Oral D-4F Causes Formation of Pre-β High-Density Lipoprotein and Improves High-Density Lipoprotein–Mediated Cholesterol Efflux and Reverse Cholesterol Transport From Macrophages in Apolipoprotein E–Null Mice

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***Background*—**These studies were designed to determine the mechanism of action of an oral apolipoprotein (apo) A-I mimetic peptide, D-4F, which previously was shown to dramatically reduce atherosclerosis in mice.

***Methods and Results*—**Twenty minutes after 500 μg of D-4F was given orally to apoE-null mice, small cholesterol-containing particles (CCPs) of 7 to 8 nm with pre-β mobility and enriched in apoA-I and paraoxonase activity were found in plasma. Before D-4F, both mature HDL and the fast protein liquid chromatography fractions containing the CCPs were proinflammatory. Twenty minutes after oral D-4F, HDL and CCPs became antiinflammatory, and there was an increase in HDL-mediated cholesterol efflux from macrophages in vitro. Oral D-4F also promoted reverse cholesterol transport from intraperitoneally injected cholesterol-loaded macrophages in vivo. In addition, oral D-4F significantly reduced lipoprotein lipid hydroperoxides (LOOH), except for pre-β HDL fractions, in which LOOH increased.

***Conclusions*—**The mechanism of action of oral D-4F in apoE-null mice involves rapid formation of CCPs, with pre-β mobility enriched in apoA-I and paraoxonase activity. As a result, lipoprotein LOOH are reduced, HDL becomes antiinflammatory, and HDL-mediated cholesterol efflux and reverse cholesterol transport from macrophages are stimulated. **(*Circulation*. 2004;109:3215-3220.)**

Key Words: cholesterol ■ lipoproteins ■ apolipoproteins

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The apolipoprotein A-I (apoA-I) mimetic peptide 4F contains only 18 amino acids, compared with apoA-I, which contains 243 amino acids. The 4F peptide was designed to contain a class A amphipathic helix with a polar and a nonpolar face that allows it to bind lipids similar to apoA-I.[1] 4F synthesized from D-amino acids (D-4F) and given orally has been shown to convert HDL from proinflammatory to antiinflammatory and to dramatically reduce atherosclerosis in mice.[2] In a mouse model of influenza infection and atherosclerosis, D-4F prevented HDL from becoming proinflammatory and dramatically decreased macrophage traffic into the aortic arch and innominate arteries.[3]

Ou et al have shown that 4F restores the balance between nitric oxide and superoxide anions in LDL-treated endothelial cells.[4] This group has also shown that 4F dramatically improves vasoreactivity in LDL receptor–null mice on a Western diet and in a mouse model of sickle cell disease.[5]

The present studies indicate that oral D-4F rapidly causes the formation of small cholesterol-containing particles with pre-β mobility that are enriched in apoA-I and paraoxonase activity, resulting in the conversion of HDL from proinflammatory to antiinflammatory. In addition, HDL-mediated cholesterol efflux and reverse cholesterol transport from macrophages are stimulated.

Methods

Materials

1-Palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphorylcholine (PAPC) (catalogue No. 850459) was from Avanti Polar Lipids. Hydroperoxyoctadecadienoic acid (13[*S*]-HPODE) was from Biomol. Rabbit

Received February 10, 2004; de novo received April 13, 2004; revision received April 13, 2004; accepted May 17, 2004.

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M.N., S.H., S.T.R., G.M.A., and A.M.F. are principals in Bruin Pharma.

This article originally appeared Online on June 14, 2004 (*Circulation*. 2004;109:r120–r125).

An online Data Supplement is available at http://www.circulationaha.org.

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Circulation **is available at http://www.circulationaha.org** **DOI: 10.1161/01.CIR.0000134275.90823.87**

