review

Structural requirements for antioxidative and anti-inflammatory properties of apolipoprotein A-I mimetic peptides

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Abstract **Recently, attention has been focused on pharmacological treatments that increase HDL cholesterol to prevent coronary artery disease. Despite three decades of extensive research of human apolipoprotein A-I (apoA-I), the major protein component of HDL, the molecular basis for its antiatherogenic and anti-inflammatory functions remain elusive. Another protein component of HDL, apoA-II, has structural features similar to those of apoA-I but does not possess atheroprotective properties. To understand the molecular basis for the effectiveness of apoA-I, we used model synthetic peptides. We designed analogs of the class A amphipathic helical motif in apoA-I that is responsible for solubilizing phospholipids. None of these analogs has sequence homology to apoA-I, but all are similar in their lipid-associating structural motifs. Although all of these peptide analogs interact with phospholipids to form peptide:lipid complexes, the biological properties of these analogs are different. Physical-chemical and NMR studies of these peptides have enabled the delineation of structural requirements for atheroprotective and anti-inflammatory properties in these peptides. It has been shown that peptides that interact strongly with lipid acyl chains do not have antiatherogenic and anti-inflammatory properties. In contrast, peptides that associate close to the lipid head group (and hence do not interact strongly with the lipid acyl chain) are antiatherogenic and anti-inflammatory. Understanding the structure and function of apoA-I and HDL through studies of the amphipathic helix motif may lead to peptide-based therapies for inhibiting atherosclerosis and other related inflammatory lipid disorders.**—Anantharamaiah, G. M., V. K. Mishra, D. W. Garber, G. Datta, S. P. Handattu, M. N. Palgunachari, M. Chaddha, M. Navab, S. T. Reddy, J. P. Segrest, and A. M. Fogelman. **Structural requirements for antioxidative and anti-inflammatory properties of apolipoprotein A-I mimetic peptides.** ***J. Lipid Res.*** **2007.** 48: **1915–1923.**

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Epidemiological studies have established an inverse relationship between HDL and coronary artery disease (1). Increasing HDL by infusion of HDL or apolipoprotein A-I (apoA-I) appears to have direct effects on the inhibition of atherosclerosis (2, 3). The protective action of apoA-I and HDL is attributed to *1*) their central role in reverse cholesterol transport [a process in which apoA-I acts as an acceptor of cholesterol from peripheral tissues, which is then transported to the liver and other tissues for excretion or steroidogenesis (4, 5)] and *2*) their anti-inflammatory properties (6). Despite three decades of extensive studies of human apoA-I, understanding of the structure-function relationship of this protein responsible for inhibiting atherosclerosis has remained elusive. Different laboratories have used different approaches to understand the structure and function of apolipoproteins. Molecular biology-based approaches (e.g., site-directed mutagenesis) of apoA-I in combination with biochemical-biophysical studies of isolated HDL and studies of apoA-I:lipid complexes have yielded important information (7–9). To better understand the secondary structural motifs responsible for lipid association, we have used de novo designed synthetic peptides, which do not have sequence homology with apoA-I but which replicate the class A amphipathic helical motif in apoA-I.

EXCHANGEABLE APOLIPOPROTEINS POSSESS THE CLASS A AMPHIPATHIC HELICAL MOTIF

Exchangeable apoA-I, apoA-II, apoC-I, apoC-II, apoC-III, and apoE from HDL associate with lipids to form protein:lipid complexes and are involved in transporting otherwise water-insoluble lipids in plasma. These proteins exhibit striking similarities in their gene structures in that

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they all possess four exons and three introns (10). A common feature among these apolipoproteins is the presence of tandem 22-mer repeating domains in exon 4. Alignment of the amino acid residues shows that proline (Pro) residues consistently appear in the first position of these 22-mers in both apoA-I and apoA-IV. When these sequences are folded into an α-helix, they produce a structure with opposing polar and nonpolar faces (11). In addition, there is a particular arrangement of the charged amino acids, with positively charged amino acids appearing at the polar-nonpolar interface and negatively charged amino acids at the center of the polar face. Such a secondary structural motif is defined as the class A amphipathic helix (11, 12). Although 70% of the protein component of HDL is apoA-I, apoA-II is the second most abundant protein. Infusion or transgenic expression of human apoA-I has been shown to protect against atherosclerosis in animal models (2, 13). On the other hand, expression of apoA-II has been shown to promote atherosclerosis (14).

