ApoA-I Mimetic Peptides with Differing Ability to Inhibit Atherosclerosis Also Exhibit Differences in Their Interactions with Membrane Bilayers*

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Two homologous apoA-I mimetic peptides, 3F-2 and 3F14, differ in their in vitro antiatherogenic properties (Epand, R. M., Epand, R. F., Sayer, B. G., Datta, G., Chaddha, M., and Anantharamaiah, G. M. (2004) J. Biol. Chem. 279, 51404–51414). In the present work, we demonstrate that the peptide 3F-2, which has more potent anti-inflammatory activity in vitro when administered intraperitoneally to female apoE null mice (20 μg/mouse/day) for 6 weeks, inhibits atherosclerosis (lesion area 15,800 ± 1000 μm², n = 29), whereas 3F14 does not (lesion area 20,400 ± 1000 μm², n = 26) compared with control saline administered (19,900 ± 1400 μm², n = 22). Plasma distribution of the peptides differs in that 3F-2 preferentially associates with high density lipoprotein, whereas 3F14 preferentially associates with apoB-containing particles. After intraperitoneal injection of 14C-labeled peptides, 3F14 reaches a higher maximal concentration and has a longer half-time of elimination than 3F-2. A study of the effect of these peptides on the motional and organizational properties of phospholipid bilayers, using several NMR methods, demonstrates that the two peptides insert to different extents into membranes. 3F-2 with aromatic residues at the center of the nonpolar face partitions closer to the phospholipid head group compared with 3F14. In contrast, only 3F14 affects the terminal methyl group of the acyl chain, decreasing the 2H order parameter and at the same time also decreasing the molecular motion of this methyl group. This dual effect of 3F14 can be explained in terms of the cross-sectional shape of the amphipathic helix. These results support the proposal that the molecular basis for the difference in the biological activities of the two peptides lies with their different interactions with membranes.

ApoA-I mimetic peptides are increasingly considered as potential effective treatments for atherosclerosis and related inflammatory disorders (1–3); therefore, knowledge of the molecular basis of their modulation of membrane properties is important for an understanding of their mechanism of action. Several studies have shown that peptides capable of enhancing the protective effects of HDL³ against inflammation also inhibit atherosclerosis (4). Not all HDL populations exhibit similar biological activities (5). The protective effect of HDL against atherosclerosis is not only dependent on a higher concentration of HDL in the blood but is also dependent on the molecular composition of the HDL. Curiously, another major protein of HDL, the apolipoprotein A-II (apoA-II), which also possesses a structural motif related to apoA-I, has proinflammatory activity, and transgenic mice expressing human apoA-II develop atherosclerosis (6). This is in contrast to transgenic mice expressing human apoA-I, which are resistant to atherosclerosis (6). Whereas apoA-I is easily exchangeable, apoA-II can displace apoA-I, indicating higher lipid affinity and nonexchangeability of this apolipoprotein (7). Furthermore, the antioxidant enzyme paraoxonase is able to associate with apoA-I-alone-containing particles and not with apoA-II-containing particles, indicating a possible reason for increased antiatherogenicity of apoA-I-alone-containing particles (8–10).

When the apoA-I mimetic peptide D-4F is administered to mice that possess inflammatory HDL, the HDL is converted to a form that has inhibitory activity against LDL-induced monocyte chemotaxis (11). We have shown that in class A amphipathic helical peptides possessing the same polar face with identical charged residue distribution, subtle differences in the arrangement of the hydrophobic residues result in only subtle differences in physical properties and interaction with lipids but result in large differences in biological properties (12, 13). This is perhaps analogous to the differences between apolipoproteins A-I and A-II. These observations prompted the present investigations to understand molecular properties of a

The abbreviations used are: HDL, high density lipoprotein; LDL, low density lipoprotein; 3F-2, Ac-DKWKAVYDKFAEAFKEFL-NH₂; 3F14, Ac-DWLKAFYDK-VAEKFKEAF-NH₂; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; CSA, chemical shift anisotropy; SSB, spinning side bands; PON, paraoxonase; HPLC, high pressure liquid chromatography; PIPES, 1,4-piperazinediethanesulfonic acid; FID, free induction decay; DMPC, dimyristoyl phosphatidylcholine; MAS, magic angle spinning.

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‡ The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S4–S7.

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pair of class A amphipathic helical peptides with opposing ability to inhibit LDL-induced monocyte chemotaxis in the coculture system.

