (15HETE-d8) per mg of LDL protein. 0.5 mg of HDL protein and 0.5 mg of the 15HETE-d8 supplemented LDL protein were then incubated at 37°C for 1.0 hr with or without L-4F. The mixes were fractionated on the FPLC. The isolated lipoprotein fractions were pooled and lipids were extracted from those pooled fractions. Supplemented LDL alone was treated similarly. The extracts were analyzed using LC/MS/MS. The figure shows the amount of 15HETE-d8 present in the isolated lipoproteins as a percentage of the amount originally present in the LDL. A 35-fold increase in the transferred 15HETE-d8 is observed in the presence of L-4F (P = 0.02) n = 3.
The increase in the binding of L-4F to HDL in the presence of LDL can be partially explained by a transfer of L-4F from LDL to HDL. This possibility was investigated by the use of purified isolated hLDL preloaded with $^{14}C$-L-4F (Fig. 2B) in incubation with mouse plasma (Fig. 3B). As shown in Fig. 3B, 50.7% of the L-4F was found in the HDL fraction of the mouse plasma, clearly indicating a possible transfer of L-4F between these lipoproteins. This process of transfer of L-4F from preloaded hLDL to mHDL was by far more efficient in vivo (see below).

LDL and HDL can exchange lipids. In the circulation, this transfer is often effected by mediating proteins such as lipid transfer protein (48), lipid transfer inhibitor protein (48), and cholesterol ester transfer protein (49). Cholesterol ester transfer protein is often bound to HDL (50, 51). However, there is evidence that lipid transfer can occur even in the absence of transfer proteins (48). Moreover, it has been shown that L-4F binds some oxidized lipids with very high affinity (32). It is, therefore, reasonable to assume that the transfer of some lipids, which are complexed to L-4F from LDL into HDL, is involved in the enhanced uptake of L-4F by HDL. In this proposed mechanism, L-4F associates with both lipoproteins in part by binding with the oxidized lipids. The L-4F-lipid complex would then be transferred from LDL to HDL efficiently, explaining the loss of L-4F from LDL and the gain by HDL. In addition, it is possible that when HDL takes up these lipids, it increases the uptake of free L-4F from the mix, explaining the synergistic increase in the binding of L-4F to HDL in the presence of LDL.

If such a mechanism, in which an oxidized lipid-L-4F adduct is transferred as a complex from LDL to HDL exists, it implies that addition of L-4F or oxidized lipid to LDL should enhance the transfer of oxidized lipid or L-4F, respectively, to HDL. These two complementing experiments are demonstrated in Figs. 4 and 5. In the first experiment, hHDL exhibited an ~2-fold increase in its association with L-4F when incubated with LDL as compared with unsupplemented LDL (Fig. 4A). If the added 15HETE had remained on LDL particles, we might have expected an increase in the amount of L-4F associated with the LDL fraction compared with unsupplemented LDL demonstrated before (Fig. 2B). However, no such increase was observed and instead, an increase in radioactivity was demonstrated strictly for hHDL. This result strongly suggests that the oxidized lipid is transferred to HDL, most likely as a complex with L-4F.

The oxidized lipid transfer hypothesis is further supported by the experiments represented in Fig. 5. In these experiments...
LDL enhances transfer of L4-F and oxidized lipids to HDL, which would not occur spontaneously in its absence. These observations also explain the synergistic increase in the binding of L-4F to HDL in the presence of LDL. LDL serves as a potential source of oxidized lipids for which HDL serves as the sink in the presence of L-4F.

Our in vivo studies establish that L-4F associates primarily with HDL in the circulation of fasting mice. Using 14C-L-4F, we have established that it eluted with almost complete