Both of these proteins possess class A amphipathic helical domains. It has been shown that whereas apoA-I is more dynamic in nature, apoA-II remains associated with HDL particles (15). Comparison of amphipathic helical domains in these two proteins indicates that apoA-II amphipathic helical domains possess broad hydrophobic faces with mostly aliphatic hydrophobic amino acids at the center of the nonpolar face (16). This suggests stronger hydrophobic interactions between the nonpolar face of apoA-II amphipathic helices and lipid acyl chains, which could account for the nonexchangeability of apoA-II compared with the dynamic nature of apoA-I (15). This dynamic nature of apoA-I is responsible for HDL subspeciation, including the formation of the most active HDL subspecies, preβ-HDL (17). Furthermore, it has been shown that paraoxonase-1 (PON-1), an antioxidant enzyme, is anchored to HDL in tight association with apoA-I (18). These results indicate that the nature of the amphipathic helical domains present in the exchangeable apolipoproteins and the protein composition of HDL may be important factors that determine the atherogenic or antiatherogenic properties of HDL (19). Our approach to understanding the structure and function of apolipoproteins present in HDL has been the study of amphipathic helical motifs by the design of peptides to mimic the structural features present in exchangeable apolipoproteins, particularly the effects of changes in the nonpolar face on their physical-chemical and biological properties.

PEPTIDE ANALOGS AS SHORT AS 18 AMINO ACID RESIDUES FORM COMPLEXES SIMILAR TO APOA-I, A 243 AMINO ACID PROTEIN

That the class A amphipathic helical structural motif is responsible for lipid association has been supported by studies of fragments of these apolipoproteins that are capable of forming amphipathic helical structures (20–22) and by de novo designed peptides (23). The first peptide designed to support the amphipathic helix hypothesis was 18A, with the sequence DWLKAFYDKVAEKLKEAF (24). This designed sequence is not present in any of the naturally occurring proteins, but when folded as an α-helix, it produces a class A amphipathic helix (**Fig. 1**). This peptide was able to solubilize multilamellar vesicles of dimyristoylphosphatidylcholine (DMPC) to form complexes that are similar to those formed by apoA-I:DMPC at a 1:1 weight ratio (**Fig. 2A**). When the charged residue positions were exchanged to place negatively charged residues at the polar-nonpolar interface and positively charged residues at the center of the polar face, the resulting peptide (KWLDAFYKDVAKELEKAF; Reverse-18A) had a lower lipid affinity than 18A (24, 25). The helix-forming propensity was increased in 18A and Reverse-18A by substituting aspartate (Asp) residues with glutamate (Glu) and alanine (Ala) residues with leucine (Leu). There was a modest increase in the lipid-associating ability in these peptides compared with 18A and Reverse-18A analogs (25). However, the peptides with lysine (Lys) at the polar-nonpolar interface were still better at associating with lipids compared with Reverse-18A analogs, in which negatively charged Glu residues are present at the polar-nonpolar interface (25).

These observations led to the "snorkel model" for interfacial Lys residues. This model was further tested using a basic amino acid, homoaminoalanine, instead of Lys at the polar-nonpolar interface of 18A (26). While Lys ε-NH_2 is attached to four CH_2- groups in the side chain, the β-NH_2 side chain of the amino acid homoaminoalanine has only two CH_2- groups (the same as the Glu side chain). The peptide analog synthesized using homoaminoalanine at the polar-nonpolar interface possessed decreased lipid-associating ability compared with 18A with Lys at the polar-nonpolar interface (Fig. 1). Because of their long acyl chains, these interfacial Lys residues in 18A analogs, when associated with phospholipids, extend (snorkel) toward the polar face of the helix to insert their charged ε-NH_2 termini into the aqueous milieu. The snorkeling of the longer hydrocarbon side chain of interfacial Lys residues in 18A provides increased hydrophobic surface area for a deeper penetration of the amphipathic helix within the hydrophobic interior of the lipid bilayer. This model thus explains the increased lipid-associating ability of class A amphipathic helices.

Protection of the N and C termini in 18A by acetyl and NH_2 groups, respectively, produced Ac-18A-NH_2, with a large increase in helicity and increased self-association. This was accompanied by an increase in lipid binding (27). This peptide [also called 2F because of the two phenylalanine (Phe) residues at the nonpolar face] was also able to mimic several properties of apoA-I (27).

The "belt model," which places apoA-I around the apoA-I:phospholipid complexes (Fig. 2B), was established based on X-ray crystallography and molecular modeling studies (28, 29). Mishra et al. (30) recently showed by proton NMR of 2F:DMPC complexes and cross-linking studies that the peptide in 2F:DMPC (1:1 weight ratio) discoidal complexes is arranged in a head-to-tail form with 16 molecules of peptides arranged with 8 peptides per