The apoA-I mimic peptides we have studied contain only 18 amino acid residues, compared with the 243 residues of apoA-I. Despite their small size, some of these peptides are as potent as apoA-I in solubilizing lipid, inhibiting hemolysis caused by lytic peptides, inhibiting oxidized phospholipid-induced monocyte chemotaxis, scavenging lipid hydroperoxides from LDL, and maintaining endothelial nitric-oxide synthase activity in the presence of atherogenic concentrations of LDL (14). Studies using these peptide analogs clearly show that in vitro anti-inflammatory activities are sensitive to the arrangement of residues on the hydrophobic face of the helix (12). Although the anti-inflammatory activities of this series of model class A amphipathic helical peptides are severalfold different, both active and inactive analogs share many of the same physical properties. They are similar in conformation, as shown by CD in both the presence and absence of lipid (12). Fluorescence studies of the Trp emission suggest that the peptides bind phospholipids to a similar extent (12). HPLC retention times and monolayer exclusion pressures indicated that there is no direct correlation of peptide function with lipid affinity. We hypothesize that the difference in anti-inflammatory activities among these peptides is a result of rather subtle differences in the penetration of the peptide into lipid. It appears that the requirements for anti-inflammatory activity are not based on a specific structure or interaction, since the peptides 3F-1, 3F-2, and 4F all are able to inhibit LDL-induced monocyte chemotaxis despite differences in their sequences (12). Furthermore, even some peptides derived from the sequence of apoA-I also have anti-inflammatory properties, although they have very different sequences (15). The present study demonstrates that one of the 3F peptides, 3F-2, which was able to inhibit LDL-induced monocyte chemotaxis (12), also protects against atherosclerotic lesion formation when administered to apoE null mice that spontaneously develop atherosclerosis, whereas the analogous peptide 3F14 (Fig. 1) that did not inhibit LDL-induced monocyte chemotaxis does not inhibit lesion formation. Using NMR, we further show that these differences in the inhibition of lesion formation are related to differences in the perturbation of lipid bilayers by these peptides. These observations are related to differences in the modulation of HDL properties in vivo by these two peptides.

EXPERIMENTAL PROCEDURES

Materials—Cholesterol and 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) were obtained from Avanti Polar Lipids (Alabaster, AL). Specifically deuterated forms of oleic acid were purchased from C/D/N Isotopes, Inc. (Pointe-Claire, Quebec, Canada). These labeled fatty acids were incorporated into POPC by chemical synthesis by Avanti Polar Lipids. The two forms of deuterio-oleoyl POPC were >99% pure with about 5% acyl migration. Peptides were synthesized by the solid phase method with a Protein Technologies PS-3 automatic peptide synthesizer using the procedures described previously (13, 16). Peptides were purified using a preparative HPLC system (Beckman Gold), and the purity of the peptides was ascertained by mass spectral analysis and analytical HPLC. For obtaining 14C-labeled peptides, during the last step of the synthesis, 14C-labeled acetic acid (American Radiolabeled Chemicals, Inc. St Louis, MO) was used to acetylate the peptide instead of normal acetic acid.

Animals—Female apoE null mice on a C57BL/6J background were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained on a chow diet (Ralston Purina). The University of Alabama at Birmingham Institutional Animal Care and Use Committee approved all animal studies.

Peptide Administration and Lesion Quantitation—Peptides 3F-2 and 3F14 were solubilized in physiological saline, and concentration was determined using A280 as described earlier (12, 13). 4-Week-old female mice were randomized into three groups. Two experimental groups received 20 μg/mouse/day peptide solutions administered intraperitoneally daily for 6 weeks, including weekends and holidays. The control group received an equal volume of saline every day for 6 weeks. At the end of the study, blood samples were taken under anesthesia by retro-orbital bleeding, and hearts were excised and subjected to histological examination and quantification of lesion area as described earlier (16).
Modulation of HDL by Antiatherogenic Peptides

Peptide-mediated Modulation of Lipoprotein Properties—Plasma distributions of 14C-labeled peptides 3F-2 and 3F14 were determined by mixing peptides with plasma from apoE null mice (5 μg of peptide/ml of plasma), incubating at 37 °C for 15 min, and then separating plasma lipoproteins using the CLiP procedure as described earlier (19). Fractions were collected, and radioactivity in each lipoprotein fraction was determined. Similar separations were done using 14C-labeled peptide-DMPC complexes (1:1 (w/w); 5 μg of peptide/ml of plasma).

For determination of changes in HDL subspecies, 20 μg of peptides were administered to female apoE null mice. Plasma was collected 5 h after peptide administration by retro-orbital bleeding. 3.5 μl of plasma was subjected to agarose electrophoresis in one dimension and nondenaturing polyacrylamide gel electrophoresis in the second dimension using the method described by us previously (20). Western analysis for apoA-I using apoA-I antibody as described previously (14).

For determining the distribution of apoA-I after peptide administration, plasma samples were first subjected to agarose gel electrophoresis in duplicates, and one agarose gel in each group was transferred and subjected to Western blot with mouse apoA-I antibody. The other agarose gel was cut into 14 equal fractions, and the apoA-I-containing fractions (as determined by the Western blot) were boiled with equal amounts of 10% SDS to extract apoA-I in these bands. These were then subjected to SDS-gel electrophoresis with isolated mouse apoA-I as a standard and subjected to Western blot with mouse apoA-I antibody. The mouse apoA-I bands were quantified as relative intensity in arbitrary units and plotted to determine the differences in the comparative amounts of apoA-I in different lipoprotein fractions.

