An apolipoprotein A-I mimetic dose-dependently increases the formation of preβ$_1$ HDL in human plasma

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Abstract **Preβ$_1$ HDL is the initial plasma acceptor of cell-derived cholesterol in reverse cholesterol transport. Recently, small amphipathic peptides composed of D-amino acids have been shown to mimic apolipoprotein A-I (apoA-I) as a precursor for HDL formation. ApoA-I mimetic peptides have been proposed to stimulate the formation of preβ$_1$ HDL and increase reverse cholesterol transport in apoE-null mice. The existence of a monoclonal antibody (MAb 55201) and a corresponding ELISA method that is selective for the detection of the preβ$_1$ subclass of HDL provides a means of establishing a correlation between apoA-I mimetic dose and preβ$_1$ HDL formation in human plasma. Using this preβ$_1$ HDL ELISA, we demonstrate marked apoA-I mimetic dose-dependent preβ$_1$ HDL formation in human plasma. These results correlated with increases in band density of the plasma preβ$_1$ HDL, when observed by Western blotting, as a function of increased apoA-I mimetic concentration. Increased preβ$_1$ HDL formation was observed after as little as 1 min and was maximal within 1 h. Together, these data suggest that a high-throughput preβ$_1$ HDL ELISA provides a way to quantitatively measure a key component of the reverse cholesterol transport pathway in human plasma, thus providing a possible method for the identification of apoA-I mimetic molecules.**—Troutt, J. S., W. E. Alborn, M. K. Mosior, J. Dai, A. T. Murphy, T. P. Beyer, Y. Zhang, G. Cao, and R. J. Konrad. **An apolipoprotein A-I mimetic dose-dependently increases the formation of preβ$_1$ HDL in human plasma.** ***J. Lipid Res.*** **2008.** 49: **581–587.**

Supplementary key words high density lipoprotein • cholesterol • reverse cholesterol transport

The ability of HDL to promote and facilitate reverse cholesterol transport is an important mechanism by which HDL protects against atherosclerosis (1–7). Furthermore, several epidemiological studies have shown a strong inverse relationship between plasma HDL-cholesterol concentrations and clinical coronary heart disease (1–7). As the initial plasma acceptor of cholesterol from cell membranes (8), preβ$_1$ HDL is of particular interest as a biomarker for therapies targeting the antiatherosclerotic capabilities of HDL. This HDL subclass exhibits preβ mobility in nondenaturing gel electrophoresis and is postulated to consist of two to three apolipoprotein A-I (apoA-I) molecules with some phospholipids and a small amount of unesterified cholesterol (9). As preβ$_1$ HDL takes on unesterified cholesterol from cell membranes, it exhibits unique epitopes not exposed in spheroidal α-HDL (10–12).

As the precursor and major protein moiety of HDL, apoA-I is thought to promote reverse cholesterol transport. Additionally, it has been shown that infusion and overexpression of apoA-I significantly reduces atherosclerosis in animal models (13–17). Recently, small, amphipathic helical apoA-I mimetic peptides composed of D-amino acids have shown similar antiatherogenic properties. Moreover, a specific apoA-I mimetic peptide, D4F, has shown improved HDL-mediated efflux and reverse cholesterol transport from macrophages, in conjunction with causing the formation of preβ HDL in apoE-null mice (18–20). It is thought that this particular D-amino acid peptide mimics the amphipathic helix of apoA-I, thus allowing it to bind lipids and interact physiologically by mechanisms similar to those of the full-length apoA-I protein (21).

Researchers from Daiichi Pure Chemicals recently developed a monoclonal mouse anti-human preβ$_1$ HDL antibody (MAb 55201) that is highly specific for apoA-I in the preβ$_1$ HDL conformation. Additionally, Daiichi now provides an ELISA kit that allows for the capture of human plasma preβ$_1$ HDL using MAb 55201 and detection via conjugated polyclonal goat anti-human apoA-I antibody (10, 11). As a research tool, this ELISA may provide the chance to better understand the activity of potential D-amino acid peptide therapies that have been shown to mimic the functionality of apoA-I in reverse cholesterol transport. In light of this possibility, we used this ELISA method to investigate the apoA-I mimetic dose-dependent formation of preβ$_1$ HDL in human plasma.

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Abbreviations: apoA-I, apolipoprotein A-I; PK, pharmacokinetic.