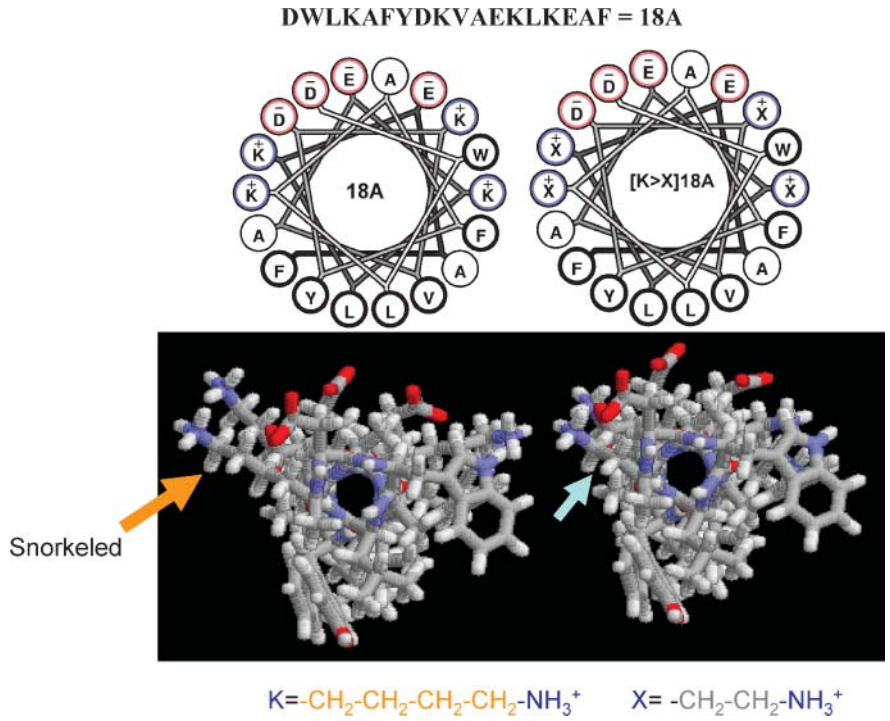

Fig. 1. Helical wheel representation and molecular modeling of 18A and [Lys→X]18A. This figure demonstrates that in 18A, Lys residues at the polar-nonpolar interface, because of their longer acyl side chains, contribute to the hydrophobicity of the hydrophobic face by extending the side chains to the polar face (snorkeling) to expose the ε-NH_2 of Lys residues (orange arrow). This is not possible by either the shorter homoaminoalanine residues (denoted X in the helical wheel representation and shown by the light blue arrow) or the Asp and Glu residues if they are at the polar-nonpolar interface.

strand (Fig. 2C). Thus, the helix axis of the self-associated peptide is oriented perpendicular to the lipid acyl chain. For the disc size of 69 Å in the peptide:lipid discoidal complex, the number of DMPC molecules is 54. The stoichiometry of peptide to lipid geometrically fits the model very well. In addition, this model satisfies not only intermolecular patterns of the nuclear Overhauser effect (which determines intermolecular close contacts between peptide side chains and lipid molecules) in high-resolution NMR studies but also satisfies the differential microenvironments of four Lys residues obtained by us in ^{13}C-NMR studies of peptide:DMPC discoidal structures (31). Thus, these peptide:lipid complexes are similar in structure to apoA-I:lipid complexes. These results are summarized in Fig. 2. It is interesting that in complexes containing two apoA-I molecules, there are eight 22-mer helices and two 11-mer helices per strand of apoA-I. Under similar conditions (1:1 weight ratio of protein:lipid), apoA-I forms slightly larger complexes (89 Å) (32) compared with the peptide 2F (69 Å) at a 1:1 weight ratio. However, despite these apparent similarities of lipid-associating properties with apoA-I, peptide 2F was not able to inhibit atherosclerosis when administered to atherosclerosis-sensitive mice (33).

What is not known is whether there is a difference in the dynamics of peptide association to lipids in class A peptide analogs. We propose that, analogous to the differences between apoA-I and apoA-II described above, the dynamics of association of the peptide with lipid (or HDL) depends upon the nonpolar face composition, as observed in studies using different class A peptide analogs. The studies described below, in which the polar face was unchanged but the hydrophobic amino acids on the nonpolar face were arranged differently, support the idea that the association of the nonpolar face with the lipid acyl chains differs, which may account for the changes in biological activities of the different peptides.

APOA-I CONSENSUS PEPTIDE ANALOGS THAT ACTIVATE LCAT TO THE SAME EXTENT AS APOA-I DID NOT INHIBIT ATHEROSCLEROSIS IN A MOUSE MODEL

Among apolipoproteins, human apoA-I is the major cofactor of the plasma enzyme LCAT (34). LCAT has two activities: *1*) phospholipase activity, in which the *sn*-2 chain from the phospholipid is cleaved; and *2*) cholesteryl esterase activity, in which the *sn*-2 acyl chain is added to the OH of cholesterol to form cholesteryl ester. The nascent discoidal HDL particles are converted into spherical HDL particles with a core of neutral cholesteryl ester formed as a result of LCAT activity. The spherical HDL particles interact with the cell surface scavenger receptor class B type I present in the liver to transfer the cholesteryl ester

