Structure/Function Relationships of Apolipoprotein A-I Mimetic Peptides

Implications for Antiatherogenic Activities of High-Density Lipoprotein

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Rationale: Apolipoprotein (apoA)-I mimetic peptides are a promising type of antiatherosclerosis therapy, but how the structural features of these peptides relate to the multiple antiatherogenic functions of HDL is poorly understood.

Objective: To establish structure/function relationships of apoA-I mimetic peptides with their antiatherogenic functions.

Methods and Results: Twenty-two bihelical apoA-I mimetic peptides were investigated in vitro for the capacity and specificity of cholesterol efflux, inhibition of inflammatory response of monocytes and endothelial cells, and inhibition of low-density lipoprotein (LDL) oxidation. It was found that mean hydrophobicity, charge, size of hydrophobic face, and angle of the link between the helices are the major factors determining the efficiency and specificity of cholesterol efflux. The peptide with optimal parameters was more effective and specific toward cholesterol efflux than human apoA-I. Charge and size of hydrophobic face were also the major factors affecting antiinflammatory properties, and the presence of cysteine and histidine residues was the main factor determining antioxidant properties. There was no significant correlation between capacities of the peptides to support individual functions; each function had its own optimal set of features.

Conclusions: None of the peptides was equally effective in all the antiatherogenic functions tested, suggesting that different functions of HDL may have different mechanisms and different structural requirements. The results do suggest, however, that rationalizing the design of apoA-I mimetic peptides may improve their therapeutic value and may lead to a better understanding of mechanisms of various antiatherogenic functions of HDL. (Circ Res. 2010;107:217-227.)

Key Words: mimetic peptides ■ high-density lipoprotein ■ atherosclerosis

Atherosclerosis underlies most cases of cardiovascular disease, which is now the major cause of morbidity and mortality in developed countries. Accumulation of cholesterol in the arterial wall and vascular inflammation are in the center of the pathogenesis of atherosclerosis, and treatments controlling delivery of cholesterol and inflammation (statins) reduce the incidence of cardiovascular disease by 30% to 40%.1 There is, however, an urgent need for further reduction of the unacceptably high remaining risk of cardiovascular disease. A most promising direction is complementing reduction in levels of the proatherogenic lipoproteins with increasing levels of the antiatherogenic lipoprotein, high-density lipoprotein (HDL), “HDL therapy.”2 It is becoming clear that success of HDL therapy critically depends on the mechanism for elevating HDL, with a straightforward, and so far the most successful, approach being direct infusion of exogenous HDL.3,4 Infusion of reconstituted (r)HDL, however, has considerable limitations because of high cost and requirement for intravenous delivery making it suitable mainly for acute treatment. An alternative to rHDL is apolipoprotein (apo)A-I mimetic peptides.

ApoA-I mimetic peptides mimic the secondary structure of the major structural element of apoA-I, 22-mer amphipathic A α-helix.5 It appears that there is no requirement for a homology between the primary structure of these peptides and apoA-I; these peptides are active as long as the secondary structure of apoA-I is replicated.5,6 ApoA-I mimetic peptides show remarkable capacity to support cholesterol efflux, share the antiinflammatory properties of HDL, and reduce development of atherosclerosis in animal models.3,5-11 ApoA-I mimetic
peptides cost a fraction of the cost of rHDL; they are safe and well tolerated, and approaches for oral delivery are being developed. Perhaps the best advantage of these peptides is the ability to modify their structure to better understand the mechanisms of atheroprotective action of HDL, with a view to further improving the atheroprotective capacity of the peptides. Relatively limited research has been done to understand structure/function relationships of apoA-I mimetic peptides. The present understanding of the structure/function relationship of the peptides can be summarized as follows:

- The amphipathic α-helix of 18 to 22 residues is essential for a peptide to mimic apoA-I and to be atheroprotective.
- There is no stereospecificity: peptides made of D-amino acids are as effective as those made of L-amino acids.
- Increasing hydrophobicity by including 2 or 4 phenylalanine residues improves the capacity of peptides to associate with lipids and antiinflammatory capacity of the peptides.
- Alignment of negative charges on the polar face increases cholesterol efflux.
- Two helices connected through proline residue work better than a single helix in cholesterol efflux and inflammation assays.
- Introducing asymmetry in bivalent peptide improves its specificity in cholesterol efflux assay and reduces toxicity.

Although the atheroprotective functions of HDL are not limited to its role in reverse cholesterol transport and inflammation, only these 2 functions have been tested in most studies, but never, to our knowledge, simultaneously. In the present study, we undertook a comprehensive analysis of the structure/function relationship of apoA-I mimetic peptides. We used bivalent peptides not only because they have shown a better antiatherosclerosis activity, but also because they offer an opportunity to mimic variability in the structure of 2 adjacent helices, a property increasing their similarity to apoA-I and improving specificity of at least some of their functions.

We investigated the impact of changes in peptide mean hydrophobicity, size of hydrophobic face, charge, type of α-helix, configuration of the bridge between 2 helices, asymmetry of the helices, and inclusion of specific residues. The impact of these changes on the capacity and specificity of cholesterol efflux, inflammatory response of monocytes and endothelium, and antioxidant properties were studied. We endeavored to determine which structural features listed above are important for the four antiatherogenic functions of the peptides and to establish optimal combination(s) of these features favoring each or all of these functions.