**Fig. 7.** Radioactivity and apoA-I from 14C-L-4F loaded hHDL injected via tail vein into C57BI/6J mice are cleared quickly from the circulation. Increasing amounts of 14C-L-4F (0 µg to 200 µg) were loaded into samples of hHDL (1.0 mg protein). Each of the samples was then tail vein-injected into separate male C57BI/6J mice. Blood was collected from each mouse immediately after injection (0 min) and then at 30, 60, and 120 min post injection. Radioactivity in plasma aliquots was determined for each collected sample (X, 0 µg 14C-L-4F; □, 5.0 µg 14C-L-4F; △, 50 µg 14C-L-4F; ○, 100 µg 14C-L-4F; ○, 200 µg 14C-L-4F). A time-dependent decline of radioactivity in the plasma is shown for each of the injected L-4F concentrations with average T1/2 = 23.6 min (SD = 5.0 min) (A). Plasma samples were also analyzed by Western blots for human-specific apoA-I using mouse albumin as a loading control. The amount of human apoA-I present in each sample is expressed as a percent of the amount at time = 0 for that mouse or, in the case of 0 µg 14C-L-4F, for those two mice (X, 0 µg 14C-L-4F; □, 5.0 µg 14C-L-4F; △, 50 µg 14C-L-4F; ○, 100 µg 14C-L-4F; ○, 200 µg 14C-L-4F). Time-dependent decrease in human apoA-I is shown for the 120 min, the longest bleed time, for all 14C-L-4F loaded samples. Western blots show human apoA-I across all four bleed times for both the mouse injected with hHDL loaded with 100 µg 14C-L-4F and for a control injection with no 14C-L-4F (B). The same plasma bleeds were probed for mouse-specific apoA-I. The amount of mouse apoA-I present in each sample is expressed as a percent of the amount at time = 0 (X, 0 µg 14C-L-4F; □, 5.0 µg 14C-L-4F; △, 50 µg 14C-L-4F; ○, 100 µg 14C-L-4F; ○, 200 µg 14C-L-4F) (C). Unlike human apoA-I, mouse A-I showed little to no decline across time in 14C-L-4F amounts of up to 100 µg and only 12% decline is observed at 200 µg 14C-L-4F. Western blots show mouse apoA-I across all four bleed times for both the mouse injected with hHDL loaded with 100 µg 14C-L-4F and for a control mouse injected with hHDL without supplementation with 14C-L-4F. Lastly, total HDL cholesterol in these same plasma samples was determined (X, 0 µg 14C-L-4F; □, 5.0 µg 14C-L-4F; △, 50 µg 14C-L-4F; ○, 100 µg 14C-L-4F; ○, 200 µg 14C-L-4F) (D). HDL cholesterol showed a time-dependent decline consistent with the decline in human apoA-I. (The figure is a representative of two separate experiments.)
overlap with the cholesterol and apoA-I peaks (see Fig. 6A). This almost exact overlap of peaks strongly suggests that $^{14}$C-L-4F associates with the HDL fraction of the plasma and thus with HDL in vivo. The FPLC profile of HDL does not reflect the heterogenous nature of HDL. In Fig. 6A, there is a small tail of peptide that does not completely coincide with apoA-I and cholesterol. Thus, the studies in Fig. 6A do not by themselves prove that the peptide is physically associated with HDL. However, these data together with the data in Fig. 7, which demonstrate that L-4F enhances the clearance of human apoA-I from the circulation, make it highly likely that most of the peptide is physically associated with HDL. Nonetheless, future studies will be required to completely prove this point. Future studies will also be needed to determine whether a particular component of HDL is responsible for L-4F association with HDL or whether the radius of the curvature of the lipoprotein and the available surface provide a favorable environment for the peptide.