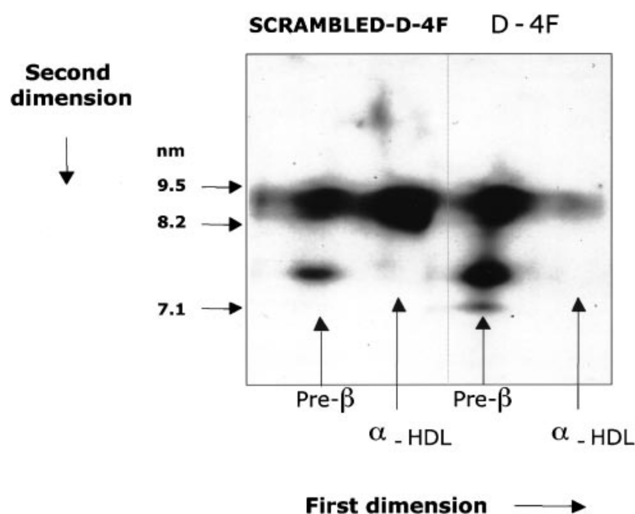

Figure 1. In vitro, in apoE-null mouse plasma, D-4F causes a major redistribution of apoA-I from α migrating to pre-β migrating particles. Both D-4F and scrambled D-4F are highly water-soluble. Two milligrams of D-4F or scrambled D-4F (Sc D-4F) was weighed and dissolved in 500 μL of apoE-null mouse plasma and diluted with additional plasma to a final concentration of 500 μg/mL and incubated for 20 minutes at 37°C with gentle mixing. Plasma was fractionated by agarose electrophoresis in first dimension, and native PAGE in second dimension, and subjected to Western analysis with anti-mouse ApoA-I. Figures are scanned images of enzyme-linked chemiluminescence film exposure. Experiment shown is representative of 3 of 3 experiments.

polyclonal anti-mouse apoA-I antibody, affinity-purified (catalogue No. K23001R) was from Biodesign International. D-4F (or scrambled D-4F with the same D-amino acids as D-4F but in a sequence that does not promote α helical formation: Ac-DWFAKDYFKKAFVEEFAK-NH_2) and D-5F were synthesized as described.[1,6] All other materials were from previously cited sources.[2]

Mice

ApoE-null and wild-type C567/BL6J mice were from The Jackson Laboratory (Bar Harbor, Me) and were maintained on a chow diet (Ralston Purina).

Plasma, Lipoproteins, Cocultures, Monocyte Chemotaxis

Lipoproteins, cocultures, and monocytes were prepared and monocyte chemotaxis assays performed as described previously[2,7] using human (after informed consent) or mouse (with Animal Research Committee approval) blood. Plasma was fractionated by gel permeation fast protein liquid chromatography (FPLC) using dual Pharmacia Superose 6 columns in series. Plasma (0.45 mL or less) was eluted with an isocratic buffer containing 154 mmol/L NaCl, 1 mmol/L EDTA, and 0.02% sodium azide, pH 8.2, at a flow rate of 0.5 mL/min, pumped by a nonmetallic Beckman high-performance liquid chromatography (HPLC) pump. Forty-two 1-mL fractions were collected. Lipoproteins isolated in the absence of EDTA contained 20 μmol/L BHT.

Measurement of Plasma Levels of D-4F

Plasma levels of peptides were determined by liquid chromatography multiple reaction monitoring as described.[8] Recovery was assessed with ^{14}C-labeled D-4F.

Sample Preparation

Aliquots (20 μL) of plasma were adjusted to d=1.215 with a d=1.33 stock solution (2.9 mol/L KBr containing 2.91 mol/L NaCl and 1 mmol/L EDTA) and brought to a total volume of 175 μL with KBr/NaCl/EDTA (1.5004 mol/L/1.04065 mol/L/0.545 mmol/L, d=1.215) and centrifuged (Beckman-Coulter Airfuge, A100/18 rotor, 148.000g, 4 hours, room temperature). The top 70 μL from 3 tubes was removed, pooled, and diluted 1:3 (vol/vol) with methanol and mixed briefly. Chloroform (210 μL) was added and mixed, yielding a single phase, which was evaporated to dryness in a vacuum centrifuge. The sample was resuspended in 1 mL water and passed over an ODS (C18) cartridge (1 mL × 100 mg, J & W Scientific) that had been equilibrated with 1 cartridge volume of methanol and 3 cartridge volumes of water. The flowthrough was passed over the cartridge a second time, and the cartridge was washed sequentially with