Plasma Turnover of the Peptides—14C-Labeled peptides (20 μg/mouse) were administered to apoE null mice intraperitoneally, and the radioactivity in plasma was determined at 5, 10, 15, 20, 30, and 45 min and at 1, 1.5, 2, 3, 4, 6, 8, and 12 h. Three samples were taken at each time point. No more than three plasma samples were taken from each animal, and time points for animals were staggered so that no two animals had identical time point assignments. Turnover parameters were calculated on all data points (rather than averages) by PKNanalyst software (MicroMath Scientific Software, Salt Lake City, UT) using a one-compartment model with first order input and first order output.

In a separate experiment, 6-week-old female apoE null mice were treated with 3F-2, 3F14, or saline for 1 week (daily intraperitoneally; 20 μg/mouse/day). Lipid hydroperoxides were determined using a spectrophotometric assay (21). Paraoxonase activity was measured as described earlier (22). Platelet-activating factor-acetylhydrolase activity was measured by using a fluorescent substrate (23).

Preparation of Samples for NMR Experiments—Lipids were dissolved in chloroform/methanol (2:1, v/v). For samples containing peptide, an aliquot of a solution of the peptide in methanol was added to the lipid solution in chloroform/methanol. The solvent was then evaporated under a stream of nitrogen with constant rotation of a test tube so as to deposit a uniform film over the bottom one-third of the tube. The last traces of solvent were removed by placing the tube under high vacuum for several h. The lipid film was then hydrated with 20 mm PIPES, 1 mM EDTA, 150 mM NaCl with 0.002% NaN3, made either at pH 7.4 in deuterium-depleted water (for 2H NMR) or in 2H2O adjusted to a pH meter reading of 7.0 (pD = 7.4) and suspended by intermittent vortexing. The samples were transferred to 45-μl Kel-F spherical inserts of an 18 × 4-mm ZrO2 rotor, attempting to pack the maximal amount of sample into the insert while maintaining it wet. The samples were then incubated 24 h at 4 °C to allow for equilibration.

Assignment of Resonances for POPC—POPC was dissolved in 2HCCl3 (Cambridge Isotope Laboratories Inc.) to a concentration of ~20 mg/ml. Chemical shifts are reported in ppm relative to TMS using the residual solvent signals at 7.26 and 77.16 ppm as internal references for the 1H and 13C spectra, respectively. All spectra were recorded on a Bruker Avance 700-MHz NMR spectrometer. Proton spectra were acquired at 700.23 MHz using a 5-mm triple resonance inverse cryoprobe with z axis gradient capability. The spectra were obtained in 16 scans in 65,536 data points over a 5.297-kHz spectral width (6.187 s acquisition time). Sample temperature was maintained at 25 °C by a Bruker BVT 3000 digital variable temperature unit. A 1.5-s relaxation delay was used between acquisitions. The free induction decay (FID) was processed using Gaussian multiplication (line broadening, ~2.0 Hz; Gaussian broadening, 0.1). The FID was also zero-filled to 131,072 before Fourier transformation.

Proton COSY two-dimensional NMR spectra were recorded in the absolute value mode using the pulse sequence 90°-t1-45°-acquire and included pulsed field gradients for coherence selection. The data were acquired in two scans for each of the 256 FIDs that contained 2,048 data points in the F2 dimension over a 5.297-kHz spectral width. The 1H 90° pulse width was 8.0 μs. A 1.2-s relaxation delay was used between acquisitions. Forward linear prediction to 512 data points and zero-filling in the F1 dimension produced a 1,024 × 1,024 data matrix with a digital resolution of 5.172 Hz/point in both dimensions. During two-dimensional Fourier transformation, a sine-bell squared window function was applied to both dimensions. The transformed data were then symmetrized.

13C NMR spectra were recorded at 176.07 MHz using the 5-mm triple resonance inverse cryoprobe with z axis gradient capability. The spectra were acquired using the J-modulated spin spin pulse sequence over a 42.373-kHz spectral width in 65,536 data points (0.773 s acquisition time). The 13C pulse width was 15.0 μs (90° flip angle). A relaxation delay of 1.0 s was used. The delay time in the J-modulated spin spin pulse sequence was set at 0.006896 s to produce an edited spectrum containing quaternary and methylene carbons with positive phase, whereas methine and methyl carbons appeared with negative phase. The FID was processed using exponential multiplication (line broadening, 4.0 Hz) and zero-filled to 131,072 before Fourier transformation.