### Methods

#### Cholesterol Efflux

Cellular cholesterol was labeled by incubation in serum-containing medium with [3H]cholesterol for 48 hours in a CO2 incubator. Cells were then washed and incubated for 18 hours at 37°C in serum-free medium in the presence or absence of TO-901317 (4 μM/L). Cells were washed and incubated for another 4 hours at 37°C (THP-1 cells) or 18 hours (H9K-1 cells) in serum-free medium containing indicated concentrations of the peptides or lipid-free apoA-I. Where indicated, cells were fixed by incubation for 20 minutes with paraformaldehyde (4%) before the efflux experiments.

#### Expression of CD11b on Human Monocytes

Resting human monocytes were stimulated with 1 μM/L phorbol-12 myristate 13-acetate (PMA) in the presence or absence of the peptides or apoA-I (final concentration, 40 μg/mL) and incubated with the FITC-conjugated antibody to the active epitope of CD11b for 15 minutes at 37°C. Cells were then fixed with 4% formaldehyde, and CD11b expression was measured by flow cytometry.

#### Expression of VCAM-1 in Mouse Endothelial Cells

SVEC4/VCAM-1 cells were washed and apoA-I, HDL or apoA-I mimetic peptides were added at the final concentration of 0.75 mg/mL. After 18 hours of incubation, tumor necrosis factor (TNF)-α was added in serum-free medium to the final concentration of 10 ng/mL. Cells were incubated for 5 hours, and luciferase activity was measured using Bright-Glo Assay.

#### Oxidation of Low-Density Lipoprotein

Freshly isolated low-density lipoprotein (LDL) (final concentration, 100 μg/mL) was incubated at 25°C for the indicated periods of time with CuSO4 (final concentration, 15 μM/L) in the presence of the peptides or apoA-I (final concentration, 100 μg/mL), and absorption was continually monitored at 234 nm.

### Results

#### Structure of apoA-I Mimetic Peptides

Twenty-two apoA-I mimetic peptides were synthesized; their sequences, physicochemical properties, and general features are shown in Table 1. Two peptides were used as prototypes, to understand how changes in their structures affect their function. The first prototype peptide was 5A (no. 1), which was described by us previously; it consists of 2 type A amphipathic α-helices connected through proline; hydrophobicity of the second helix was reduced by substituting hydrophobic amino acids with alanine. Four derivatives of 5A were synthesized (peptides 19 to 22) to test the impact of the introduction of 2 amino acids known to be antioxidants, cysteine and histidine, on its properties. The second prototype peptide was ELK (no. 2), which is made of just 3 amino acids: glutamic acid, leucine, and lysine. It consists of 2 identical canonical type A amphipathic α-helices with 180° hydrophobic face and neutral net charge; helices are connected with a proline residue. ELK peptide was used to make sixteen modifications (peptides 3 to 18) testing the impact of modification affecting the following features: (1) net charge, because it may affect interaction with cellular receptors and lipids; (2) mean hydrophobicity and size of hydrophobic face, because they may affect the interaction with lipids and cellular receptors; (3) type of helix and configuration of the
proline bridge between the 2 helices, because it may affect interaction with lipoprotein particles and cellular receptors, as well as properties of complexes of peptides with lipids after they acquire the latter from cells; and (4) asymmetry, because it was shown to affect specificity of cholesterol efflux. Some of these properties are interdependent (eg, charge and hydrophobicity), requiring testing several peptides with combinations of these features.

Peptides were tested in lipid-free form to mimic the interaction of lipid-free apoA-I with cells and to exclude the confounding effects of lipid-binding properties of the peptides and variations in size of “rHDL” particles.

**Efficiency of Cholesterol Efflux From Human Monocyte Cell Line THP-1**

To test the capacity of cholesterol efflux to the apoA-I mimetic peptides, human monocytic cells THP-1 were differentiated into macrophages, activated or not with liver X receptor (LXR) agonist TO-901317, which induces expression of ABC transporters, labeled with [3H]cholesterol and incubated with various concentrations of peptides for 4 hours. THP-1 cells not activated with LXR agonist contain low levels of ABC transporters, therefore, the efflux from nonactivated cells was considered to represent the component of the efflux that was not mediated by these transporters. The difference between the efflux in the presence and absence of LXR agonist was therefore defined as ABCA1-mediated cholesterol efflux. The dose-dependencies of the efflux from THP-1 cells are presented in Online Figures I through III (see the Online Data Supplement, available at http://circres.ahajournals.org). Figure I shows the efflux from the cells activated with TO-901317, Figure II shows the efflux from cells not-activated with TO-901317, and Figure III shows a difference between the effluxes from activated and nonactivated cells, ie, ABCA1-dependent efflux. To quantitate cholesterol efflux, the areas under the dose dependence curves were calculated, as well as the contribution of ABCA1 for cholesterol efflux at nonsaturating concentration of 20 μg/mL. These parameters are shown in Table 2. Analysis of the structure/function relationships, as related to the capacity of the peptides to support ABCA1-