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MATERIALS AND METHODS

Sample preparation

Freshly obtained human EDTA plasma from normal healthy volunteers was incubated with the apoA-I mimetic peptide D4F (21) or other similar peptides at concentrations ranging from 0 to 500 μg/ml. Human apoA-I (Academy Bio-Medical) diluted to 100–500 μg/ml in plasma was prepared as a positive control, whereas untreated plasma and vehicle (PBS) in plasma provided baseline negative controls. The resulting treated plasma samples and controls were incubated at 37°C for 0–4 h, followed by a 1:10 dilution of the plasma into a solution of 50% sucrose in MilliQ water to stabilize preβ$_1$ HDL. During this step, the samples were diluted to 1:9 sample-50% sucrose solution. Afterward, samples were stored at −20°C before subsequent analysis. **Figure 1** shows an overall flow diagram for the subsequent analyses described in more detail below.

Western blotting analysis of human plasma samples

Human plasma samples treated as above were diluted 1:2 with sample buffer (60% sucrose, 0.1% bromophenol blue) and resolved by nondenaturing, nonreducing one-dimensional 12% polyacrylamide gel electrophoresis at 175 V for ~90 min. Colored molecular weight markers (Invitrogen) were run on each gel. Afterward, the gels were transferred to nitrocellulose for 2 h at 100 V. After transfer, the membrane was blocked with TBS-casein (Pierce) and washed three times (10 min each) with TBST (10 mM Tris, pH 7.4, 150 mM NaCl, and 0.1% Tween 20). The membrane was then probed with 2 μg/ml polyclonal HRP-labeled goat anti-human apoA-I antibody (Academy Bio-Medical) for 1 h at room temperature. Afterward, the blots were washed as above with TBST followed by an additional 10 min wash with TBS. After washing, the blots were developed with ECL reagent (Amersham), and after drying, they were exposed to Bio-Max X-ray film (Kodak).

ELISA determination of preβ$_1$ HDL levels

Stored samples (1:10 in 50% sucrose) were diluted an additional 1:100 in dilution buffer (1% BSA in PBS) for a final dilution of 1:1,000. A standard curve was established by reconstitution of lyophilized apoA-I standard in dilution buffer to 200 ng/ml, followed by serial 1:2 dilutions down to 1.5 ng/ml. Next, preβ$_1$ HDL ELISA kits (Daiichi Pure Chemicals, Inc.) were used to measure preβ$_1$ HDL as described previously (10, 11) with minor modifications. Briefly, 50 μl of sample and standard were added in duplicate to the wells of the MAb 55201-precoated, preblocked 96-well plate. Wells were incubated for 1 h at room temperature, followed by three washes with 100 μl of wash solution (0.1% BSA-PBS). Bound preβ$_1$ HDL was detected with HRP-labeled polyclonal goat anti-apoA-I antibody for 1 h at room temperature followed by four additional washes with BSA-PBS. After washing, wells were incubated with 50 μl of substrate (*o*-phenylenediamine in citrate buffer) for ~15 min and measured for absorbance at 492 nm using a Spectromax 96-well plate reader. Raw absorbance data were entered into Sigma Plot for subsequent standard curve modeling and sample preβ$_1$ HDL concentration determinations. This ELISA method is highly specific for apoA-I in the preβ$_1$ HDL conformation and has been shown to correlate extremely well with preβ$_1$ HDL levels as measured by two-dimensional gel electrophoresis (10, 11).

Pharmacokinetic analysis of apoA-I mimetic peptides, including preparation and extraction of plasma standards, samples, controls, and liquid chromatography-tandem mass spectrometry

Plasma standards were prepared from 1 to 10,000 ng/ml. For sample analysis, a 100 μl aliquot of each plasma sample, standard, and control plasma was used. A 50 μl aliquot of an internal standard solution (125 ng/ml in water) was added to each sample aliquot. The plasma samples were then transferred to a Waters (Milford, MA) Sep-PAK tC18 microelution solid-phase extraction