Inverse detected 1H,13C two-dimensional chemical shift correlation spectra were acquired in the phase-sensitive mode using the pulsed field gradient version of the HSQC pulse sequence. The FIDs in the F2 (1H) dimension were recorded over a 5.297-kHz spectral width in 2,048 data points. The 256 FIDs in the F1 (13C) dimension were obtained over a 29.499-
kHz spectral width. Each FID was acquired in two scans. The fixed delays during the pulse sequence were a 1.2-s relaxation delay and 1.724-ms delay for polarization transfer. The 90° 1H pulse width was 8.0 μs, whereas the 13C 90° pulse width was 15.0 μs. The data were processed using a sine-bell squared window function shifted by π/2 in both dimensions. Forward linear prediction to 512 data points followed by zero-filling to 1024 data points was also performed in the F1 dimension. Inverse detected 1H, 13C two-dimensional chemical shift correlation spectra through two- and three-bond coupling interactions were acquired in the absolute value mode using the pulsed field gradient version of the Heteronuclear Multiple Bond Correlation pulse sequence. The FIDs in the F2 (1H) dimension were acquired over a 5.297-kHz spectral width in 2,048 data points. The 256 FIDs in the F1 (13C) dimension were recorded over a 29.499-kHz spectral width. Each FID was acquired in two scans. The fixed delays during the pulse sequence were a 1.2-s relaxation delay and an 80.0-ms delay to allow evolution of the long range couplings. The 90° 1H pulse width was 8.0 μs, whereas the 13C 90° pulse width was 15.0 μs. The data were processed using a sine-bell window function in both dimensions. Forward linear prediction to 512 data points followed by zero-filling to 1024 data points was also performed in the F1 dimension.

2H Static Powder Pattern—Deuterium powder pattern spectra at a frequency of 92.12 MHz were acquired using a fixed tuned deuterium high resolution (saddle coil) probe on a Bruker AVANCE 600 spectrometer. 100 μl of the suspension in a 5-mm Shigemi NMR tube was centered in the coil of the probe. The quadrupole echo sequence (90°−τ1−90°−τ2-acquire) was used with a 90° pulse length of 9 μs and a relaxation delay of 0.1 s. The value of τ1 was 50 μs, and that of τ2 was very short, so that the top of the echo could be manually found by 22 left shifts of the data. 4,096 data points were acquired with a sweep width of 303.03 kHz for an acquisition time of 0.00681 s. Once the top of the echo was set at the start of the data, the set was zero-filled to 8,192, an exponential multiplication corresponding to a line broadening of 100 Hz was applied, and the data were Fourier transformed and phased. Quadrupole coupling values were measured from the separation of the peaks in the powder pattern.

MAS/NMR Measurements—The solid state NMR spectra were recorded on a Bruker AVANCE 500 spectrometer equipped with a standard bore 11.7-tesla magnet, giving a 500.12 MHz 1H base frequency. The spectrometer was equipped with a 4-mm broad band tunable MAS probe, and the spectra were recorded at ambient temperature. Magic angle spinning was controlled using a Bruker model H2620 pneumatic MAS controller.

13C T1 Measurements—The 13C NMR T1 times were determined using the saturation recovery method together with power-gated decoupling of the protons. The samples were spun at a rate of 5500 Hz. The spectra were recorded at a carbon resonance frequency of 125.77 MHz with a sweep width of 43 kHz, and the acquisition time was set to 0.5 s. Because the saturation recovery method permits rapid repetition, the recycle delay was set to 0.1 s.

At least eight different variable delay times were chosen, and their order was randomized in order to eliminate any systematic errors. For each variable delay time, 4,096 transients were co-added to give sufficient signal to noise. The raw data were zero-filled to 32,768 points, and exponential multiplication (broadening factor lb = 20 Hz) was applied. The integrated intensities of the peaks were plotted versus delay time, and an exponential fit of the data as per Equation 1 yielded the T1 results.

\[
I(t) = I(0) \cdot \left(1 - \exp\left(-\frac{t}{T_1}\right)\right) \quad \text{(Eq 1)}
\]

SSB of 31P—The 31P NMR spectra were recorded at 202.45 MHz over a 25-kHz sweep width. Raw spectra were recorded using 16,384 data points (acquisition time less than 0.3 s), and the data were processed without zero filling and with exponential multiplication (lb = 50 Hz). At least three different MAS spinning speeds were used for each sample. The chemical shift anisotropies (CSAs) of the 31P NMR from the phospholipid samples were determined using the solid line shape analysis routine from version 1.3 of Bruker’s Topspin program. This program calculates the CSA following the Herzfeld-Berger method (24). The CSAs were determined from the 31P NMR spectra recorded at at least three different spinning speeds, and the average result is reported.

RESULTS

Effect of Peptide Administration on Plasma Cholesterol—In all of the three groups, there was no difference in the total plasma cholesterol values. Fractionation of plasma on two serially connected Superose 6 FPLC columns yielded essentially identical lipoprotein profiles (results not shown).