### Table 1. Sequences and Structural Features of ApoA-I Mimetic Peptides

<table>
<thead>
<tr>
<th>No.</th>
<th>Peptide</th>
<th>Sequence</th>
<th>Mean Hydrophobicity</th>
<th>Charge</th>
<th>Key Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5A</td>
<td>DWLKFYDVAELKKE-PEWAKAAYDKAEEKA</td>
<td>−0.57</td>
<td>0</td>
<td>Asymmetrical</td>
</tr>
<tr>
<td>2</td>
<td>ELK</td>
<td>EKLKELLEKLEK-PEELKELLEKLEK</td>
<td>−0.4</td>
<td>0</td>
<td>Canonical type A helix with 180° hydrophobic face and 0 net charge</td>
</tr>
<tr>
<td>3</td>
<td>ELK-3E3L</td>
<td>EELKELLEKLEK-PEELKELLEKLEK</td>
<td>−1.1</td>
<td>−6</td>
<td>3× (K-E, L-K) substitutions; decreased hydrophobic face and 3 additional negative charges per helix</td>
</tr>
<tr>
<td>4</td>
<td>ELK-3E3K</td>
<td>EELKELLEKLEK-PEELKELLEKLEK</td>
<td>−0.76</td>
<td>−2</td>
<td>3× (K-E, E-A, L-K) substitutions. Decreased hydrophobic face and 1 additional negative charges per helix</td>
</tr>
<tr>
<td>5</td>
<td>ELK-2A</td>
<td>EKLKELLEKLEK-PEELKELLEKLEK</td>
<td>0.12</td>
<td>+4</td>
<td>2× E-A substitutions; increased hydrophobic face and 2 negative charges per helix less</td>
</tr>
<tr>
<td>6</td>
<td>ELK-1W</td>
<td>EWLKELLEKLEK-PEELKELLEKLEK</td>
<td>−0.19</td>
<td>−2</td>
<td>K-W substitution; 1 positive charge per helix less</td>
</tr>
<tr>
<td>7</td>
<td>ELK-2F</td>
<td>EKLKELLEKLEK-PEELKELLEKLEK</td>
<td>−0.43</td>
<td>0</td>
<td>2× (L-F) substitutions; increased hydrophobic face</td>
</tr>
<tr>
<td>8</td>
<td>ELK-1L1K</td>
<td>EKLKELLEKLEK-PEELKELLEKLEK</td>
<td>−0.01</td>
<td>+2</td>
<td>K-L and E-K substitutions; 1 negative charge per helix less</td>
</tr>
<tr>
<td>9</td>
<td>ELK-1K1E</td>
<td>EKLKELLEKLEK-PEELKELLEKLEK</td>
<td>−0.39</td>
<td>0</td>
<td>L-K, E-A, and K-E substitutions; decreased hydrophobic face</td>
</tr>
<tr>
<td>10</td>
<td>ELK-1A</td>
<td>EKLKELLEKLEK-PEELKELLEKLEK</td>
<td>−0.1</td>
<td>+2</td>
<td>E-A substitution; 1 negative charge per helix less</td>
</tr>
<tr>
<td>11</td>
<td>ELK-1F</td>
<td>EKLKELLEKLEK-PEELKELLEKLEK</td>
<td>−0.35</td>
<td>0</td>
<td>L-F substitution; increased hydrophobic face</td>
</tr>
<tr>
<td>12</td>
<td>ELK-2A2K2E</td>
<td>EKLKELLEKLEK-PEELKELLEKLEK</td>
<td>−0.47</td>
<td>0</td>
<td>2× (E-A, L-K and K-E) substitutions; optimal hydrophobicity and charge</td>
</tr>
<tr>
<td>13</td>
<td>ELK-3E3E</td>
<td>EELKELLEKLEK-PEELKELLEKLEK</td>
<td>−0.31</td>
<td>0</td>
<td>3× (K-E, E-K) substitutions. G-helix</td>
</tr>
<tr>
<td>14</td>
<td>ELK-2E2K</td>
<td>EELKELLEKLEK-PEELKELLEKLEK</td>
<td>−0.31</td>
<td>0</td>
<td>2× (K-E, E-K) substitutions; Y-helix</td>
</tr>
<tr>
<td>15</td>
<td>ELK-PB</td>
<td>EKLKELLEKLEK-PEELKELLEKLEK</td>
<td>−0.2</td>
<td>0</td>
<td>A-P substitution in the link</td>
</tr>
<tr>
<td>16</td>
<td>ELX-P2A</td>
<td>EKLKELLEKLEK-PEELKELLEKLEK</td>
<td>−0.16</td>
<td>0</td>
<td>2A-P substitution in the link</td>
</tr>
<tr>
<td>17</td>
<td>ELX-PA</td>
<td>EKLKELLEKLEK-PEELKELLEKLEK</td>
<td>−0.49</td>
<td>0</td>
<td>ELK-2A2K2E peptide with 5A substitution in second helix. Asymmetrical</td>
</tr>
<tr>
<td>18</td>
<td>ELX-CH2</td>
<td>EKLKELLEKLEK-PEELKELLEKLEK</td>
<td>−0.46</td>
<td>0</td>
<td>ELK-2A2K2E peptide with C+H substitution in the second helix</td>
</tr>
<tr>
<td>19</td>
<td>5A-CH1</td>
<td>DHLKFYDVAELKKE-PEWAKAAYDKAEEKA</td>
<td>−0.47</td>
<td>0</td>
<td>C+H substitution in the first helix</td>
</tr>
<tr>
<td>20</td>
<td>5A-CH2</td>
<td>DHLKFYDVAELKKE-PEWAKAAYDKAEEKA</td>
<td>−0.52</td>
<td>0</td>
<td>C+H substitution in the second helix</td>
</tr>
<tr>
<td>21</td>
<td>5A-C1</td>
<td>DHLKFYDVAELKKE-PEWAKAAYDKAEEKA</td>
<td>−0.44</td>
<td>0</td>
<td>C substitution in the first helix</td>
</tr>
<tr>
<td>22</td>
<td>5A-H1</td>
<td>DHLKFYDVAELKKE-PEWAKAAYDKAEEKA</td>
<td>−0.61</td>
<td>+1</td>
<td>H substitution in the first helix</td>
</tr>
</tbody>
</table>
dependent cholesterol efflux, allowed for the following conclusions.