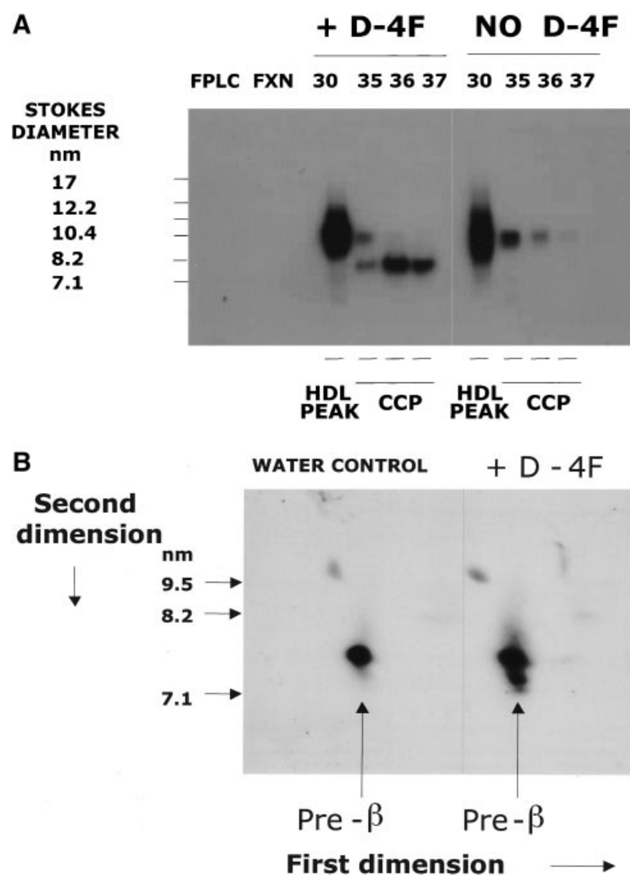

Figure 2. A, Western blot for mouse apoA-I. Female ApoE-null mice at 8 weeks of age (4 per group) were given 500 μg D-4F in 200 μL of water (+ D-4F) or were given 200 μL of water (no D-4F) by stomach tube. Twenty minutes later, mice were bled, and plasma was separated by FPLC and fractions 30 (fraction containing main peak of mature HDL), 35, 36, and 37 (fractions in which pre-β HDL would be expected) were analyzed by native-PAGE and Western blotting using antisera to mouse apoA-I. Diameter of particles is shown on left, as determined from markers run on native PAGE (not shown in Figure). (FPLC Fxn indicates FPLC fraction number; HDL Peak, fraction 30; and CCP, fractions 35 to 37). Experiment shown is representative of 3 of 3 experiments (2 in female mice and 1 in male mice). B, ApoE-null mice at 9 weeks of age (n=4 per group) were given by stomach tube 500 μg D-4F (+ D-4F) or same volume of water that D-4F was dissolved in (Water Control), and blood was drawn 20 minutes later. Plasma samples were fractionated by FPLC, and post-HDL fractions in which pre-β HDL would be expected were subjected to agarose electrophoresis in first dimension and native PAGE in second dimension and Western blotted. Image is a Western blot that was probed with antisera to mouse apoA-I. Experiment shown is representative of 3 of 3 experiments (2 in female mice and 1 in male mice).