Effect of Peptide Administration on Lesion Formation—Mean lesion cross-sectional areas are presented in Fig. 2. Mice administered peptide 3F-2 showed a significant decrease in lesion area compared with both the control group and the 3F-14-administered group. However, administration of the peptide 3F-14 did not show any difference compared with the control group (Fig. 2).
**TABLE 1**

| [14C]Peptide turnover parameters |
|----------------------------------|
| Plasma turnover parameters of 14C-labeled 3F-2 or 3F14 were determined as described under “Experimental Procedures.” Kinetic parameters were determined using a one-compartment model with first order input and first order output. T1/2, half-life; Cmax, maximum concentration. |
| 3F-2 | 3F14 |
|---|---|
| T1/2 of absorption (h) | 0.09 | 0.11 |
| T1/2 of elimination (h) | 0.54 | 2.10 |
| Time to Cmax (h) | 0.28 | 0.48 |
| Cmax (% injected cpm) | 5.52% | 12.29% |

**Distribution of 14C-labeled peptides in different plasma lipoproteins.** 5 μg of 14C-labeled 3F-2, 3F14, or peptide-DMPC complexes (1:1, w/w) were mixed with 1 ml of apoE null mouse plasma and incubated at 37 °C for 15 min. 200-μl plasma samples were separated into lipoprotein fractions using Superose 6 column chromatography. Fractions were counted for radioactivity. The percentages of total lipoprotein-associated counts were plotted for each lipoprotein fraction. Dark bars, 3F-2; double hatched bars, 3F-2-DMPC; gray bars, 3F14; single hatched bars, 3F14-DMPC; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein.

**Kinetics of Plasma Turnover of 14C-Radiolabeled Peptides—** Administration of 14C-labeled 3F-2 and 3F14 was done in order to determine the bioavailability of peptides. The results are summarized in Table 1. The half-time of absorption of 3F-2 was somewhat faster than for 3F14, and the maximal concentration of 3F14 was higher. Peptide 3F14 had a slower half-time of clearance compared with 3F-2.

**Distribution of Peptides 3F-2 and 3F14 in Plasma Lipoprotein Fractions—** Plasma from apoE null mice was incubated with 14C-labeled peptides 3F-2 or 3F14 or with peptide-DMPC complexes, and lipoprotein fractions were separated by FPLC using Superose 6 column chromatography. The radioactivity present in different lipoprotein fractions was measured, and the percentage of the total lipoprotein-associated radioactivity was plotted (Fig. 3). Results indicate that peptide 3F-2 preferentially associates with HDL-sized particles, with comparatively lesser association with the very low density lipoprotein and intermediate density lipoprotein/LDL peaks. However, peptide 3F14 associated primarily with the very low density lipoprotein and intermediate density lipoprotein/LDL peaks, with proportionally less associated with HDL. These results suggest that the peptide 3F14 was not able to modulate HDL as much as the peptide 3F-2.

Plasma samples at 5 h were subjected to two-dimensional gel electrophoresis and subjected to Western blot using polyclonal antibody for mouse apoA-I. Results show that 3F-2 caused increased immunoreactivity of apoA-I in post-α particles compared with 3F14 (Fig. 4), indicating that epitopes of apoA-I are exposed in the HDL samples from 3F-2-treated animals, perhaps accounting for increased antiatherogenic properties. This was supported by SDS-gel electrophoresis of total plasma and Western blotting with mouse apoA-I antibody in which the bands for apoA-I were of equal intensity, suggesting that neither peptide altered apoA-I levels (results not shown). These results support the concept that 3F-2 and not 3F14 modulates HDL properties.

The SDS bands of agarose electrophoresis gel fractions show a pattern similar to the two-dimensional gel electrophoresis (Fig. 5). Whereas the HDL fractions in 3F-2-administered samples possessed most of the apoA-I, the fractions in 3F14-administered agarose gel fractions showed less apoA-I in HDL fractions and comparatively more apoA-I in apoB-containing lipoprotein fractions. The 3F-2 agarose gel fractions also showed significantly more apoA-I in HDL fractions compared with fractions from 3F14-administered plasma, indicating that the differences seen in two-dimensional gel electrophoresis was not due to differences in the amounts of apoA-I present in these groups of mice but rather due to differences in the distribution of apoA-I among lipoprotein fractions.

**Lipid Hydroperoxides and Enzymatic Activities—** In mice treated with peptides 3F-2 or 3F14 for 1 week intraperitoneally (as described under “Experimental Procedures”), differences in lipid hydroperoxide levels, PON-1 activity, or platelet-activating factor-acetylhydrolase activity were not found between the groups (data not shown).

**2H Nuclear-Quadrupole Coupling Constants—** The splittings of the 2H NMR spectra of phospholipids with deuterated acyl chains provides a good measure of the orientation of the acyl chain with respect to the bilayer normal as well as its motional properties. We have measured the nuclear-quadrupole coupling constants of POPC labeled with either perdeuteropalmitate, 11,11-d2-oleate, or 9,10-d2-oleate. The static powder patterns were recorded for the pure lipid as well as the lipid in the
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presence of 1 or 2 mol % 3F-2 or 3F-14 at 15 and 25 °C. The nuclear-quadrupole coupling constants for the perdeuterated palmitoyl chain are compared for the two peptides and the pure lipid at two different peptide concentrations and at two temperatures (Fig. 6). The actual values of the coupling constants of the palmitoyl chain are given in supplemental Tables 4 and 5. In addition, we have measured nuclear-quadrupole coupling constants for specifically deuterated positions on the oleoyl chain (Table 2). These results show significant changes in the coupling constants on both the palmitoyl and the oleoyl chains of POPC with the addition of only 1 or 2 mol % peptide at both 15 and at 25 °C. The results with the pure lipid are in reasonable agreement with earlier measurements by Seelig and Waespe-Sarcevic (25), who has also assigned the splittings to specific positions on the acyl chain.