Hydrophobicity and Charge
The relationship between mean hydrophobicity and the capacity of the peptides to support cholesterol efflux is shown in Figure 1A. It appears that the relationship is characterized by a sharp peak around a mean hydrophobicity value of \( \mu \approx 0.5 \).

Adding charges inevitably changes hydrophobicity, making it difficult to investigate the effect of the charge independently of hydrophobicity. It appears, however, that peptides carrying positive (squares) and negative (triangles) charges have lesser capacity to support cholesterol efflux and an overall neutral charge is optimal. The peptide ELK-A2K2E was synthesized after this initial analysis, creating a peptide with neutral charge and optimal hydrophobicity. Indeed this peptide showed exceptional capacity to support cholesterol efflux, even exceeding that of apoA-I, supporting our conclusions.

Four neutral charged peptides that had average hydrophobicity of approximately \( \mu \approx 0.5 \) but still failed to support cholesterol efflux (Figure 1A) all had other features strongly detrimental for the efflux capacity, such as the inclusion of histidine and/or cysteine residues or asymmetry in ELK peptides or both (see below).

Size of Hydrophobic Face
Increasing the size of hydrophobic face was beneficial, as long as overall hydrophobicity and charge were maintained (ELK-2F, ELK-1F).

Type of Helix
Changing the type of helix from the type A helix found in ELK to type G and Y helices, as was done for peptides ELK-3E3EK and ELK-2E2K, had a small beneficial effect on the capacity of the peptides to support cholesterol efflux.

Proline Bridge
Substitution of Ala for Pro in the bridge, as was done for peptide ELK-PA, was detrimental for the efflux capacity; however, substitution of Ala-Ala for Pro (peptide ELK-P2A), which generated half of the angle generated by proline, restored the efflux capacity.

Asymmetry
Asymmetry had a significant beneficial effect for the peptide 5A as compared to the parent symmetrical peptide L37PA20;
however, the same feature tested on ELK peptides had a strong detrimental effect (ELKA versus ELK).

**Inclusion of Cys and His**

This was tested on the derivatives of asymmetrical peptide 5A. Inclusion of Cys or His and especially Cys+His in the first (hydrophobic) helix (peptides 5A-CH1, 5A-H1, and 5A-C1) was detrimental for the efflux, whereas inclusion of these amino-acids in the second (less hydrophobic) helix (peptide 5A-CH2) was beneficial.

With few exceptions, the contribution of ABCA1 transporter for cholesterol efflux was proportional to the overall capacity of the peptides to support cholesterol efflux (Table 2 and Online Figure IV), resulting in a statistically significant correlation between these 2 parameters ($r=0.66$, $P<0.001$) (Figure 1B). This finding confirms that changes in the peptide structure affected specifically the ABCA1-dependent component of the efflux.

Finally, we analyzed the effect of complexing the most active peptide, ELK-2A2K2E with phospholipid on cholesterol efflux. Complex ELK-2A2K2E/POPC was significantly more effective in supporting cholesterol efflux from THP-1 cells (Table 2, last row). Cholesterol efflux to lipiddated particles is mediated by several mechanisms, including ABCG1 and SR-B1, as well as by aqueous diffusion; and whereas the former is activated by LXR agonists, the latter are not; thus, the contribution of specific transporters could not be tested using this design.

**Specificity of Cholesterol Efflux From Human Monocyte Cell Line THP-1**

The amphipathic nature of the peptides is essential for their capacity to support cellular cholesterol efflux and form lipoprotein particles; however, it can potentially cause cytotoxicity by damaging the plasma membrane. To analyze the contribution of the potentially cytotoxic “nonspecific” efflux, we compared cholesterol efflux to the peptides at saturating concentration (80 $\mu$g/mL) from live THP-1 cells and cells fixed with paraformaldehyde, a method previously to analyze cytotoxic properties of peptides 5A and L37PA. The data for cholesterol efflux are shown in Online Figure V, the absolute values of nonspecific efflux (ie, efflux from fixed cells) is shown in Figure 2A and the “specificity” of the efflux (ie, efflux from live cells minus efflux from fixed cells/efflux from live cells) is shown in Figure 2B. Analyzing features of the peptides responsible for the high nonspecific efflux, we excluded from consideration peptides with low overall capacity to support efflux from live cells (marked with arrows in Figure 2). The rationale for this exclusion was that analyzing efflux properties of the peptides that do not support total cholesterol efflux would not provide meaningful information about specificity of the efflux. Two features of the peptides associated with the high level of nonspecific efflux were the following: (1) net positive charge of the peptide (peptides with charge $\geq+2$ denoted with fine crosshatched bars in Figure 2); and (2) replacement of the proline a bridge with a single alanine (peptide ELK-PA denoted with coarse cross-hatched bar in Figure 2).

Thus, these 2 features should be avoided to avert toxicity of the peptides. The analysis also pointed to the 2 peptides with exceptional specificity, ELK-2A2K2E and ELK-1W; their
peptides or apoA-I (final concentration 40 µg/mL). The expression of CD11b was assessed by flow cytometry and is shown in Figure 4A. All peptides, with the exception of ELK-1F and ELK-2A, inhibited the expression of CD11b on activated human monocytes; there was a 6-fold difference in the magnitude of inhibition between the most and the least efficient peptides. Analysis of structure/function relationships as related to the inhibition of CD11b expression led to the following conclusions:

1. The structural feature having the most impact on the capacity of peptides to inhibit CD11b expression was asymmetry: all 3 asymmetrical peptides, 5A, ELK-A, and ELKA-CH2, were most active in inhibition of CD11b expression on monocytes.