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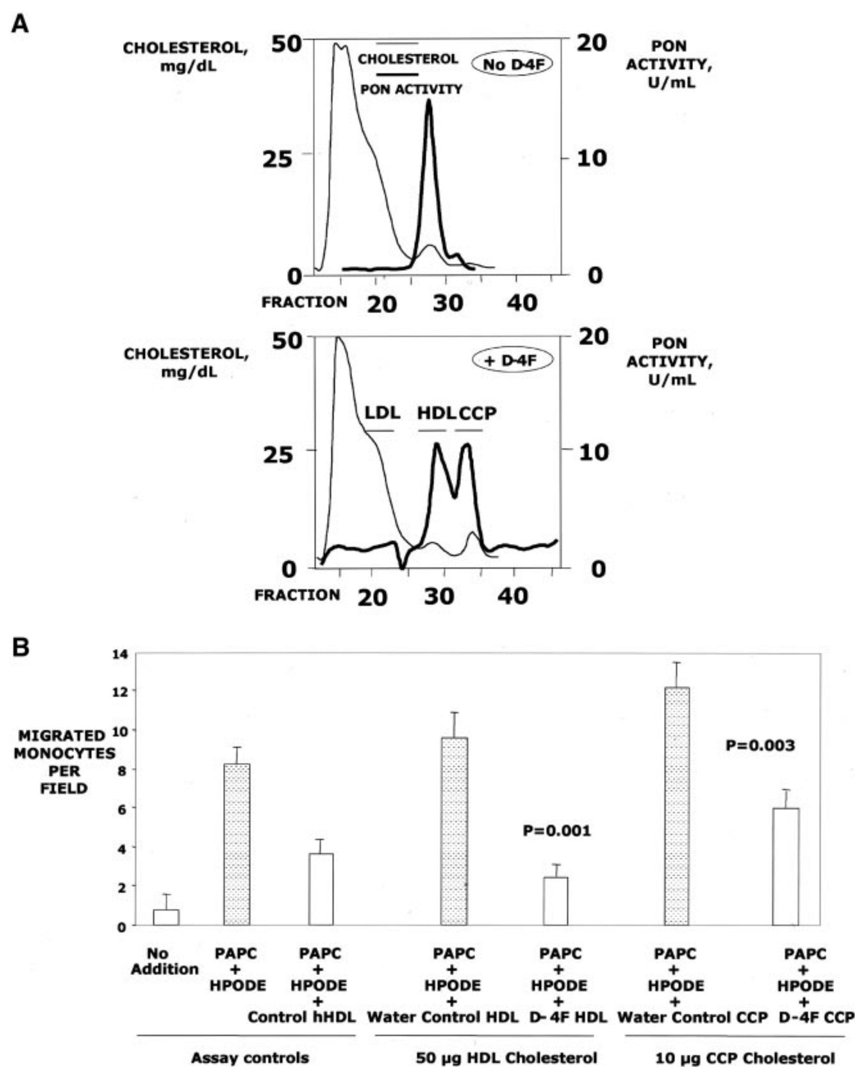

Figure 3. Oral D-4F causes formation of small CCPs rich in paraoxonase activity, which inhibit oxidized phospholipid-induced monocyte chemotactic activity. A, Twenty minutes after instilling water (No D-4F, top) or 500 μg D-4F (+ D-4F, bottom) into stomachs of apoE-null mice at 10 weeks of age (4 per group), mice were bled, and their plasma was fractionated by FPLC and cholesterol (thin black line) and paraoxonase (PON) activity (thick black line) determined in fractions. Bottom, Fractions in which pre-β HDL would be expected that appeared to right of HDL after D-4F. As shown at bottom, these latter fractions also contained PON activity after administration of D-4F. Experiment shown is representative of 3 of 3 experiments (2 in female mice and 1 in male mice). B, Particles formed after oral D-4F in FPLC fractions in which pre-β HDL would be expected are biologically active. Water or 500 μg of D-4F was instilled into stomachs of apoE-null mice at 9 weeks of age (4 per group). Twenty minutes later, mice were bled, and their plasma was fractionated by FPLC. Fractions 28 to 30 contained larger mature HDL, and fractions 35 to 37 are those in which pre-β HDL would be expected. PAPC 20 μg was added together with 1 μg/mL of HPODE to cocultures of human artery wall cells. Human HDL (hHDL) was added at 350 μg/mL cholesterol, or no addition was made to cocultures (No Addition), or mouse HDL isolated by FPLC (fractions 28 to 30) from mice given water alone (Water Control HDL) or D-4F (D-4F HDL) at 50 μg/mL HDL cholesterol, or FPLC fractions in which pre-β HDL would be expected (fractions 35 to 37) after water alone (Water Control CCP), or after D-4F (D-4F CCP) at 10 μg/mL cholesterol was added to cocultures. After 8 hours of incubation, supernatants were collected and assayed for monocyte chemotactic activity. Data are mean±SD of number of migrated monocytes in 9 fields for triplicate samples. Experiment shown is representative of 3 of 3 experiments (2 in female mice and 1 in male mice).

water (1 mL) and water/acetonitrile (90/10, vol/vol, 1 mL), and eluted with water/acetonitrile (50/50, vol/vol, 1 mL). The eluate was taken to dryness in a vacuum centrifuge. Some plasma samples were spiked with 10 ng of D-4F for recovery and cochromatography experiments.