We also studied the nuclear-quadrupole splittings of equimolar mixtures of perdeuteropalmitoyl-POPC and cholesterol in the presence and absence of each of the two peptides. As expected, the splittings were greater in the presence of cholesterol than with the pure POPC, but there was still a measurable effect of 1 mol % 3F-14 but not of 3F-2 (see supplemental Table 6). The 13C resonance of the palmitoyl group, that has a significantly different chemical shift from that of the oleoyl, was not observed, probably because of its greater rigidity. The order of groups in both Fig. 7 and supplemental Table 7 is from the polar head group to the terminal methyl group of the acyl chain. The relaxation rates in supplemental Table 7 are given in terms of NT1, where N is the number of directly bonded hydrogens on a particular carbon atom (except for the carboxyl group, where N would be 0). The acyl chain positions show a gradient of motion, with the terminal methyl group having the greatest mobility. The C=C is somewhat more rigid because of the lack of rotation around the double bond. The peptides have significant but different effects on the motional rates.

CSA of 31P—we calculated the CSA of the phosphate atom of POPC in the presence and absence of peptides from measurements of the spinning side bands (SSB) using MAS/NMR at several different spinning speeds. An example showing the difference in the SSB pattern for the POPC in the presence of the two peptides is shown in Fig. 8. A series of such spectra, measured using different spinning speeds, is used to calculate the CSA. The CSA measured from SSB are much more precise than those measured from static powder patterns. In all cases, the static powder pattern corresponded to that of a lipid bilayer with rapid axial rotation (not shown). In addition to 3F-2 and 3F-14, we also measured the CSA in the presence of the other two members of the 3F family of peptides previously studied (12). All of the peptides reduced the CSA compared with the lipid alone, indicating that they were disorganizing the membrane/water interface, resulting in a more rapid axial rotation of the phosphatidic group. However, the two more biologically active peptides, 3F-1 and 3F-2, caused a greater decrease in the CSA. In particular, for the present study, 3F-2 and 3F-14 represent the 3F peptides causing the greatest and the least change, respectively, in the CSA among the four peptides studied.

DISCUSSION

The peptide 3F-2, but not the peptide 3F-14, has been shown to inhibit LDL-induced monocyte chemotaxis (12). We have now shown that a peptide that is able to inhibit LDL-induced monocyte chemotaxis, 3F-2, also inhibited atherosclerosis in apoE null mice. The mechanism we have proposed for these
observations is that the active peptide 3F-2, due to π-electrons at the center of the nonpolar face (that results in a trapezoidal cross-sectional shape), is not able to bury deeply within the lipid acyl chains (26).

To determine the differences in the effects of these two peptides on plasma lipoproteins and apoA-I distribution, we added 14C-labeled peptides to plasma from apoE null mice and determined the distribution of radioactivity among lipoproteins after fractionation of lipoproteins by Superose 6 column separation (Fig. 3). This was done both with free peptide and peptide-phospholipid complexes. These results indicated that peptide 3F-2 preferentially associated with HDL. In contrast, peptide 3F14 associated preferentially with apoB-containing particles. These results suggest that 3F-2 may significantly modulate HDL to a greater degree than 3F14.

It must be considered that differential bioavailability of peptides may account for their different antiatherogenic effects. In order to investigate this possibility, peptides were synthesized with 14C label in the acetyl group, avoiding the potential effects on the hydrophobic face by a bulky 125I group. As shown in Table 1, the nonatheroprotective peptide 3F14 had both a greater maximal blood concentration and a longer plasma residence time than did the protective peptide 3F-2. Thus, absolute bioavailability does not account for the difference in properties of the peptides. However, relative bioavailability differs, in the sense that peptide 3F-2 is present predominantly in HDL, whereas 3F14 is predominantly associated with apoB-containing particles. Thus, association with, and presumably perturbation of, HDL may be required for peptide-mediated atheroprotection.

The relatively short residence times of these peptides suggest that atheroprotective effects are either rapid or do not require the presence of the peptide to be maintained. We did not observe differences in lipid hydroperoxides, PON-1, or platelet-activating factor-acetylhydrolase. It is possible that such changes occurred and were transient or that some other atheroprotective mechanism may be involved.