2. Second feature having considerable impact on the capacity of the peptides to inhibit CD11b expression was the size of the hydrophobic face. An increase of the size of hydrophobic face of >180° was detrimental for the antiinflammatory property of the peptides (peptides ELK-2F, ELK-1F and ELK-2A).

3. Third feature affecting this antiinflammatory property was charge. Two or more additional positive charges were detrimental (peptides ELK-2A, ELK-1A and ELK-1L1K); however, additional negative charges were neither detrimental nor beneficial (peptides ELK-1W, ELK-3E3LK and ELK-3E3K3A).

4. Changing the type of helix to type G or Y was detrimental for the antiinflammatory capacity of the peptides (peptides ELK-2E2K and ELK-3E3EK).

5. Introduction of Cys and His residues or manipulation with proline bridge between the 2 helices had limited impact on the antiinflammatory properties of the peptides.

Thus, the optimal structural features of an effective antiinflammatory peptide are an asymmetrical pair of type A α-helices with hydrophobic face less than 180° and with neutral or negative charge.

We also analyzed the effect of complexing of the most active peptide, ELK-CH2, with phospholipid on its capacity to inhibit CD11b expression. Complex ELK-CH2/POPC had a higher capacity to inhibit CD11b expression compared to lipid-free peptide (Figure 4A). Similar effects were observed for the peptide, 5A (not shown).

**Antinflammatory Properties: Endothelium**

HDL and apoA-I affect expression of adhesion molecules on endothelial cells and this function may contribute significantly to the antiinflammatory properties of HDL. To test the capacity of the peptides to mimic antiinflammatory function of apoA-I toward endothelium we used a mouse endothelial cell line (SVEC4) stably transfected with luciferase under control of human VCAM-1 promoter. It was originally suggested that whereas apoA-I reconstituted with phospholipid or native HDL was a potent inhibitor of VCAM-1 expression, lipid-free apoA-I may not be as effective ascribing endothelial antiinflammatory property of HDL to its lipid constituents. This was not confirmed in our studies, with mouse endothelial cells: lipid-free apoA-I was just as effective inhibitor of VCAM-1 expression as HDL, inhibiting 90% of VCAM-1 expression (HDL inhibited...
VCAM-1 expression by 95%, $P>0.05$ versus apoA-I) (Figure 4B). Peptides were tested in lipid-free form; cells were activated with TNF-$\alpha$ and incubated with apoA-I, HDL or the peptides at the final concentration of 0.75 mg/mL, which was found to be a nonsaturating concentration of the peptides for this response. Analysis of structure/function relationships as related to the inhibition of VCAM-1 expression is shown in Figure 4B and led to the following conclusions:

1. Increased size of hydrophobic face (peptides ELK-2A, ELK-1F, ELK-2F) was beneficial for the inhibition of the VCAM-1 expression.
2. Negative charge (peptides ELK-1W, ELK-3E3LK, ELK-3E3K3A) was detrimental for the inhibition of VCAM-1 expression.
3. Inclusion of a combination of Cys+His residue was detrimental independently of their location (peptides 5A-CH2, 5A-CH1), whereas inclusion of Cys residue into the first helix of asymmetrical peptide was beneficial (peptide 5A-C1).
4. Hydrophobicity, changing helix type, disruption of the proline bridge, and asymmetry had limited impact on the capacity of the peptides to inhibit VCAM-1 expression.

Thus, to be effective in inhibition of VCAM-1 expression the peptide ideally should have a larger hydrophobic face, positive or neutral charge, and may contain a Cys residue. We also analyzed the effect of complexing of the most active peptide, ELK-2A, with phospholipid on its capacity to inhibit VCAM-1 expression. Unsurprisingly, complex ELK-2A/POPC did not inhibit VCAM-1 expression (Figure 4B). Complexing ELK-2A with another phospholipid, dimyrisoylphosphatidyl choline (DMPC), partially restored the capacity of the peptide to inhibit
VCAM-1 expression (Figure 4B). Similar effects were observed for the peptide, 5A (not shown).

**Antioxidant Properties**

The antioxidant properties of the peptides were assessed in an LDL oxidation assay. Human plasma LDL was incubated in the presence of Cu²⁺ and apoA-I mimetic peptides or apoA-I (final concentration 100 μg/mL); time course of diene formation was monitored by measuring absorption at 234 nm. Duration of lag phase and maximum diene formation were used to quantitate the rate of LDL oxidation as described by Pinchuk et al.²⁶ The time course curves for LDL oxidation are shown in Figure 5. All peptides, with the exception of ELK-1K1A1E, ELK-1F and ELK-1L1K inhibited oxidation of LDL by Cu²⁺; there was a 5-fold difference in the magnitude of inhibition between the most and least effective peptides. Analysis of structure/function relationships as related to the inhibition of LDL oxidation led to the following conclusions.

1. As expected, presence of Cys and/or His residue (peptides 5A-CH1, 5A-C1, 5A-H1, 5A-CH2, ELKA-CH2) significantly increased the antioxidant capacity of the peptides. The exact position of the residues had limited impact.

2. Unexpectedly, changes affecting secondary structure of the peptides had beneficial effect: asymmetrical peptides, peptides comprising type G and type Y helices (peptides ELK-1K1A1E, ELK-1F and ELK-1L1K inhibited oxidation of LDL by Cu²⁺); there was a 5-fold difference in the magnitude of inhibition between the most and least effective peptides. Analysis of structure/function relationships as related to the inhibition of LDL oxidation led to the following conclusions.