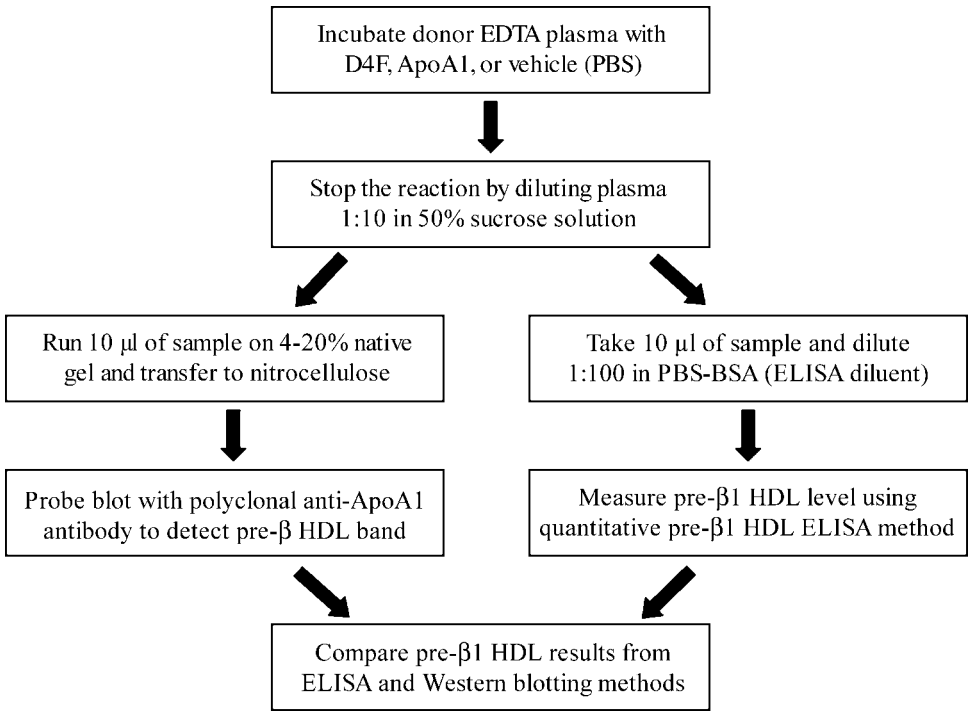


Fig. 1. Flow diagram for Western blotting and ELISA methods. Human plasma was incubated with D4F, apolipoprotein (apoA-I), or vehicle and processed for preβ$_1$ HDL analysis by Western blotting and ELISA, as shown.

plate. The plasma samples, standards, and controls were washed three times with 400 μl of water before being eluted with 180 μl of 0.1% trifluoroacetic acid in 40:60 water-acetonitrile. The eluate was dried under nitrogen and then reconstituted with 80 μl of 0.1% acetic acid.

Reconstituted samples (20 μl) were injected onto a 35 × 2.1 mm Capcell PAK UG 300A liquid chromatography column packed with 5 μM C18 resin (Phenomenex, Torrance, CA). The mobile phases used for chromatographic separation consisted of 0.1% acetic acid (0.5:500, v/v, acetic acid-water) (mobile phase A) and 1% acetic acid in acetic acid-water-methanol-acetonitrile (5:50:325:125, v/v/v/v) (mobile phase B). The analytes and internal standard were eluted from the column using a flow rate of 150 μl/min and a programmed binary gradient starting at 10% mobile phase B for 0.5 min, then a linear ramp to 90% mobile phase B at 2.5 min. The column was maintained at 90% mobile phase B for 3.0 min and then returned to 10% mobile phase B. The cycle time from one injection to the next was 5.0 min. The liquid chromatography column was coupled to a TSQ Quantum tandem mass spectrometer (Thermo Electron, San Jose, CA). Analytes were ionized using positive ion electrospray and detected using selected reaction monitoring. Quantitative determination was performed by regressing detected analytes against their respective plasma standard curves. Integration and quantitation were performed using LCquan 2.0 (Thermo Electron).

Peptide dosing in mice and murine preβ HDL detection

All animal experiments were approved by the Institutional Animal Care and Use Committee of Eli Lilly and Co. Eight week old male C57BL6/J mice were purchased from Harlan (Indianapolis, IN) and acclimated for 1 week before the start of the studies. Mice were provided Purina 5001 chow ad libitum and had free access to water throughout the experiments. The mice were individually caged. Light was controlled on a 12 h on/12 h off light/dark cycle. Peptide was dosed subcutaneously in PBS. Mice were bled at appropriate time points under isoflurane anesthesia, euthanized at 1 h after dose by CO_2 asphyxiation, and bled by cardiac puncture. All samples were kept on ice until centrifuged. Nondenaturing gel electrophoresis was used to detect the murine preβ HDL particles as described previously (22). Briefly, gels were prerun at 125 V for 30 min at 4°C. Next, 0.2 μl of each mouse plasma sample (diluted in 10% sucrose and 0.016% bromophenol blue) was loaded into each well. Electrophoresis was performed with the following step voltage scheme: 25 V for 15 min, 50 V for 15 min, 75 V for 15 min, and 250 V for 20 h. Proteins were transferred from the gel to nitrocellulose membrane at 200 mA for 12 h at 4°C. Murine preβ HDL particles on the membrane were detected using rabbit anti-mouse apoA-I antibody (BioDesign, Inc.).