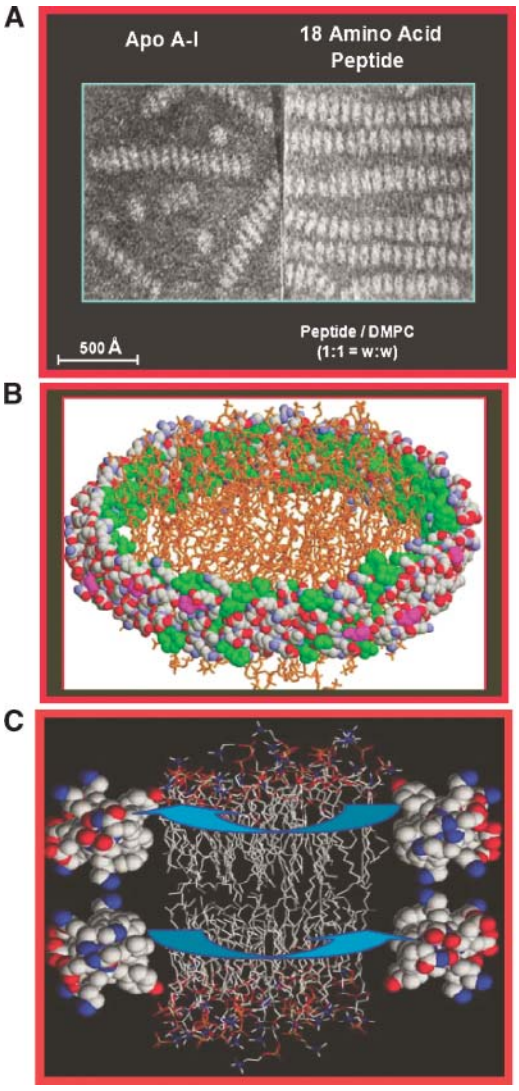

Fig. 2. A: Comparison of negative stain electron micrographs of apolipoprotein A-I:dimyristoylphosphatidylcholine (apoA-I:DMPC) and 18A:DMPC complexes. Complexes were prepared by mixing protein (or peptide) with multilamellar vesicles of DMPC at a 1:1 weight ratio. B: The belt model for the apoA-I:lipid discoidal complex. Two molecules of apoA-I are arranged in a head-to-tail belt manner on the disc edge of the protein:lipid complex (9, 29). Protein is shown in the space-fill model, and lipids are shown in the stick model. The green-shaded patch (in the interior of the ring) shows the hydrophobic face of the two strands of apoA-I molecules facing the lipid acyl chains. Charged residues face the aqueous environment on the outer edge of the two protein antiparallel strands. C: Molecular model showing two average energy-minimized molecules of 2F. The peptide is oriented parallel to the plane of the membrane in a head-to-tail manner. This model is in agreement with the results obtained by proton NMR in combination with cross-linking studies of the peptide:lipid complexes (30) and by observed sided pk_a values of the four Lys residues in 2F (31). As shown, two Lys residues (Lys-9 and Lys-13) in two different strands are close to each other (in a basic environment) and thus possess pk_a values (9.5) less than the pk_a value for Lys (10.1), and the outer two Lys residues (Lys-4 and Lys-15) in two strands stretch toward the aqueous environment and thus possess higher pk_a values of 10.3 and 11, respectively. Arrows indicate the two protein or two head-to-tail strands of peptides at the edge of the discoidal structure of 2F (or apoA-I):DMPC complexes.

for excretion or steroidogenesis. Thus, LCAT activation by apoA-I is considered an important step mediating reverse cholesterol transport. As mentioned above, exon 4 of human the apoA-I gene contains eight 22-mers and two 11-mers, which appear to have evolved by tandem duplications of a primordial sequence of 22 amino acid residues (10). Most of the tandem 22-mer repeats possess Pro as the first amino acid. Each of these tandem repeats, when folded as an α-helix, produces the amphipathic helix motif. By aligning the eight 22-mer amino acid sequences, we designed the following 22-mer consensus sequence: Pro-Val-Leu-Asp-Glu-Phe-Arg-Glu-Lys-Leu-Asn-Glu-X-Leu-Glu-Ala-Leu-Lys-Gln-Lys-Leu-Lys (referred to as A-ICon). This sequence, when folded as an α-helix, forms a class A amphipathic helix (when X is substituted by Ala, Leu, and other hydrophobic amino acids). Because position 13 from the N terminus, denoted by X, had a marked polymorphism in the 22-mer sequences of apoA-I, several peptides were synthesized with X = Glu, Lys, Arg, His, or Ala.

Monomers or tandem homodimers and heterodimers of these peptides were tested in vitro for their ability to activate LCAT using discoidal and vesicular assay systems with tracer amounts of radiolabeled cholesterol. The cholesteryl ester formed upon the addition of purified LCAT was separated from free cholesterol using thin-layer chromatography and quantified. In these two assay systems, the dimer peptide that possesses Glu at position 13 was essentially as effective as apoA-I in activating LCAT. This peptide had LCAT-activating ability equal to that of apoA-I at lower amounts of the peptide (35). The peptide 18A-Pro-18A exhibited low LCAT-activating ability at lower concentrations of the peptide. However, at higher concentrations, 18A-Pro-18A exhibited much more LCAT-activating ability than apoA-I (36). In these studies, it was shown that at higher concentrations of the peptide, the vesicular egg phosphatidylcholine (PC):cholesterol substrate was converted into a discoidal structure. Because both apoA-I and [Glu^{13}]A-ICon dimer do not convert the substrate into discoidal forms, it was proposed that, whereas 18A-Pro-18A did not exhibit LCAT-activating properties similar to those of apoA-I, the peptide [Glu^{13}]A-ICon dimer was a mimic of apoA-I in LCAT-activating properties (35).