The same studies also indicate that the complexes of L-4F and HDL are cleared quickly from the circulation. Direct injection of L-4F resulted in a time-dependent decline in the amount of L-4F associating with the HDL fraction of the plasma (see Fig. 6B). The radioactive signal associated with the HDL fraction was validated as $^{14}$C-L-4F by LC/MS/MS (data not shown). The time-decrease in L-4F in the HDL fraction was associated with an appearance of lower size radioactively eluting material (Fig. 6B, Fractions 41–45), which did not consist of $^{14}$C-L-4F as determined by LC/MS/MS. Although detailed analysis to elucidate the structure(s) of the radioactive materials in these fractions was not done, it is assumed that they represent degradation products of the $^{14}$C-L-4F. In this experiment, the calculated $T_{1/2}$ for the decline is 16 min, indicating a quick removal of the $^{14}$C-L-4F from the circulation (Fig. 6C). Comparable results were obtained when a complex consisting of hHDL that had been preloaded with L-4F was injected directly into the circulation of C57BL/6J mice. Here again, the $^{14}$C-L-4F was cleared quickly from the circulation (Fig. 7A). The average $T_{1/2}$ for the decline in Fig. 7A was calculated to be 23.6 min, which is somewhat longer than the clearance time of the $^{14}$C-L-4F from the mHDL complex. Watson et al. (52) reported that the $T_{1/2}$ for L-4F in humans was $\sim$1.5 h after intravenous administration and was $\sim$2.5–3.0 h after subcutaneous administration. We do not know if the difference between the values reported here ($T_{1/2} < 30$ min) is due to the difference between mice and humans or is because we relied on measurements of radioactivity for our determination of $T_{1/2}$ and Watson et al. (52) relied on mass measurements using LC-MS/MS. The time-dependent decline of $^{14}$C-L-4F from the plasma of these mice was associated with a time-dependent decline in human apoA-I, strongly indicating a relationship between the presence of L-4F and the clearance of the hHDL. The importance of L-4F in this clearance is indicated by the lack of decline in human apoA-I from native hHDL devoid of L-4F (Fig. 7B). We cannot rule out a decaployment of $^{14}$C-L-4F in the HDL as a mechanism that may explain the difference in the rate of clearance of $^{14}$C-L-4F radioactivity and human apoA-I. The differential effect of the injected $^{14}$C-L-4F-hHDL complex on the clearance of human apoA-I as compared with mouse apoA-I strongly indicate a selective clearance of the L-4F bound HDL. The 12% decline in the mouse apoA-I at the highest $^{14}$C-L-4F level of 200 µg after 120 min may represent an overflow of the high amount of L-4F from the hHDL to the mHDL. Whatever the explanation, this represents only a small fraction of cleared mHDL compared with the $\sim$40% decrease in hHDL in the same
LDL enhances transfer of L4-F and oxidized lipids to HDL

14C-L-4F associated with HDL was cleared quickly from the circulation with a decline of 85% being observed between 3 and 60 min. Here again, we cannot exclude deacylation and rapid loss of the radiolabel in vivo compared with in vitro as the explanation for the seemingly different results of Fig. 3B and Fig. 8. Recently, Navab et al. (28) reported that dose rather than the plasma level of 4F is predictive of its efficacy. Oral administration of D4F (4.5 mg/kg) to apoE null mice significantly improved biomarkers of atherosclerosis, even though plasma levels of peptide did not exceed 20 ng/ml. By contrast, subcutaneous (SQ) injection of 4F failed to improve biomarkers even when plasma levels over 700 ng/ml were achieved (SQ dose of 0.45 mg/kg). SQ injections were effective at doses similar to the doses effective orally i.e. ≥4.5 mg/kg. Although less likely, the results by Navab et al. suggest that the effects of 4F on plasma lipoproteins may not be a major mechanism of 4F peptides. A currently accepted mechanism of action of 4F peptides is their ability to bind and remove oxidized lipids (40) from the circulation and tissue (39). Our studies support this mechanism for the circulation. Future studies will determine whether the effects of 4F on the intestine, which was proposed to be their major site of action by Navab et al., also involve the removal of oxidized lipids via lipoproteins similar to those in the circulation. It should be noted that the intestine is the second major site of lipoprotein synthesis (~30%) (53–55).

We report here that L-4F associates with HDL in both human plasma and in the circulation of C57BL/6J mice. Both observations are consistent with prior reports.
(13, 29, 47) and with a previous report from our laboratory (19). However, Wool et al. (41) have recently argued against the conclusion that 4F associates with HDL in vivo. Their studies, employing direct injection of biotinylated 4F into the circulation of C57BL/6J mice, resulted in two peaks of biotinylated 4F associated with plasma fractions, neither of which directly overlapped the peak of HDL as determined by apoA-I and cholesterol. Based on this observation and their assumption that the effect of the biotin moiety on the physical properties of 4F is negligible, they concluded that 4F itself does not associate with HDL in vivo. However, our in vivo association data, using 14C-labeled L-4F, appear to counter the central conclusion of Wool et al. that 4F does not associate with HDL in vivo. We do not have a satisfactory explanation for the discrepancy in our observations but it could be due to the chemical modification of the L-4F by biotinylation, which alters its mass by $\sim 10\%$ (addition of 241 daltons). Although the addition of biotin to the N-terminal of L-4F may not affect the ability of immobilized b4F to bind both native and oxidized 1-palmitoyl-2-arachidonoyl phosphatidylcholine (PAPC) in vitro (32), comparison to our studies is difficult because we demonstrate binding of L-4F to intact lipoproteins in the circulation. It is possible that the addition of the comparatively large moiety affects the interaction, within the circulation, between L-4F and lipoproteins. It is entirely possible that other chemical modifications of L-4F for the purpose of detection (e.g., 125I-tyrosine modification) may alter the amphipathicity of the 4F a helix as well and result in similar change in binding properties to lipoproteins. Hence, the choice in the use of 14C-L-4F in our present study.