Cholesterol efflux and paraoxonase assays

Mouse macrophages (RAW 264.7; American Type Culture Collection catalog number TIB-71) were maintained in 75 mm flasks, according to American Type Culture Collection recommendations. After seeding at 0.8–1 million/ml in the same medium with 10% FBS, cells were cultured to ~85% confluence. Subsequently, culture medium was replaced for 24 h with medium containing 0.2% BSA, 50 μg/ml acetylated LDL, and 1 μCi/ml [^{3}H]cholesterol (Perkin-Elmer) in DMEM. After cholesterol loading, cells were washed twice with serum-free medium. For the next 24 h, cells were incubated in DMEM with 0.2% fatty acid-free BSA with or without 0.3 mM Br-cAMP (Sigma). After another wash with serum-free medium, cells were incubated for the next 4 h in DMEM with 0.2% fatty acid-free BSA and 2.5% plasma collected from animals used in the study at 4 h after administration of either compound or vehicle. Subsequently, medium was removed and filtered through a 0.45 μm glass fiber filter to remove cellular debris. Both medium and cell lysate (0.1% Triton X-100 in PBS) were mixed separately with scintillation cocktail and counted. Cholesterol efflux was calculated as the percentage of radioactivity associated with medium over that for the sum of radioactivity from medium and cell lysate. The cAMP-dependent component of the efflux was calculated as the difference between cholesterol efflux measured in the presence or absence of Br-cAMP, with the change in cholesterol efflux capacity determined in comparison with animals treated with vehicle only.

Paraoxonase activity was measured using the EnzCheck Paraoxonase Assay Kit (Molecular Probes, Invitrogen) according to the protocol of the manufacturer. Plasma from animals treated with 30 mg/kg peptide or vehicle was collected for paraoxonase measurement and was diluted to 2% in the final reaction mixture. Intensity of emitted light was determined using Victor2 (EG&G Wallac) using 360 and 450 nm filters for excitation and emission, respectively. Enzymatic activity was calculated by converting fluorescent signal into activity using a standard curve.

Data analysis

SigmaPlot version 8.0, using a four parameter logistic model, was used for fitting of the calibration curves of the ELISA. Data were plotted using version 2.98 of the program FigP (Biosoft, St. Louis, MO). Statistical analysis was performed using the same program.

RESULTS

Preβ$_1$ HDL has been hypothesized to contain two to three molecules of apoA-I protein. To better understand the apoA-I content of preβ$_1$ HDL, apoA-I or D4F was incubated with human plasma and samples were analyzed by one-dimensional, nondenaturing, nonreducing Western blotting. These results were also compared with those obtained after incubating apoA-I with buffer alone. **Figure 2** shows the results from this series of experiments. When D4F was added to plasma, the formation of a preβ$_1$ HDL band at ~67 kDa was observed. In contrast, when apoA-I was added to buffer only, a somewhat lower band was observed, and when apoA-I was added to plasma, a broader intermediate band was observed. Because the molecular mass of human apoA-I is ~29 kDa, these results suggested that plasma preβ$_1$ HDL contains two molecules of apoA-I per particle, with some additional lipid, and that D4F increased preβ$_1$ HDL, rather than simply displacing apoA-I from HDL to cause an increase in lipid-free apoA-I.

Next, we used one-dimensional, nondenaturing, nonreducing Western blotting to examine the effect of the apoA-I mimetic D4F on preβ$_1$ HDL formation. As **Fig. 3** demonstrates, under baseline conditions, preβ$_1$ HDL was unable to be visualized in human plasma by direct Western blotting. The apoA-I mimetic D4F, however, dose-dependently increased the density of a 67 kDa preβ$_1$ HDL band when it was incubated with human plasma. The intensity of this band dramatically increased in response to increasing D4F concentrations. These data suggested that HDL remodeling was taking place as a dose-dependent response to the apoA-I mimetic peptide.