Based on these initial observations, Dasseux et al. (37) designed additional analogs. The sequences of their analogs were similar to that of A-ICon, with changes at positions 5 (Glu→Leu) and 9 (Lys→Leu). Thus, the primary sequence was Pro-Val-Leu-Asp-Leu-Phe-Arg-Glu-Leu-Leu-Asn-Glu-X-Leu-Glu-Ala-Leu-Lys-Gln-Lys-Leu-Lys. X was changed to either Leu (to obtain A-IConA) or glycine (Gly) (to obtain A-IConB). These two peptides associated with phospholipid well and were as effective as apoA-I in activating the plasma enzyme LCAT in the two assay systems described above. However, as shown in **Fig. 3**, these two peptides, when administered daily (50 μg/day for 16 weeks) to C57BL/6J mice on the atherogenic Paigen diet (which contains cholate) (38), did not significantly inhibit atherosclerosis compared with that in control mice.

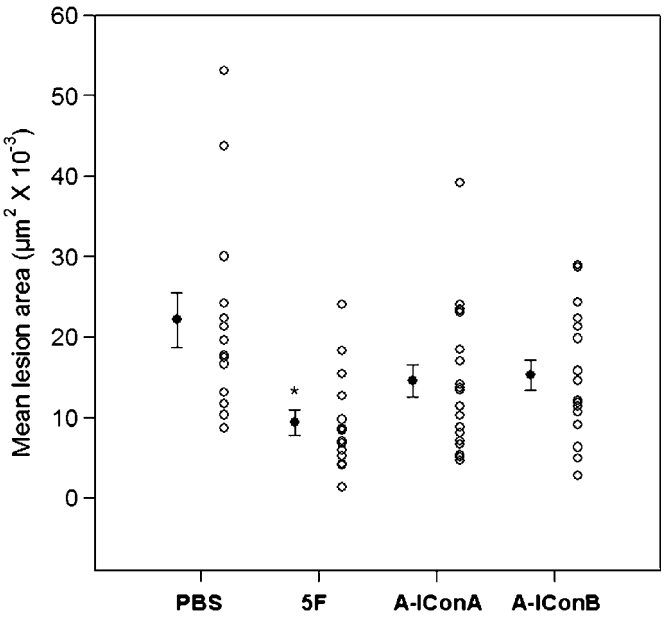

Fig. 3. Differential effect of four peptide analogs on the inhibition of lesion formation in C57BL/6J mice fed an atherogenic diet. Female C57BL/6J mice fed the Paigen diet (38) were injected with peptide (20 μg/mouse/day for peptide 5F and 50 μg/mouse/day for the A-ICon peptides, intraperitoneally) or with the vehicle PBS daily for 16 weeks. Aortic sinus lesion formation was assessed at the end of 16 weeks. The two peptides A-IConA and A-IConB, which are potent activators of the plasma enzyme LCAT, did not inhibit diet-induced lesion formation. In contrast, the peptide 5F, which possesses LCAT-activating ability of <30% of that of apoA-I or the A-ICon peptides, significantly inhibited lesion formation. * $P <$ 0.001 versus PBS. Data shown represent individual animals (opened circles) and mean (closed circles) ± SEM.

LIPID BINDING BY APOA-I MIMETICS IS NECESSARY BUT NOT SUFFICIENT TO CONFER THE ANTIATHEROGENIC AND ANTI-INFLAMMATORY PROPERTIES OF APOA-I MIMETICS

In search of a peptide that mimics apoA-I in inhibiting atherosclerosis, hydrophobic amino acids on the nonpolar face of the peptide 2F were systematically changed to Phe residues to synthesize 3F to 7F, where the number refers to the number of Phe residues present on the nonpolar face (33). Tryptophan and Phe are the most hydrophobic amino acids on the hydrophobicity scale of Wimley and White (39); Phe was selected because it is resistant to acid treatment. The peptides were tested for their retention time on a C-18 reverse-phase HPLC column, ability to penetrate an egg PC monolayer, solubility in aqueous solution, percentage helicity, ability to associate with lipids, ability to clarify egg PC multilamellar vesicles, ability to activate LCAT, and ability to inhibit LDL-induced monocyte chemotaxis in cultures of human aortic cells (33). As shown in **Table 1**, all of the peptides had exclusion pressures higher than apoA-I (34 dynes/cm) and also solubilized egg PC spontaneously. Increase in the number of Phe residues increased exclusion pressure from 38 to 45 dynes/cm (33).