HPLC Chromatography

Dried samples were redissolved in 100 μL of water/acetonitrile/formic acid (95/5/0.1, vol/vol/vol) and centrifuged (16 000g, 30 s), and 50 μL of the supernatant was injected onto a reverse-phase HPLC column (C18, BetaBasic-18, Thermo Hypersil-Keystone; 20×2.1 mm, 0.5 μm, 150A pore size) equilibrated with water/acetonitrile/formic acid (95/5/0.1) and eluted (40°C) with an increasing concentration of acetonitrile (min/% acetonitrile: 0/5, 10/50, 12/100 at 150 μL/m, then for a further 5 minutes at 500 μL/m).

Mass Spectrometry

The effluent HPLC column was directed into the Ionspray source of a Perkin-Elmer Sciex API III+ triple-quadripole instrument operated at maximum sensitivity such that there was no resolution between the ^{13}C-satellite ions of the polypropylene glycol calibrant. Data were recorded in the positive-ion tandem mass spectrometry (MS/MS) mode using multiple-reaction monitoring. Recordings were made of the intensity of the transitions m/z 770.7 (triply charged parent) to 159.2 (the most intense signal recorded in the fragment ion spectrum of the m/z 770.7 parent, assigned as $[z1+1]^+$) for D-4F, and m/z 810.1 (triply charged parent) to 159.2 (the most intense signal recorded in the fragment ion spectrum of the m/z 810.1 parent, assigned as $[b1+1]^+$) for the D-5F internal standard when used.

HDL-Mediated Cellular Cholesterol Efflux

Experiments were performed as described by Remaley et al,[9] with minor modifications. Human monocytes were obtained as described previously[2,7] and converted to macrophages in DMEM high-glucose medium supplemented with 10% FBS. Cells were plated at a density of 5×10^5 cells per mL in 24-well culture dishes. Twenty-four hours later, the medium was replaced overnight with medium containing 10% lipoprotein deficient serum. The cells were washed, and 1 μCi/mL ^{3}H-labeled cholesterol and 50 μg/mL of acetylated LDL were added and incubated for 48 hours. The cells were washed 3 times and incubated in medium containing 1% BSA to allow cell cholesterol pools to equilibrate. HDL-mediated cholesterol efflux was determined by incubating the test HDL with labeled cells for 4 hours at 37°C. Radioactivity in the supernatants and total cell extracts were measured and expressed as the percentage of total radioactive counts removed from the cells during the efflux period.

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In Vivo Reverse Cholesterol Efflux From Macrophages

Two-month-old apoE-null female mice were maintained in metabolic cages, and 100 μg/mL of D-4F or scrambled D-4F was added to their drinking water. Twelve hours later, the mice were injected intraperitoneally with 5×10^6 J774 cholesterol-loaded macrophages containing 1.5×10^6 cpm of ^{3}H cholesterol as described by Zhang et al.[10] Twenty-four hours later, the mice were killed, and lipid extracts from plasma, liver, and feces were analyzed for ^{3}H tracer as described.[10]

Other Procedures

Paraoxonase activity was measured as described previously.[3] Lipoprotein cholesterol concentrations were determined by use of a Cholesterol-20 kit (Sigma). For Western analyses, plasma (0.5 μL) was subjected to SDS-PAGE (4% to 20% Tris glycine from Novex) and Western-transferred (semidry onto nitrocellulose from Amersham). The blots were treated sequentially with rabbit anti-mouse apoA-I (Biodesign International K23500R), followed by horseradish peroxidase–labeled anti-rabbit IgG $F(ab')_2$ (Jackson) and Amersham enzyme-linked chemiluminescence reagent, and exposed to film (Amersham). Two-dimensional agarose/native PAGE was performed as described previously.[11] 15-Hydroxyeicosatetraenoic acid (15-HETE) was measured by mass spectrometry.[12] Negative staining of lipoproteins[13] and determination of lipoprotein lipid hydroperoxides[14] were as described previously. Statistical significance was determined by use of model I ANOVA, and significance was defined as a value of $P<0.05$.