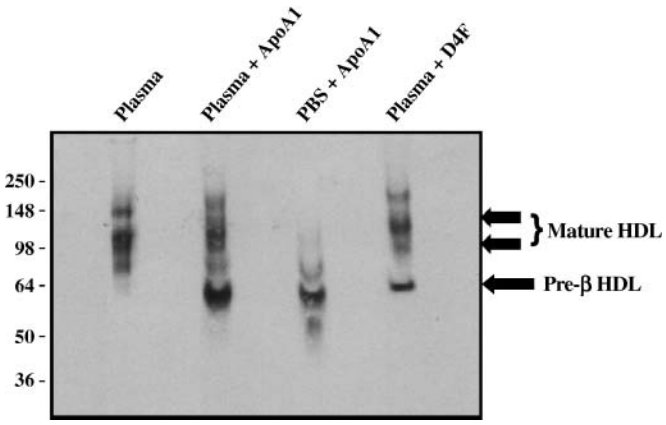


Fig. 2. ApoA-I increases plasma preβ$_1$ HDL as visualized by Western blotting. Human plasma was incubated with 500 μg/ml apoA-I or D4F, and PBS buffer was incubated with 500 μg/ml apoA-I. After incubation at 37°C for 1 h, reactions were stopped by diluting the samples 1:10 in 50% sucrose. Samples were separated using one-dimensional electrophoresis and analyzed by nonreducing, nondenaturing Western blotting with polyclonal HRP-labeled goat anti-human apoA-I antibody. Results are representative of two independent experiments.

These results indicated that one-dimensional, nondenaturing, nonreducing Western blotting could be used to assess preβ$_1$ HDL levels in human plasma in response to the addition of apoA-I or an apoA-I mimetic such as D4F. Because Western blotting is limited by its relatively low throughput, we investigated whether a novel Daiichi ELISA kit could accurately measure preβ$_1$ HDL levels. This ELISA uses a unique capture antibody that recognizes an epitope of apoA-I that is only exposed when apoA-I is in the preβ$_1$ HDL state. **Figure 4A** shows the results from these experiments, in which the ELISA detected increases

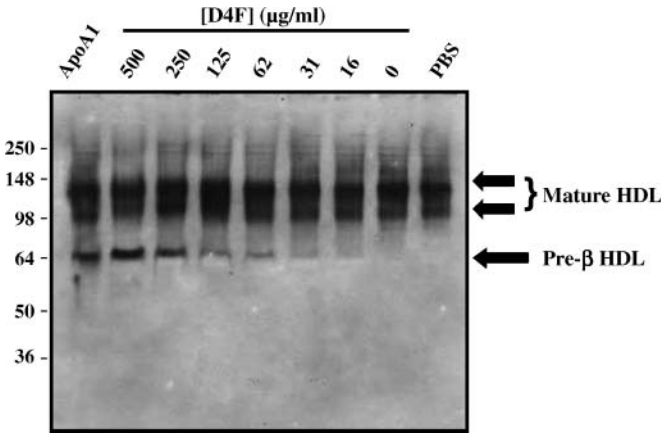


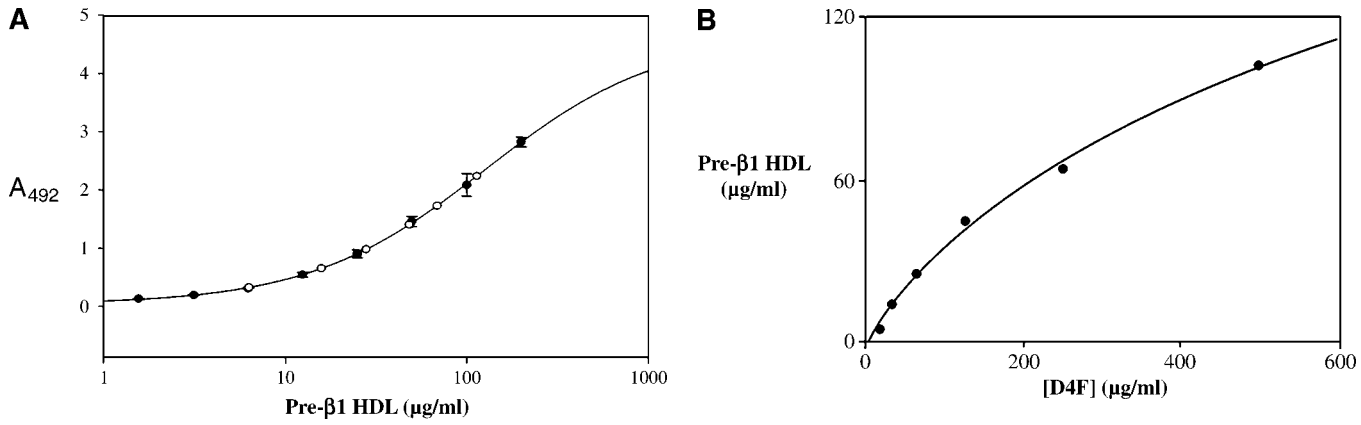
Fig. 3. D4F increases plasma preβ$_1$ HDL as visualized by Western blotting. Human plasma samples were incubated with either 100 μg/ml apoA-I or 0–500 μg/ml of the apoA-I mimetic D4F. After incubation at 37°C for 1 h, the reaction was stopped by diluting the samples 1:10 in 50% sucrose. Afterward, samples were separated electrophoretically and transferred to nitrocellulose for subsequent Western blotting with polyclonal HRP-labeled goat anti-human apoA-I antibody. Results are representative of two independent experiments.