Two-dimensional gel electrophoresis (Fig. 4) further supports the concept that 3F-2 causes changes in smaller HDL particles (as determined by apoA-I immunoreactivity) that are lacking in 3F14-administered mouse plasma 5 h after peptide administration. It should be noted that administration of the peptide 4F causes a different modification of HDL than 3F-2, with the formation of pre-β-like-HDL subfractionation that is mostly apoA-I-containing with small amounts of phospholipid (27); this may reflect differences in potency of atheroprotection between 3F-2 and 4F. The results further support our model that π-electron-containing aromatic residue clustering is

### TABLE 2

Nuclear quadrupole splitting of oleoyl C-H in POPC

| Oleoyl carbon | Lipid alone | +1 3F14 | +1 3F-2 | +2 3F14 | +2 3F-2 |
|--------------|-------------|---------|---------|---------|---------|
| 9 15°C | 13.93 | 13.61 | 13.94 | 13.53 | 13.85 |
| 10 15°C | 2.60 | 1.93 | 2.59 | 1.76 | 2.55 |
| 11 15°C | 7.84 | 6.64 | 7.62 | 6.49 | 7.69 |
| 9 25°C | 13.19 | 12.84 | 13.14 | 12.71 | 12.84 |
| 10 25°C | 2.32 | 1.71 | 2.26 | 1.39 | 2.23 |
| 11 25°C | 6.92 | 5.76 | 6.55 | 5.47 | 6.52 |

14C-labeled peptides to plasma from apoE null mice and determined the distribution of radioactivity among lipoproteins after fractionation of lipoproteins by Superose 6 column separation (Fig. 3). This was done both with free peptide and peptide-phospholipid complexes. These results indicated that peptide 3F-2 preferentially associated with HDL. In contrast, peptide 3F14 associated preferentially with apoB-containing particles. These results suggest that 3F-2 may significantly modulate HDL to a greater degree than 3F14.

It must be considered that differential bioavailability of peptides may account for their different antiatherogenic effects. In order to investigate this possibility, peptides were synthesized with 14C label in the acetyl group, avoiding the potential effects on the hydrophobic face by a bulky 125I group. As shown in Table 1, the nonatheroprotective peptide 3F14 had both a greater maximal blood concentration and a longer plasma residence time than did the protective peptide 3F-2. Thus, absolute bioavailability does not account for the difference in properties of the peptides. However, relative bioavailability differs, in the sense that peptide 3F-2 is present predominantly in HDL, whereas 3F14 is predominantly associated with apoB-containing particles. Thus, association with, and presumably perturbation of, HDL may be required for peptide-mediated atheroprotection.

The relatively short residence times of these peptides suggest that atheroprotective effects are either rapid or do not require the presence of the peptide to be maintained. We did not observe differences in lipid hydroperoxides, PON-1, or platelet-activating factor-acetylhydrolase. It is possible that such changes occurred and were transient or that some other atheroprotective mechanism may be involved.

Two-dimensional gel electrophoresis (Fig. 4) further supports the concept that 3F-2 causes changes in smaller HDL particles (as determined by apoA-I immunoreactivity) that are lacking in 3F14-administered mouse plasma 5 h after peptide administration. It should be noted that administration of the peptide 4F causes a different modification of HDL than 3F-2, with the formation of pre-β-like-HDL subfractionation that is mostly apoA-I-containing with small amounts of phospholipid (27); this may reflect differences in potency of atheroprotection between 3F-2 and 4F. The results further support our model that π-electron-containing aromatic residue clustering is
responsible for remodeling HDL, thereby altering its function. 3F14, with Leu residue at the center of the nonpolar face, interacts with the acyl chains of the phospholipid and thus is not available for modulation of HDL properties. This is supported by the NMR experiments.

Recently, it was proposed that HDL-3C, a post-α-migrating HDL subspecies in two-dimensional electrophoresis (28), is rich in proteins and enzymes, such as PON-1 (29). We propose that the peptide 3F-2 and not 3F14 is producing HDL-like particles that are responsible for antiatherogenic properties shown by the peptide analog 3F-2. This is evident in plasma samples from 3F-2-treated mice (with modulated immunoreactivity in post-α-migrating particles) (Fig. 4).

This model of the insertion of 3F-2 into lipid is consistent with the present findings of the changes in lipid properties induced by the peptide. 3F-2 has little effect on the acyl chain order parameters (Fig. 6), and it causes a greater increase in the \( T_1 \) of the choline methylene in the lipid head group compared with 3F14, whereas 3F-2 has no effect on the \( T_1 \) of the terminal methyl group of the acyl chain (Fig. 7). In addition, the greater effect of 3F-2 on interfacial properties is also demonstrated by its large effect on the \(^{31}\)P CSA (Table 3). With 3F14, the situation is opposite, with the lipid acyl chain motion being governed by the strong interaction of the nonpolar face of the peptide with the lipid acyl chain, resulting in a deeper burial of the peptide into the bilayer. This is shown by the observations that 3F14 affects both the deuterium order parameters (Fig. 6) and the \( T_1 \) of \(^{13}\)C (Fig. 7) down to the terminal methyl group of the acyl chain.

For the pure lipid, a decrease in the order parameter is accompanied by an increase in NT \(_1\), indicative of groups being displaced away from the bilayer normal and having increased rates of motion. In the presence of 3F14, these two parameters go in opposite directions, suggesting that 3F14 causes displacement of the acyl chain, but at the same time it increases the packing density (lateral pressure) at the center of the membrane, thereby slowing molecular motion.