3. Charge, hydrophobicity, and size of hydrophobic face had limited impact on antioxidant capacity of the peptides.

Thus, an effective antioxidant peptide should contain Cys residue and preferably contains a non–type A α-helix, such as type G or type Y. We also analyzed the effect of complexing of the most active peptide, 5A-CH1, with phospholipid on its capacity to inhibit LDL oxidation. Complex 5A-CH1/POPC had a higher capacity to inhibit LDL oxidation compared to lipid-free peptide (Figure 5).

**Relationships Between Different Antiatherogenic Properties of the Peptides**

The finding that different apoA-I mimetic peptides have a wide range of efficiencies toward various antiatherogenic properties made it possible to investigate if any of these functions are related to each other. We found, however, no significant positive relationship for the various assays. In fact, the analysis of the structural features shows that features beneficial for one function may be detrimental for another (Table 3). For example, increased size of hydrophobic face was beneficial for cholesterol efflux, but detrimental for the monocyte antiinflammatory function. Maintaining the proline bridge was essential for the efflux, but was detrimental for the antioxidant function and did not affect monocyte antiinflammatory function. Although several peptides were superior to apoA-I for some of the individual functional assays, none of them was better than apoA-I in all tested functional assays. These findings are consistent with a suggestion that the various functions of apoA-I may have different structural requirements and are determined by different regions of the protein.

**Discussion**

In this study, we analyzed the structure/function relationship of 22 bihelical apoA-I mimetic peptides to identify structural features of these peptides that enable them to better mimic the various antiatherogenic functions of HDL. We also aimed at establishing if the various antiatherogenic properties of apoA-I are dependent on the same structural features and, if so, whether they share the same underlying mechanism.

The critical features of the peptides for cholesterol efflux capacity and ABCA1 specificity were hydrophobicity, size of the hydrophobic face, charge, and angle between 2 helices. Some of these features, such as requirement for a proline bridge¹⁸,¹⁹ or size of hydrophobic face,⁸ were previously investigated and our data are consistent with the results of these studies; however, this is the first systematic analysis of multiple structural modifications on cholesterol efflux. Fol-
Peptides active in inhibiting monocyte CD11b were asymmetrical peptides with a smaller hydrophobic face and a negative charge. In contrast, peptides active in inhibiting expression of endothelial VCAM-1 had relatively large hydrophobic faces and were positively charged. As the mechanisms of the antiinflammatory effects of apoA-I on monocytes and endothelial cells are not known, it is premature to speculate on how these structural features are translated into differences in the expression of adhesion molecules.

Another interesting finding of this study was that different atheroprotective functions of the peptides were determined by different structural features. No consistent correlation was found between the capacities of the peptides to mediate the various functions. No specific structural feature equally benefitted all functions. Furthermore, time and dose dependencies of the effects of the peptides and apoA-I on specific functions varied dramatically from one function to another: it took less than 15 minutes to inhibit the expression of CD11b and almost 24 hours to inhibit expression of VCAM-1. The saturating concentration of the peptides significantly differed for the different assays. Approximately 20 μg/mL of the peptides was required for maximum cholesterol efflux, whereas 100 μg/mL was needed for antioxidant capacity and >750 μg/mL for inhibition of VCAM-1 expression in endothelium. These findings suggest that different antiatherogenic functions of apoA-I have different mechanisms. This is consistent with findings of Wool et al., who demonstrated that modification of the peptides that favors HDL remodeling have negative impact on antioxidant function. Although a number of peptides were better than apoA-I in supporting individual functions, none of them could match the versatility of apoA-I when all the functions were taken into consideration. Possibly, different parts of apoA-I are responsible for different antiatherogenic functions and mimicking just one or 2 structural features of apoA-I is insufficient to create a peptide active in the many antiatherogenic facets of apoA-I. Although it may, therefore, be difficult to duplicate all of the biological properties of apoA-I in a single peptide of limited length, it may be possible to use a combination of peptides, an option that is currently being tested. The uncoupling, in the peptides, of the different antiatherogenic properties of HDL, however, creates a unique opportunity to investigate the relative contribution of the different antiatherogenic activities of HDL by testing them in animal models of atherosclerosis.