In summary, the results of the studies reported here suggest that i) LDL promotes the association of L-4F with HDL and ii) in the presence of L-4F, oxidized lipids in LDL are rapidly transferred to HDL allowing these oxidized lipids to be acted upon by the enzymes in HDL and/or cleared from the circulation.

REFERENCES

1. Anantharamaiah, G. M., J. L. Jones, C. G. Brouillet, C. F. Schmidt, B. H. Chung, T. A. Hughes, A. S. Bhowm, and J. P. Segrest. 1985. Studies of synthetic peptide analogs of the amphipathic helix. Structure of complexes with dimyristoyl phosphatidylcholine. J. Biol. Chem. 260: 10248–10255.
2. Anantharamaiah, G. M. 1986. Synthetic peptide analogs of apolipoproteins. Methods Enzymol. 128: 627–647.
3. Segrest, J. P., R. L. Jackson, J. D. Morrisett, and A. M. Goto, Jr. 1988. A molecular theory of lipid-protein interactions in the plasma lipoproteins. FEBS Lett. 238: 247–258.
4. Segrest, J. P., M. K. Jones, H. De Loof, C. G. Brouillet, Y. V. Venkatachalapathi, and G. M. Anantharamaiah. 1992. The amphipathic helix in the exchangeable apolipoproteins: a review of secondary structure and function. J. Lipid Res. 33: 141–166.
5. Anantharamaiah, G. M., V. K. Mishra, D. W. Garber, G. Datta, W. G. Mani, J. S. Frank, G. M. Anantharamaiah, et al. 2000. Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: a randomized controlled trial. JAMA 283: 2292–2300.
6. Anantharamaiah, G. M., V. K. Mishra, D. W. Garber, A. M. Fogelman, and J. P. Segrest. 2004. Antinflammatory properties of HDL. Circ. Res. 95: 764–772.
7. Plump, A. S., C. J. Scott, and J. L. Breslow. 1994. Human apolipoprotein A-I gene expression increases high density lipoprotein and suppresses atherosclerosis in the apolipoprotein E-deficient mouse. Proc. Natl. Acad. Sci. USA. 91: 9607–9611.
8. Chiesa, G., and C. R. Sirtori. 2002. Use of recombinant apolipoproteins in vascular diseases: the case of apoA-I. Curr. Opin. Investig. Drugs. 3: 420–426.
9. Nissen, S. E., T. Tsunoda, E. M. Tuzcu, P. Schoenhagen, C. J. Cooper, M. Yasim, G. M. Eaton, M. A. Lauer, W. S. Sheldon, C. L. Grines, et al. 2003. Effect of recombinant ApoA-1 Milano on coronary atherosclerosis in patients with acute coronary syndromes: a randomized controlled trial. JAMA 290: 2292–2300.
10. Dricer, P. J., S. Nicholls, K-A. Rye, G. M. Anantharamaiah, M. Navab, and A. M. Fogelman. 2004. Antiinflammatory properties of HDL. Circ. Res. 95: 764–772.
11. Navab, M., S. Y. Hama, C. J. Cooke, G. M. Anantharamaiah, M. Chaddha, L. Jin, G. Subhanagounder, K. F. Faull, S. T. Reddy, N. E. Miller, et al. 2000. Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: a randomized controlled trial. J. Lipid Res. 41: 1481–1494.
12. Navab, M., S. Y. Hama, G. M. Anantharamaiah, K. Hassan, G. P. Hough, A. D. Watson, S. T. Reddy, A. Sevanian, G. C. Fonarow, and A. M. Fogelman. 2000. Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: steps 2 and 3. J. Lipid Res. 41: 1495–1508.