in preβ$_1$ HDL concentration after apoA-I mimetic incubation. Untreated plasma and vehicle baseline controls demonstrated a preβ$_1$ HDL concentration of 3 ± 1 μg/ml after a 1 h incubation at 37°C. Incubation of the same plasma with 31 μg/ml of the apoA-I mimetic increased the plasma preβ$_1$ HDL concentration to 25 ± 6 μg/ml. Moreover, further increases in mimetic concentration produced dramatic increases in the formation of preβ$_1$ HDL (Fig. 4B). Figure 4C demonstrates that these ELISA data correlated well with the Western blotting data, suggesting that the ELISA provides a robust method for quantitating human preβ$_1$ HDL levels.

In light of the fact that paraoxonase is inhibited by EDTA and that D4F has been proposed to work in part by increasing cholesterol-containing particles with preβ mobility enriched in paraoxonase (19), an additional dose curve experiment with D4F was performed with heparinized plasma compared with EDTA plasma. The results observed with heparinized plasma were similar to those obtained with EDTA plasma across the range of D4F concentrations tested, with 500 μg/ml D4F resulting in a level of preβ$_1$ HDL of 111 ± 2 μg/ml in EDTA plasma versus 112 ± 11 μg/ml in heparinized plasma. Preβ$_1$ HDL concentrations across the rest of the dose curve of concentrations of D4F were also similar for EDTA and heparinized plasma (data not shown). Furthermore, in light of a previous report that a level of D4F of 0.322 μg/ml or less was able to generate preβ HDL in apoE knockout mice (19), we performed an additional experiment to examine the dose effect of D4F from 0 to 1 μg/ml. At these levels of D4F, the ELISA was unable to detect a significant effect on preβ$_1$ HDL levels, with levels being 5 ± 1 μg/ml at doses of 0, 250, 500, and 1,000 ng/ml D4F.

We next investigated the time course of D4F-induced increases of preβ$_1$ HDL in human plasma. **Figure 5** shows the results of these experiments, in which human plasma was treated with 500 μg/ml D4F peptide for 0–4 h. D4F-induced preβ$_1$ HDL formation occurred quickly (as early as 1 min after treatment of the plasma with the peptide), with a plateau reached after ~1 h, indicating that D4F-induced preβ$_1$ HDL formation is rapid and sustained.

To correlate D4F-induced preβ$_1$ HDL increases as measured by ELISA and the function and composition of HDL particles in vitro and in vivo, we first intravenously injected 250 μl of a solution containing 3 mg/ml D4F peptide into C57BL6/J mice (weighing ~25 g) and measured the pharmacokinetic (PK) concentration, which was ~0.5 mg/ml. Estimating that plasma constituted 50% of the volume of the blood, this PK level was consistent with almost complete recovery, indicating that the peptide was likely localized to the plasma. Next, we identified a D-amino acid apoA-I mimetic peptide similar to D4F with regard to its ability to increase preβ$_1$ HDL formation in human serum (data not shown), injected a comparable amount into C57BL6/J mice, and measured the effect on HDL fractions in vivo using capillary isotachophoresis (23). The response to peptide administration showed marked differences between individual HDL peaks. Specifically, the fHDL peak (corresponding to



C

Sample	Apo A1	D4F 500	D4F 250	D4F 125	D4F 62	D4F 31	D4F 16	D4F 0	PBS
Western Result									
A_{492}	1.53	2.12	1.66	1.35	0.93	0.62	0.31	0.14	0.16
μg/ml	54 ± 3	139 ± 21	89 ± 15	66 ± 12	42 ± 9	25 ± 6	7 ± 2	3 ± 1	3 ± 1