It is important to recognize several potential limitations of this study. First, HDL constituents other than apoA-I most
likely also contribute to the antiatherogenic properties of HDL. Size of HDL affects its ability to support cholesterol efflux,30 paraoxonase has a significant contribution to the antioxidant function,31 phospholipids may contribute to the antiinflammatory effects of HDL to endothelium25 and various pro-and antiinflammatory factors carried on HDL may contribute to the HDL antiinflammatory effects.32 Furthermore, a number of HDL functions were not investigated in this study, such as antithrombotic activity, suppression of apoptosis, regulation of endothelial function, insulin secretion and glucose oxidation. Although these functions may contribute to the antiatherogenic properties of HDL, most available data suggest that involvement of HDL in cholesterol efflux, inflammation and oxidation are the major determinants of its atheroprotective potential and therefore are the main targets of “HDL therapy.” Finally, peptides were examined in their lipid-free form. This was a deliberate strategy, as the physicochemical properties of the peptides will have a significant impact on the lipid content of rHDL particles assembled with the peptides, the size of these particles and on the number of peptide molecules per particle. These factors may have a significant confounding influence on many antiatherogenic properties of the peptides. The lipid-free peptides may also better simulate the process by which apoA-I interacts with cells and lipid-free peptides were shown to be as effective as their phospholipid complexes in preventing atherosclerosis in vivo.33 It is likely, however, that these peptides, like apoA-I, will readily acquire in vivo various lipids and/or will recombine with endogenous HDL; therefore, future in vivo studies will be needed to fully assess the effect of these peptides on atherosclerosis. Lipidated peptides (rHDL) would also interact with endogenous HDL and undergo remodeling and therefore would not be a better representation of the in vivo situation than lipid-free apoA-I. Acknowledging however that in vivo apoA-I mimetic peptides are likely to be lipidated, we examined the effect of complexing the most active peptides with phospholipid on their activity. As expected, lipidation increased peptide cholesterol efflux capacity, most likely by complementing ABCA1-dependent efflux with the efflux through other pathways. Lipidation further improved peptide capacities to prevent LDL oxidation and to inhibit CD11b expression on monocytes. Surprisingly, lipidation had a profound effect on the capacity of peptides to inhibit VCAM-1 expression in endothelium. Whereas lipidation of apoA-I into HDL did not alter its antiinflammatory capacity, lipidation of the peptides with DMPC reduced this capacity and lipidation with POPC (palmitoyloleoyl phosphatidyl choline) abolished it. In vivo, however, lipidated peptide 5A did inhibit expression of VCAM-1 in rabbit arteries.34 Clearly, this property of the peptides may be affected by lipid constituency.

In summary, by examining a panel of amphipathic bihelical peptides, it was found that the different antiatherogenic features of these peptides requires different structural features. This is relevant to the design of apoA-I mimetic peptides for HDL therapy and may lead to new insights into what properties of HDL are the most relevant for its ability to reduce cardiovascular disease.

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Disclosures

None.

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Novelty and Significance

**What Is Known?**

- Apolipoprotein (apo)A-I mimetic peptides are active in a number of antiatherogenic functions of high-density lipoprotein (HDL) and can protect against atherosclerosis.

- Structurally, ApoA-I mimetic peptides reproduce secondary structure of apoA-I, an array of 22-mer amphipathic α-helices, without close homology to the apoA-I primary structure.

- Knowledge on structure/function relationship of the peptides is limited to the facts that peptides with 2 helices work better than single helix peptides and that increasing peptide hydrophobicity improves their functionality.

**What New Information Does This Article Contribute?**

- Using a panel of related peptides, we established optimal structural requirements for the activity of the peptides in cholesterol efflux, antiinflammatory, and antioxidant activities.

- Different antiatherogenic properties of apoA-I have different, and sometimes conflicting, structural requirements pointing to independent mechanisms.

- Different parts of apoA-I may be responsible for different antiatherogenic functions, and it may not be possible to combine all the features in one peptide of limited length.

- ApoA-I mimetic peptides are a promising therapeutic approach attempting to reproduce the antiatherogenic properties of HDL. These peptides support cholesterol efflux and have antiinflammatory and antioxidant activities. Structurally, they are comprised of one or two 18- to 22-mer canonical amphipathic α-helices. ApoA-I, however, is comprised of “almost” canonical, as well as “imperfect” α-helices, and such mixture may be required for the variety of antiatherogenic functions of apoA-I. By changing the structure of one or both helices in bilevel peptides, we were able to “fine tune” the peptides to be more active and more specific in individual functions compared with apoA-I. However, structural requirements for the individual functions were often conflicting with each other, and although a number of peptides have high activity in all assays, we were unable to design a peptide that was more active than apoA-I in all its functions. These findings provide a rationale for designing more complex formulations that would combine multiple antiatherogenic activities. We have also designed a number of peptides that are active in one but inactive in other antiatherogenic functions, providing a powerful tool to study the relative contribution of various HDL functions to its overall antiatherogenic capacity.
Structure/Function Relationships of Apolipoprotein A-I Mimetic Peptides: Implications for Antiatherogenic Activities of High-Density Lipoprotein
Wilissa D'Souza, John A. Stonik, Andrew Murphy, Steven J. Demosky, Amar A. Sethi, Xiao L. Moore, Jaye Chin-Dusting, Alan T. Remaley and Dmitri Sviridov

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SUPPLEMENTAL MATERIAL FOR THE MANUSCRIPT:

Structure-function relationships of apolipoprotein A-I mimetic peptides: implications for anti-atherogenic activities of high density lipoprotein

Wilissa D’Souza, MSc., John A. Stonik, Andrew Murphy, PhD, Steven J. Demosky, Amar A. Sethi, PhD, Xiao L. Moore, PhD, Jaye Chin-Dusting, PhD., Alan T. Remaley, PhD., Dmitri Sviridov, MD, PhD.
Methods:

Peptide synthesis

All peptides were synthesized by a solid-phase procedure, using a Fmoc/DIC/HOBt protocol on a Biosearch 9600 peptide synthesizer and were purified to greater than 99% purity by reverse-phase HPLC on an Aquapore RP-300 column, as assessed by MALDI-TOF-MS (Bruker UltraFlex). Retention time of peptides was determined after injection of 1 mg of purified peptide on a C-18 reverse phase HPLC column and elution with a 25-85% gradient of acetonitrile, containing 0.1% TFA. The mean hydrophobic moment was calculated as the vectorial sum of all the hydrophobicity indices, divided by the number of residues using DNA Star software.