None of the peptides was able to activate LCAT by >30% compared with human apoA-I at lower concentrations, at which the vesicular substrate of LCAT was not affected (33). Of all the parameters tested, only the ability of the peptides to inhibit LDL-induced monocyte chemotactic activity predicted their ability to inhibit atherosclerosis in mouse models (33, 40). In an initial experiment, 5F (20 μg/mouse/day for 16 weeks) was administered to C57BL/6J mice on an atherogenic diet. Compared with the control group, administration of 5F significantly decreased diet-induced lesions (Fig. 3). Under similar conditions, peptide 2F was unable to inhibit diet-induced atherosclerosis. The inhibition of lesion formation by 5F administration occurred without any change in plasma cholesterol levels (41). To determine a possible mechanism for this inhibition, the F-series peptide analogs were screened for their ability to inhibit LDL-induced monocyte chemotaxis in the coculture system. It has been shown that this inhibition is correlated to the ability of apoA-I or HDL to scavenge oxidized lipids from LDL (42). Although peptide 2F was only marginally effective at inhibiting LDL-induced monocyte chemotaxis, peptides 4F, 5F, and 6F were all effective in their ability to inhibit LDL-induced monocyte chemotaxis (33). Based on the higher aqueous solubility of 4F, it was chosen for further testing in mouse models of atherosclerosis. These studies indicated that neither the LCAT-activating ability nor the ability to bind to nonoxidized lipids predicts the antiatherosclerotic property(ies) of these peptides. The best predictor of the ability of the peptides to inhibit atherosclerosis in mouse models was the LDL-induced monocyte chemotactic activity in cocultures of human aortic wall cells.

ANTIATHEROGENIC PROPERTIES OF APOA-I MIMETIC PEPTIDES DEPEND ON THE NATURE OF INTERACTION WITH MEMBRANES

The structural differences between the peptides 2F and 4F might appear to be minimal. Both the Leu residues on the hydrophobic face of peptide 2F were replaced by Phe residues to obtain 4F (33). The resulting changes in the peptides with these conservative amino acid substitutions are shown in **Fig. 4** for 2F and 4F. As shown in Fig. 4A, the molecular volume of the Leu and Phe residues differs significantly. In addition, the Phe ring also possesses π-electrons. As illustrated in Fig. 4A, a cluster of aromatic residues can modify the cross-sectional area of the nonpolar face. In 4F compared with 2F, the center of the nonpolar face is rich in π-electrons and the area occupied by these amino acids is larger, resulting in a cross-sectional shape that is entirely different from that of 2F. Therefore, we propose that insertion of 4F into the membrane would possess a slightly polar π-electron cluster in the hydrophobic milieu. This could allow the sequestering of water and more polar lipids such as oxidized phospholipids (Fig. 4B). In contrast, the nonpolar face of 2F (with two Leu residues at the center of the nonpolar face) would strongly interact with the lipid acyl chain, preventing the entry of water or more polar lipids such as oxidized phospholipids. If this hypothesis is correct, then the arrange-

TABLE 1. Properties of homologous peptides (33)

Peptide	Sequence[a]	Hydrophobicity[b]	Monolayer Exclusion Pressure (π_e)	LCAT[c]	
				5 μg[d]	20 μg[d]
			dynes/cm	*% activation by apoA-I*	
2F	Ac-18A-NH_2	2.05	38	11	40
$3F^3$	Ac-[$F^3$18A]NH_2	2.20	38	10	38
$3F^{14}$	Ac-[F^{14}18A]NH_2	2.20	39	10	38
4F	Ac-[$F^{3,14}$18A]NH_2	2.35	40	14	45
5F	Ac-[$F^{11,14,17}$18A]NH_2	2.81	45	25	80
6F	Ac-[$F^{10,11,14,17}$18A]NH_2	2.96	46	22	75
7F	Ac-[$F^{3,10,11,14,17}$18A]NH_2	3.15	45	10	35

ApoA-I, apolipoprotein A-I.
[a] The baseline sequence of 18A is DWLKAFYDKVAEKLKEAF.
[b] Hydrophobicity is expressed as the hydrophobicity per residue on the nonpolar face.
[c] LCAT activation is expressed as a percentage of that obtained by apoA-I. The activation is dependent on the concentration of the peptide. Here, we report LCAT activation obtained by 5 and 20 μg of peptide.
[d] Egg phosphatidylcholine:cholesterol vesicular substrate was not altered at 5 μg of the peptide but was altered at 20 μg.

ment of aromatic residues on the nonpolar face might be more important for anti-inflammatory properties than the overall hydrophobicity of the peptides.