Fig. 4. D4F increases plasma preβ$_1$ HDL as assessed by ELISA, with results correlating to those obtained via Western blotting. A: After the Western blotting analysis shown in Fig. 3, the same samples (preserved in 50% sucrose) were analyzed using a preβ$_1$ HDL ELISA after an additional dilution of 1:100 in 1% BSA-PBS (final dilution = 1:1,000). The ELISA standard curve was established by reconstitution of lyophilized apoA-I standard in dilution buffer at a concentration of 200 ng/ml, followed by serial 1:2 dilutions (closed circles). At a 1:1,000 dilution, all samples (open circles) were well within the standard curve of the ELISA. Results are representative of two independent experiments. A_{492}, absorbance at 492 nm. Results indicate ± SEM. B: Plasma preβ$_1$ HDL concentrations were plotted as a function of apoA-I mimetic concentration. An increase in preβ$_1$ HDL formation was observed in response to increasing concentrations of D4F. Results are representative of two independent experiments. C: Direct comparison between the Western blotting results from Fig. 3 and the ELISA results from A and B. D4F ELISA results are expressed as means ± SEM and represent n = 4 from two independent experiments.

mature HDL) showed a significant dose-dependent decrease, whereas the slower migrating sHDL and chylomicron peaks (which contain preβ$_1$ HDL) showed significant dose-dependent increases (data not shown), suggesting that the peptide was remodeling mature α HDL into preβ HDL.

Next we attempted to use the ELISA to measure increases in preβ$_1$ HDL in the mice. Unfortunately, however, it was observed that the ELISA was not able to recognize rodent preβ$_1$ HDL (data not shown). As a result, an additional series of experiments was performed with the D-amino acid peptide described above. The peptide was again spiked into human plasma, resulting in a dose curve on the preβ$_1$ HDL ELISA similar to that of D4F (data not shown). Next, the peptide or vehicle was added in vitro into mouse plasma at a concentration of 300 μg/ml or was

Time (min)	0	1	5	15	30	60	120	240
Western Result								
A_{492}	0.31	2.20	2.35	2.60	2.78	2.88	2.77	2.67
μg/ml	4 ± 1	67 ± 6	80 ± 3	90 ± 10	107 ± 20	125 ± 26	116 ± 18	110 ± 11

Fig. 5. Time course of D4F-induced increases in plasma preβ$_1$ HDL. Human plasma was incubated with 500 μg/ml D4F for 0–4 h. Afterward, the reaction was stopped by diluting the samples 1:10 in 50% sucrose. Samples were diluted an additional 1:100 in 1% BSA-PBS (final dilution = 1:1,000) and analyzed as described for Fig. 4. A direct comparison between the Western blotting results and the ELISA results is shown. D4F ELISA results are expressed as means ± SEM and represent n = 4 from two independent experiments. A_{492}, absorbance at 492 nm.

injected into mice (n = 5 for each group) in amounts (20 or 30 mg/kg) that gave corresponding PK concentrations of ~200–300 μg/ml in the recovered plasma from the injected animals. Subsequently, these plasma samples were assayed for their ability to increase cAMP-dependent, ABCA1-mediated cholesterol efflux using RAW cells with a final plasma concentration of 2.5% (v/v). Results from this series of experiments indicated that at levels consistent with those required for preβ$_1$ HDL formation in vitro in human plasma, there were significant increases in cAMP-dependent, ABCA1-mediated cholesterol efflux. Specifically, a 48 ± 25% increase was observed with plasma from mice treated with 20 mg/kg, an 82 ± 19% increase was observed with plasma from mice treated with 30 mg/kg, and a 45 ± 21% increase was observed in the spiked plasma (all efflux results expressed as means ± SD). Paraoxonase activity was also measured in the 30 mg/kg and control groups and was found not to be increased significantly by administration of the peptide. Control plasma had 32 ± 1 U/ml paraoxonase activity, whereas plasma from peptide-treated animals had 33 ± 1 U/ml paraoxonase activity.

In light of the fact that the ELISA method was unable to recognize mouse preβ$_1$ HDL, we also performed additional experiments to measure preβ HDL formation in vivo. C57BL6/J mice were injected with D4F peptide (~5 mg/kg), and plasma samples were collected at time points ranging from 10 to 60 min. Preβ HDL was then measured via one-dimensional electrophoresis (22) followed by Western blotting with rabbit anti-mouse apoA-I antibody. **Figure 6** shows the results from these experiments, in which D4F increased the formation of preβ HDL in a time-dependent manner in the mice. Maximal increases were observed at 10–30 min, with some preβ HDL still detectable at 60 min, suggesting that in vivo the preβ HDL was being converted into mature HDL.