Results

In Vitro Studies

The addition of 500 μg/mL of D-4F (but not scrambled D-4F) for 20 minutes to apoE-null plasma in vitro resulted in a dramatic shift of apoA-I from α migrating to pre-β migrating particles (Figure 1). Addition of D-4F (but not scrambled D-4F) to wild-type C57BL/6J mouse plasma in vitro increased apoA-I in pre-β migrating particles (data not shown). FPLC fractions of apoE-null mouse plasma after addition of D-4F in vitro revealed a decrease in HDL cholesterol and an increase in cholesterol-containing particles (CCPs) found in the trailing peak after the bulk HDL peak, where pre-β HDL were found (see Figure 3A above, and data not shown). ApoE-null mouse plasma contains increased lipid peroxidation products.[15] After addition of D-4F to apoE-null mouse plasma in vitro, 15-HETE was found to be concentrated in the fractions in which pre-β HDL would be expected (data not shown).

In Vivo Studies

Twenty minutes after 500 μg of D-4F (dissolved in 200 μL of water and given by stomach tube) was administered to apoE-null mice, the plasma concentration of D-4F was ≈60 pmol/mL, or ≈138 ng of D-4F/mL as measured by liquid chromatography multiple reaction monitoring, and 85% of the D-4F was found in HDL in the experiments shown in Figures 2 through 4. In other experiments, the highest plasma concentration observed 20 minutes after administration of 500 μg of D-4F (dissolved in 200 μL of water and given by stomach tube) to apoE-null mice was 140 pmol/mL, or ≈322 ng of D-4F/mL plasma. Twenty minutes after administration of 500 μg of scrambled D-4F, no peptide was detected in the plasma. As shown in Figure 2A, 20 minutes after oral D-4F, apoA-I was found in smaller particles (7 to 8 nm) in the FPLC fractions of mouse plasma in which pre-β HDL would be expected. The formation of these small HDL-like particles was confirmed by negative staining electron microscopy (data not shown). Twenty minutes after oral D-4F, there was a marked increase in apoA-I with pre-β mobility (Figure 2B). A similar increase in apoA-I with pre-β mobility was seen 20 minutes after oral D-4F in wild-type C57BL/6J mice (data not shown). Twenty minutes after oral D-4F, the FPLC fractions in which pre-β HDL would be expected also contained increased cholesterol and paraoxonase activity (Figure 3A). Twenty minutes after oral D-4F, HDL and the FPLC fractions in which pre-β HDL would be expected were converted from proinflammatory to antiinflammatory (Figure 3B). Paralleling the improvement in HDL inflammatory/antiinflammatory properties, 20 minutes after oral D-4F (but not scrambled D-4F), there was also a significant improvement in the ability of apoE-null HDL to mediate cholesterol efflux from human

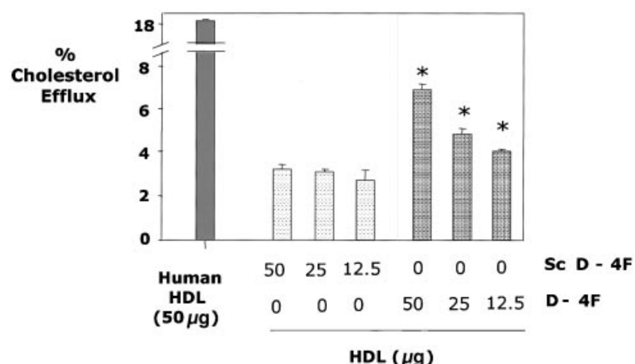

Figure 4. Oral D-4F (but not scrambled D-4F) improves HDL-mediated cholesterol efflux from macrophages. ApoE-null mice at 11 weeks of age (4 per group) were given 500 μg of D-4F or 500 μg of scrambled D-4F (Sc D-4F) by stomach tube. Twenty minutes later, mice were bled, their lipoproteins were separated by FPLC, and indicated concentration of HDL cholesterol was added to human monocyte macrophages and percent of ^{3}H cellular efflux determined as described in Methods. Values are mean±SD. *$P<0.05$. Experiments shown are representative of 4 of 4 experiments (3 in female mice and 1 in male mice).