13. Garber, D. W., G. Datta, M. Chaddha, M. N. Palgunachari, S. V. Harun, M. Navab, A. C. Wagner, J. P. Segrest, and G. M. Anantharamaiah. 2001. A new synthetic class A amphipathic peptide analogue protects mice from diet-induced atherosclerosis. J. Lipid Res. 42: 545–552.
14. Navab, M., G. M. Anantharamaiah, S. T. Reddy, S. Hama, G. Hough, V. R. Grijalva, N. Yu, B. J. Ansell, G. Datta, D. W. Garber, et al. 2005. Apolipoprotein A-I mimetic peptides. Arterioscler. Thromb. Vasc. Biol. 25: 1325–1331.
15. Venkatachalapathi, Y. V., M. C. Phillips, R. M. Epand, R. F. Epand, E. M. Tyler, J. P. Segrest, and G. M. Anantharamaiah. 1993. Effect of end group blockage on the properties of a class A amphipathic helical peptide. Proteins. 15: 349–359.
16. Getz, G. S., G. D. Wool, and C. A. Reardon. 2009. Apoprotein A-I mimetic peptides and their potential anti-atherogenic mechanisms of action. Curr. Opin. Lipidol. 20: 171–175.
17. Van Lenten, B. J., A. C. Wagner, M. Navab, G. M. Anantharamaiah, S. Hama, S. T. Reddy, and A. M. Fogelman. 2007. Lipoprotein inflammatory properties and serum amyloid A levels but not cholesterol levels predict lesion area in cholesterol-fed rabbits. J. Lipid Res. 48: 2344–2353.
18. Navab, M., G. M. Anantharamaiah, S. Hama, D. W. Garber, M. Chaddha, G. Hough, R. Lallone, and A. M. Fogelman. 2002. Oral administration of an Apo A-I mimetic peptide synthesized from D-amino acids dramatically reduces atherosclerosis in mice independent of plasma cholesterol. Circulation. 105: 290–292.
19. Navab, M., I. Shechter, G. M. Anantharamaiah, S. T. Reddy, B. J. Van Lenten, and A. M. Fogelman. 2010. Structure and function of HDL mimetics. Arterioscler. Thromb. Vasc. Biol. 30: 164–168.
20. Van Lenten, B. J., A. C. Wagner, G. M. Anantharamaiah, M. Navab, S. T. Reddy, G. M. Buga, and A. M. Fogelman. 2009. Apolipoprotein A-I mimetic peptides. Curr. Atheroscler. Rep. 11: 52–57.
21. Navab, M., G. M. Anantharamaiah, and A. M. Fogelman. 2008. The effect of apolipoprotein mimetic peptides in inflammatory disorders other than atherosclerosis. Trends Cardiovasc. Med. 18: 61–66.
22. Garver, D. W., Schoeneman, C. G., Brouillet, G. C., Hama, M., Navab, G. S. Park, B. J. Van Lenten, A. C. Wagner, A. M. Fogelman, and E. Brah. 2008. Treatment with an apolipoprotein A-I mimetic peptide in combination with pravastatin inhibits collagen-induced arthritis. Cln. Immunol. 127: 234–244.
23. Buga, G. M., J. S. Frank, G. A. Mottino, A. Hakhamian, A. Narasimha, A. D. Watson, B. Yekta, M. Navab, S. T. Reddy, G. M. Anantharamaiah, et al. 2008. D-FP reduces E06 immunoreactivity, SREBP-1c mRNA levels, and renal inflammation in LDL receptor-null mice fed a Western diet. J. Lipid Res. 49: 192–205.
24. Van Lenten, B. J., A. C. Wagner, G. M. Anantharamaiah, D. W. Garber, M. C. Fishbein, L. Adhikary, D. P. Nayak, S. Hama, M. Navab, and A. M. Fogelman. 2002. Inflammation infection promotes macrophage traffic into arteries of mice that is prevented by D-FP, an apolipoprotein A-I mimetic peptide. Circulation. 106: 1127–1132.
LDL enhances transfer of L4-F and oxidized lipids to HDL