Finally, an additional D-amino acid peptide (similar in length to D4F but different from D4F, consisting of some amino acids not present in the D4F linear sequence) was identified that was inactive in increasing cAMP-dependent, ABCA1-mediated cholesterol efflux either in vitro or in vivo. This peptide was evaluated using the preβ$_1$ HDL ELISA after being spiked into human plasma at concentrations ranging from 0 to 500 μg/ml. The baseline concentration of preβ$_1$ HDL in this human plasma was 6 μg/ml. Compared with 500 μg/ml D4F, which caused a >40-fold increase in preβ$_1$ HDL levels in this human plasma to 244 μg/ml, the inactive peptide at the same concentration (500 μg/ml) only minimally increased the preβ$_1$ HDL level to 16 μg/ml (barely a 2-fold increase).

DISCUSSION

Our results demonstrate that a preβ$_1$ HDL ELISA method can provide insight into the mechanism of action of apoA-I mimetic peptides. Using the highly selective MAb 55201 capture antibody in this sensitive and robust ELISA format, we were able to observe dose-dependent increases in human plasma preβ$_1$ HDL after treatment of human plasma with the D4F apoA-I mimetic peptide. These increases corresponded well with the increased density of the preβ$_1$ HDL band observed via one-dimensional nondenaturing, nonreducing Western blotting.

Our findings with human plasma are in agreement with those described by Navab et al. (19), which linked D4F administration to preβ HDL formation in apoE-null mice via two-dimensional gel electrophoresis methods. Compared with two-dimensional gel electrophoresis and the earlier gel filtration (8, 9, 19), however, the Daiichi ELISA offers increased throughput. The ELISA also has a relatively broad dynamic range.

Because cardiovascular disease remains a leading cause of mortality worldwide, it is likely that increased efforts will be made toward the development of HDL-modulating therapies. As it has been shown that overexpression and direct infusion of apoA-I have antiatherogenic effects in numerous animal models, treatment with apoA-I analogs presents an enticing possibility as a pharmacological approach to modulating HDL. The large size of apoA-I combined with the high doses that would be required, however, necessitate the need to explore alternative options, such as apoA-I mimetic peptides.

These mimetics are currently being examined by numerous researchers as possible therapeutic agents (2, 24). The apoA-I mimetic peptides are composed of D-amino acids and are much smaller than native and full-length recombinant apoA-I, thus likely providing more favorable

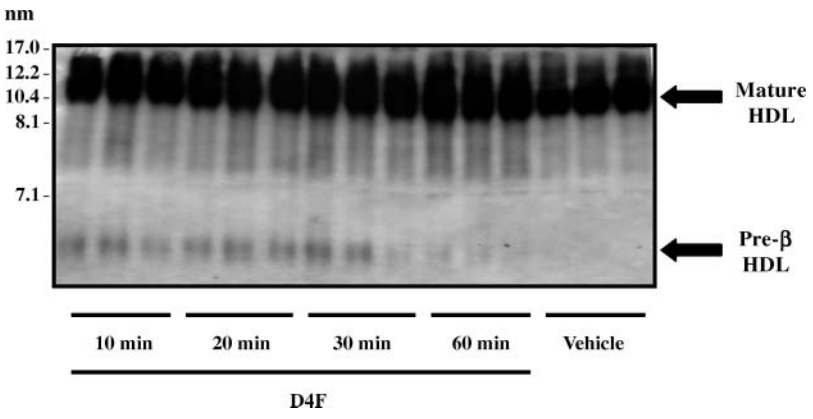


Fig. 6. D4F increases plasma preβ HDL in vivo. C57BL6/J mice (three per group) were injected with D4F peptide, and plasma samples were collected at time points ranging from 10 to 60 min. Plasma samples were diluted in sucrose, separated stepwise electrophoretically as described in Methods, and transferred to nitrocellulose for subsequent Western blotting with polyclonal rabbit anti-mouse apoA-I antibody. Results are representative of two independent experiments.

possibilities with regard to administration, synthesis, and production cost. For any of these compounds to ultimately become a drug, however, it will be very helpful to have a high-throughput assay that provides the potential for determining whether apoA-I mimetic peptides can associate with preexisting HDL particles in plasma to form preβ_1 HDL. Such an assay could also be adapted as a high-throughput screen to identify new apoA-I mimetic peptides. Based on our results in this study, we believe that preβ_1 HDL measurement by ELISA will provide this type of information by using a practical, high-throughput method capable of measuring the ability of apoA-I mimetic peptides to induce the formation of preβ_1 HDL.

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