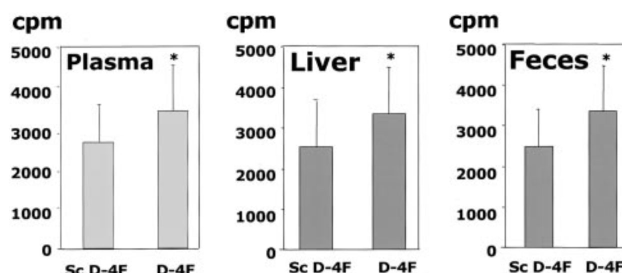

Figure 5. Oral D-4F stimulates reverse cholesterol transport from macrophages in apoE-null mice. Two-month-old apoE-null mice (8 per group) were maintained in metabolic cages, and 100 μg/mL of D-4F or scrambled D-4F was added to their drinking water. Twelve hours later, mice were injected intraperitoneally with 5×10^6 J774 cholesterol-loaded macrophages containing 1.5×10^6 cpm of ^{3}H cholesterol. Twenty-four hours later, mice were killed, and lipid extracts from plasma, liver, and feces were analyzed for ^{3}H tracer. Figure shows ^{3}H content of lipid extracts. Values are mean±SD; *$P<0.05$. Experiments shown are representative of 3 of 3 experiments and represent pooled data from all 3 experiments.

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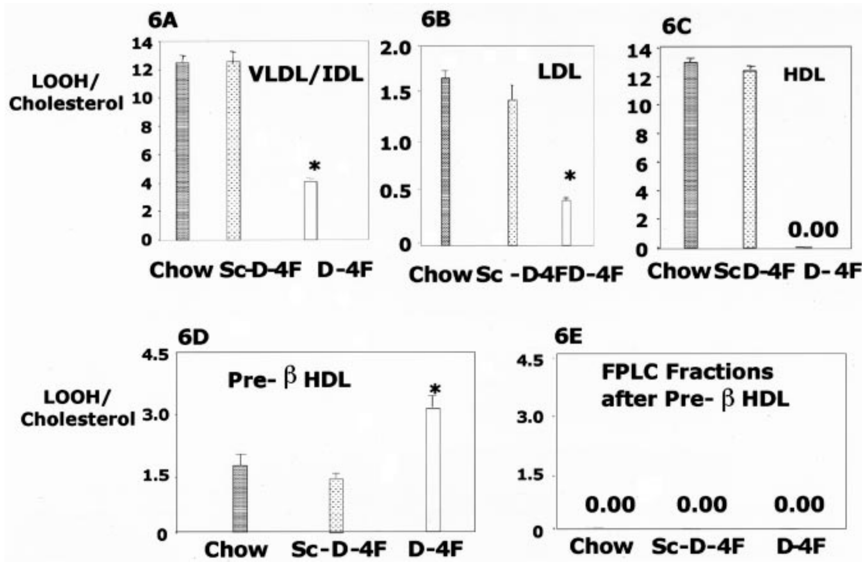

Figure 6. Oral D-4F reduces lipoprotein lipid hydroperoxides. ApoE-null mice at 10 weeks of age (4 per group) were given or not given D-4F or scrambled D-4F (Sc D-4F) added to mouse chow (200 μg/g chow). In the morning, mice were bled, and their plasma was fractionated by FPLC and lipid hydroperoxides determined. Values shown are lipid hydroperoxide content (mean±SD) normalized to cholesterol content of lipoprotein fractions. *$P<0.05$. Experiment shown is representative of 4 of 4 experiments (3 in female mice and 1 in male mice).

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monocyte macrophages (Figure 4). The data shown in Figures 2, 3, and 4 represent experiments with female apoE-null mice. Similar results were found in male apoE-null mice, and no differences were observed when scrambled D-4F or water alone was used as the control (data not shown).

Oral D-4F significantly stimulated reverse cholesterol transport from intraperitoneally injected macrophages into plasma, liver, and feces in vivo (Figure 5). ApoE-null lipoproteins contain elevated levels of oxidized lipids.[14] If oral D-4F causes the formation of pre-β HDL enriched in apoA-I and paraoxonase, one might expect oral D-4F to decrease lipoprotein lipid hydroperoxides. This was indeed the case for oral D-4F (but not for scrambled D-4F) for VLDL/IDL (Figure 6A), LDL (Figure 6B), and HDL (Figure 6C). After oral D-4F, in the female apo E-null mice shown in Figure 6C, there were no detectable lipid hydroperoxides in HDL. In contrast, as shown in Figure 6D, there was a significant increase in lipid hydroperoxides 20 minutes after oral D-4F (but not after scrambled D-4F) in the FPLC fractions in which pre-β HDL would be expected. Total plasma lipid hydroperoxides were decreased after oral D-4F (data not shown). In male apoE-null and in wild-type C57BL/6J mice, there was a very significant decrease in HDL lipid hydroperoxides 20 minutes after oral D-4F, but they were detectable (data not shown). In male apoE-null and in wild-type C57BL/6J mice, there was also a significant increase in pre-β HDL lipid hydroperoxides (data not shown). As expected, FPLC fractions coming off the column after pre-β HDL, which do not contain lipoproteins, did not contain lipid hydroperoxides (Figure 6E). There was also an increase in paraoxonase activity after oral D-4F in the HDL and pre-β HDL FPLC fractions, and the total plasma paraoxonase activity was increased (data not shown). Moreover, in the coculture assay, the pre-β HDL fractions were anti-inflammatory, despite the increased lipoprotein lipid hydroperoxides (LOOH) content (data not shown).