Studies using 3F analogs support this hypothesis. The amino acids on the nonpolar face of 2F were replaced to have three Phe residues on the nonpolar face (42). In one case, the aromatic residues are at the center of the nonpolar face (similar to 4F) to produce the peptide 3F-2, whereas another analog (3F-1) has aromatic residues clustered at the polar-nonpolar interface (42). Based on the molecular volume occupied by the aromatic residues, one would expect that the two newly designed peptides would have overall cross-sectional shapes different from those of the earlier biologically inactive 3F analogs $3F^3$ and $3F^{14}$, which are described in Ref. 33. In agreement with this, both of the newly designed peptides had a relatively larger hydrophobic face (similar to 4F) that makes these two analogs (and the most active 4F analog) cylindrical (42). Thus, it was anticipated that these two peptides (3F-1 and 3F-2) would interact differently with membrane lipid acyl chains compared with $3F^3$ and $3F^{14}$, which have an overall wedge cross-sectional shape. The 3F-1 and 3F-2 peptides were effective at inhibiting LDL-induced monocyte chemotaxis (42).

Because LDL-induced monocyte chemotaxis has been shown to depend on lipid hydroperoxides in LDL (43, 44), we compared the ability of these peptides to scavenge oxidized lipids from plasma of Watanabe rabbits, which possess increased LDL levels and increased amounts of lipid hydroperoxides. The 4F peptide was used as a positive control. Peptides 3F-1, 3F-2, and 4F (and not $3F^3$ and $3F^{14}$) were able to scavenge lipid hydroperoxides from LDL (42). Based on their ability to inhibit LDL-induced monocyte chemotaxis, one would predict that 3F-2 would inhibit atherogenesis in apoE null mice and $3F^{14}$ would not, and this was indeed the case (45). Additionally, the plasma distribution of the peptides differed in that 3F-2 preferentially associated with high density lipoprotein, whereas $3F^{14}$ preferentially associated with apoB-containing particles (45). After intraperitoneal injection of ^{14}C-labeled peptides, $3F^{14}$ reached a higher maximal plasma concentration and had a longer half-time of elimination than 3F-2 (45). The longer half-time of elimination of $3F^{14}$ indicates a stronger association with lipoproteins. Despite this, the peptide $3F^{14}$ was not effective at inhibiting atherosclerosis. This is in accordance with our hypothesis that lipid association is necessary but not sufficient to confer antiatherosclerotic properties. A study of the effect of these peptides on the motional and organizational properties of phospholipid bilayers, using several NMR methods, demonstrated that the two peptides insert to different extents into membranes. Peptide 3F-2, with aromatic residues at the center of the nonpolar face, partitioned closer to the phospholipid head group compared with $3F^{14}$ (45). In contrast, $3F^{14}$, but not 3F-2, affected the terminal methyl group of the membrane acyl chains, decreasing the ^{2}H order parameter and at the same time also decreasing the molecular motion of this methyl group (45). This dual effect of $3F^{14}$ can be explained in terms of the cross-sectional shape of the amphipathic helix (45). These results support the proposal that the molecular basis for the differences in the biological activities of the two peptides lies with their different interactions with membranes.

Because 4F is the most effective anti-inflammatory peptide in the series (33), it has been studied extensively. Similar to 3F-2, 4F associates with HDL and forms preβ-HDL particles with increased PON-1 activity (46). Peptide 4F interacts with existing HDL to recruit phospholipids and apoA-I into these particles, thus producing apoA-I-containing particles (with no apoA-II) with preβ-mobility. As described previously, apoA-I-only particles are good anchors for the enzyme PON-1 (18). This peptide has also been shown to inhibit lipopolysaccharide-induced inflammation (47) and to improve endothelial function (48). Peptide 4F has been shown to inhibit atherosclerosis in apoE null mice on normal chow and LDL receptor null mice on the Western diet (40). The 4F peptide has also been shown to reduce brain arteriole inflammation and to improve cognitive function in LDL receptor null mice fed a Western diet (49).