In the presence of cholesterol, the effects of the two peptides are smaller, and there is less difference between them (see supplemental Table 6). In addition, the effect of 3F14 is somewhat greater close to the end of the acyl chain but before the terminal methyl group. These results are consistent with \(^1\)H MAS/NOESY measurements that demonstrated that in pure POPC 3F14 inserted deeper into the membrane than 3F-2 and that cholesterol inhibited the entry of both peptides into the membrane (26).

The changes seen in the acyl chain order parameters are not restricted to the palmitoyl chain, but the carbons around the double bond of the oleoyl chain are also affected by 3F14 (Table 2). The low value of the nuclear-quadrupole coupling for the C-10 position of the oleoyl chain is a consequence of the geometry of the cis double bond that causes the acyl chain to be displaced from the bilayer normal. There is a dramatic difference between the two peptides in their effect on the C-10 position, with 3F14 being much more potent than 3F-2. The order parameter is dependent on both the deviation of the acyl chain from the bilayer normal and the molecular motion.

It is clear that these two peptides, 3F-2 and 3F14, have different effects on the lipid by several independent NMR criteria. Most of these effects are explicable as a consequence of the

![Figure 7](image7.png)

**FIGURE 7.** \( T_1 \) of POPC in the presence and absence of peptide. The ratio of \( T_1 \) in the presence to that in the absence of peptide is shown for various positions in the phospholipid. The actual values of \( T_1 \) are given in the supplemental material. An increase of the ratio is indicative of the peptide causing an increase of the rate of motion at that position in the phospholipid. The position in the phospholipid is identified by its chemical shift and is indicated along the abscissa.

![Figure 8](image8.png)

**FIGURE 8.** \(^{31}\)P SSB of POPC with 10 mol % peptide. The spinning side bands of samples of POPC with 10 mol % 3F-2 or 3F14 are shown using a 1000-Hz spinning speed in a 4-mm MAS rotor with 45-μl inserts. These spectra illustrate the clear differences between the effects of 3F-2 and 3F14 on these spectra, reflecting differences in the motional properties of the phosphate. Several such spectra using different spinning speeds were recorded, from which the CSA was calculated and is presented in Table 2.

### Table 3

| Sample                  | CSA \( \text{ppm} \) |
|-------------------------|---------------------|
| Pure POPC               | 47.0                |
| 3F-2 10 mol % in POPC   | 33.6                |
| 3F-2 10 mol % in POPC   | 37.0                |
| 3F14 10 mol % in POPC   | 43.0                |

These results represent the average CSA determined from fitting the spectral data from three different spinning speeds of roughly 1, 3, and 5.5 kHz. The pure POPC was also measured at a fourth spinning speed of 2 kHz.
lower degree of penetration of 3F-2 into the membrane compared with 3F14. We also suggest that the mode of insertion of these peptides into the membrane and their effects on membrane properties explain their different biological activities. This suggestion is supported by the finding that the CSA of 31P correlates with anti-inflammatory potency. Thus, the two more active peptides (12), 3F-1 and 3F-2, have the smallest CSA (Table 3), whereas the two inactive peptides, 3F3 and 3F14, cause less reduction in the CSA. By this criterion and for this relatively small group of peptides having identical amino acid compositions, there is a good correlation between the change in CSA and the biological activity.

There are likely to be multiple mechanisms by which these peptides exert their antiatherosclerotic activity. We suggest that the active peptides form complexes with lipids that have favorable biological properties. In normal animals, there is already a preponderance of endogenous HDL containing apoA-I that has anti-atherosclerotic activity. In order to further enhance the protection against atherosclerosis provided by HDL, these peptides must have greater activity than apoA-I itself or potentiate the activity of apoA-I. It is not likely that this can occur simply by having the peptides substitute for apoA-I, because they are in so much lower concentration than the native protein. Our hypothesis is that the peptides modify HDL, either to form new HDL-like particles that can recruit proteins, cholesterol, and oxidized lipids or to change the properties of HDL itself. These peptides could alter the interfacial properties of a membrane in such a manner that enzymes such as PON-1 can be activated. There is evidence that this enzyme plays an important role in protecting against atherosclerosis because of its ability to attenuate the oxidation of lipoproteins (30). When peptide D-4F is administered to mice, the HDL is found to possess increased PON-1 activity and inhibited recruitment of monocytes as well as formation of atherosclerotic lesions (11, 18). The presence of PON-1 on HDL is thought to be a major factor contributing to the antiatherogenic properties of this lipoprotein (17). That we did not observe changes in PON-1 may reflect transient changes or may be due to peptide 3F-2 acting through different mechanisms from 4F.

The effects we find with NMR, indicating that the more protective peptide 3F-2 is more effective in modifying the interfacial properties of the lipid, would correlate with this peptide forming novel lipid-peptide complexes with altered physical properties that have enhanced activity in destroying oxidized lipids and also facilitating reverse cholesterol transport.

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