Discussion

In vitro, D-4F caused a dramatic redistribution of apoA-I from α migrating to β migrating particles in apoE-null mouse plasma (Figure 1), suggesting that D-4F is acting directly on HDL or some plasma component that in turn remodels HDL. In vivo, in apoE-null mice, despite the small amount of D-4F absorbed 20 minutes after an oral dose, D-4F rapidly caused the formation of CCPs (Figure 3A) with pre-β mobility (Figure 2B) that were enriched in apoA-I (Figure 2A) and paraoxonase activity (Figure 3A). Twenty minutes after oral D-4F, proinflammatory HDL became antiinflammatory (Figure 3B), and the ability of the HDL to mediate cholesterol efflux from macrophages in vitro was enhanced (Figure 4). Oral D-4F also stimulated reverse cholesterol transport from macrophages in vivo in apoE-null mice (Figure 5). After oral D-4F (but not scrambled D-4F), LOOH decreased in all lipoprotein fractions except those in which pre-β HDL would be expected (Figure 6). The fractions in which pre-β HDL would be expected were antiinflammatory 20 minutes after oral D-4F (Figure 3B). After feeding D-4F overnight, LOOH was increased in the pre-β HDL FPLC fractions (Figure 6D). At this time, paraoxonase activity was also increased in the pre-β HDL FPLC fractions, and these fractions were anti-inflammatory in the coculture assay (data not shown). Thus, the increase in paraoxonase activity must have more than compensated for the increased LOOH in terms of reducing the proinflammatory-oxidized phospholipids in these fractions. The failure to detect scrambled D-4F (which does not bind lipids) in the plasma 20 minutes after oral administration may indicate that the lipid-binding properties of D-4F are critical to its absorption. Alternatively, the scrambled D-4F may have been absorbed, but because of its inability to bind lipids, it may have been rapidly cleared from plasma, as is the case for lipid-poor apoA-I in Tangiers disease. Reverse cholesterol transport is considered to be important in preventing the buildup of lipids that predisposes to atherosclerosis.[16] Reddy and colleagues[17] demonstrated in vitro that an increase in cellular cholesterol levels caused human artery wall cells to increase the formation of LDL-derived oxidized phospholipids that are thought to initiate the inflammatory response in atherosclerosis.[18] Pre-β HDL is generally considered to be the most active HDL fraction in promoting reverse cholesterol

transport, and the cycling of cholesterol through pre-β HDL is generally considered to be protective against atherosclerosis.[19] The data presented in this article suggest that oral D-4F provides a novel method for stimulating the formation pre-β HDL and stimulating reverse cholesterol transport from macrophages. These properties of oral D-4F may account for its remarkable ability to reduce lesions in mouse models of atherosclerosis.[2] The ability of oral D-4F to cause pre-β HDL formation in wild-type C57BL/6J mice on a chow diet, to decrease LOOH in HDL, and to increase LOOH in pre-β HDL in these mice (data not shown), together with the ability of oral D-4F to render HDL antiinflammatory and dramatically reduce atherosclerosis in LDL receptor-null mice,[2] suggests that these properties are not dependent on the absence of apoE.

Acknowledgments

This work was supported in part by US Public Health Services grants HL-30568 and HL-34343 and the Laubisch, Castera, and M.K. Grey Funds at UCLA. We thank Dr Kym Faull and Dr Julian Whitelegge for their expert valuable assistance with mass spectrometry analyses.

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Circulation. 2004;109:3215-3220; originally published online June 14, 2004;
doi: 10.1161/01.CIR.0000134275.90823.87
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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