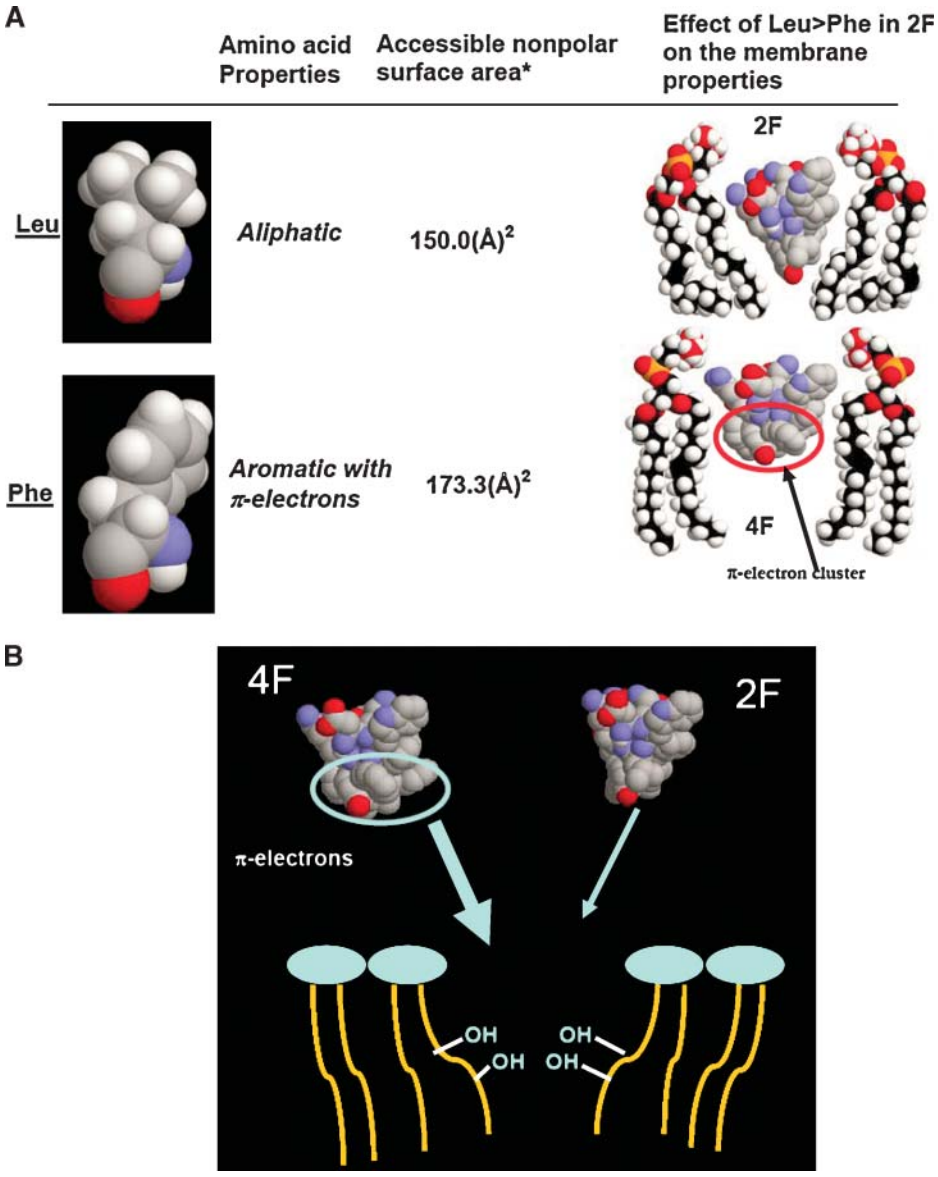

Fig. 4. A: Modulation of lipid-associating properties of 2F upon substitution of two Leu residues at the center of the nonpolar face with Phe residues. Leu possesses an aliphatic side chain and is able to interdigitate with the lipid acyl chain. Phe has an aromatic side chain with π-electrons. In addition, compared with Leu, Phe has a larger nonpolar accessible surface area (55). These two properties allow the 4F analog to reside close to the lipid head group, positioning the π-electron cloud in the hydrophobic milieu to allow for the sequestration of water or polar lipid hydroperoxides in the membrane. B: Differential association of 4F and 2F with oxidized lipid-containing membranes or lipoproteins (as denoted by the differences in the size of the arrows). As described elsewhere, 4F inhibits oxidized LDL-induced monocyte chemotaxis much more effectively than 2F (33), in agreement with our recent observations that partitioning of 4F (with the π-electron cluster at the center of the nonpolar face) increases more (compared with 2F) into multilamellar vesicles of POPC in the presence of oxidized 1-Palmitoyl-2-arachidonoyl-sn-phosphocholine (V. K. Mishra et al., unpublished results).

Recently it was found that increases in HDL cholesterol levels per se may not be beneficial. Indeed, in recent studies testing the effect of an inhibitor of cholesteryl ester transfer protein (CETP) on atherosclerosis in humans, there was a >50% increase in HDL levels and a marked decrease in LDL levels. Despite this, the CETP inhibitor, when given with a statin, was not able to either reduce atherosclerosis, as studied by intravascular ultrasound, or prevent the progression of atherosclerosis, as studied by change in mean intima-media thickness in the common carotid artery (50, 51). Ansell et al. (52) have studied HDL from patients with coronary heart disease (or heart disease equivalents) who possessed normal or high HDL cholesterol levels. HDL from these patients did not reduce LDL-induced monocyte chemotaxis, as did HDL from normal volunteers who were age- and gender-matched. Peptide 4F,

which associates with HDL when administered to monkeys, removed lipoprotein lipid hydroperoxides and rendered their HDL able to inhibit LDL-induced monocyte chemotaxis (53). Recent phase I clinical studies with this peptide also showed improvement in the ability of HDL from patients with coronary heart disease (or equivalent) to inhibit LDL-induced monocyte chemotaxis after a single oral dose of 4F (54). Thus, peptides designed to interact with lipoproteins and membranes to remove and sequester polar lipids (such as oxidized phospholipids) appear to have therapeutic potential.

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