Peptides were reconstituted with palmitoyloleoyl phosphatidyl choline (POPC) or dimyrisoylphosphatidyl choline (DMPC), at a molar ratio of 1:7. Peptides and POPC or DMPC were complexed by co-lyophilization after first being dissolved in glacial acetic acid. The resultant lyophilized cakes were reconstituted with 20 mM NaHCO₃, 0.15 M NaCl and heated to 50°C in a water bath for 3 min and then allowed to cool at room temperature for 3 min for a total of three cycles.

Lipoproteins

LDL (1.006-1.063 g/ml) and HDL (1.083-1.21 g/ml) were isolated from human plasma (pooled plasma supplied by Red Cross) by sequential centrifugation. Apolipoprotein A-I was isolated from HDL as described previously 1.

Cholesterol efflux from THP-1 cells

THP-1 cells were maintained in RPMI medium supplemented with 10% FBS. Cells were differentiated into macrophage-like cells by incubation in RPMI supplemented with 10% FBS and 100 nMol/L phorbol 12-myristate 13-acetate (PMA) for 72 h. Cellular cholesterol was labeled by incubation in serum-containing medium with [1α,2α(n)-3H]-cholesterol (GE Health-Amersham, final radioactivity 0.5 MBq/ml) for 48 h in a CO₂ incubator. Cells were then washed and incubated for 18 h at 37°C in serum-free medium in the presence or absence of the Liver X Receptor (LXR) agonist TO-901317 (4 μmol/L). Cells were washed and incubated for another 4 h at 37°C in serum-free medium containing indicated concentrations of the peptides or lipid-free apoA-I. The medium was collected, centrifuged for 15 min at 4°C at 10,000 x g and aliquots of supernatant were counted in a β-counter. Cells were harvested and cell-associated radioactivity was counted. Cholesterol efflux was expressed as the proportion of [3H]cholesterol transferred from cells to medium. Non-specific efflux (i.e. the efflux in the absence of an acceptor) was subtracted. Where indicated, cells were fixed by incubation for 20 min with paraformaldehyde (4%) prior to the efflux experiments. All experiments were done in quadruplicates; intra-assay variability was < 5%. Efflux to each peptide was assessed in 2-3 independent experiments. Inter-assay variability was assessed by including apoA-I and peptide 5A in all experiments;
this variability was up to 30% therefore the results from different experiments were combined after normalization to the efflux to apoA-I and 5A.

**Cholesterol efflux from BHK cells**

BHK cells stably transfected with human ABCA1 under the mifepristone-inducible promoter were a kind gift of Dr. J. Oram and were handled as described. Cellular cholesterol was labeled by incubation with 
$[^3H]$-cholesterol for 48 h in a CO$_2$ incubator. Cells were then washed and incubated for 18 h at 37°C in serum-free medium and then for another 24 h at 37°C in serum-free medium containing 20 μMol/ml (or approximately 90 μg/ml) of the peptides. The medium was collected, centrifuged for 15 min at 4°C at 10,000 x g and aliquots of supernatant were counted in a $\beta$-counter. Cells were harvested and cell-associated radioactivity was counted. Cholesterol efflux was expressed as the proportion of $[^3H]$cholesterol transferred from cells to medium. Non-specific efflux (i.e. the efflux in the absence of an acceptor) was subtracted. All peptides were tested in the one experiment done in quadruplicates and repeated twice, inter-assay variability was <10%.

**Expression of CD11b on human monocytes**

Resting human monocytes were isolated from blood of healthy volunteers by density centrifugation with Lymphoprep followed by Dynal Negative Monocyte Isolation kit as described previously. The CD11b assay was described previously. In brief, monocytes were stimulated with 1μmol/L phorbol-12 myristate 13-acetate (PMA) (Sigma, Australia) in the presence or absence of the peptides or apoA-I final concentration 40 μg/mL) and incubated with the FITC conjugated Ab to the active epitope of CD11b (eBiosciences, USA, Clone CBRM1/5) for 15min at 37°C. Cells were then fixed with 4% paraformaldehyde. Samples were controlled for by using the isotype matched negative control (FITC-anti-mouse IgG, Serotec, USA, Clone W3/25). CD11b expression was measured by flow cytometry using FACS Calibur (Becton Dickinson). Analysis was conducted using the Cell Quest Pro software. Results were expressed as percentage of the CD11b expression compared to cells stimulated with PMA in the presence of a vehicle. Due to considerable inter-assay variability (mainly due to various levels of activation of monocytes from different donors) each peptide was tested with monocytes from at least three different donors and results were expressed relative to CD11b expression after stimulation with PMA.

**Expression of VCAM-1 in mouse endothelial cells**

SVEC4-10 cells, a mouse endothelial cell line, was stably transfected with the Pgl3 plasmid, containing the cDNA for firefly luciferase, with 2.27 kbp of the proximal promoter of the human VCAM-1 gene. Cells were co-transfected with PsvNeo and selected with 100 μg/mL of G418.

Transfected SVEC4 cells were seeded into 96 well plates at the final density of 0.25x10$^5$ cells per well. After 24 h cells were washed and apoA-I, HDL or apoA-I mimetic peptides were added at the final concentration of 0.75 mg/ml. After 18 h incubation cells were washed and tissue necrosis factor (TNF-α) was added in serum-free medium to the final concentration of 10 ng/ml. Cells were incubated for 5 h and luciferase activity was measured using Bright-Glo Assay (Promega). Data were
expressed per milligram of cellular protein and related to the luciferase activity in
cells incubated with a vehicle instead of the peptides. All peptides were tested in the
one experiment done in quadruplicates and repeated twice, inter-assay variability was
<10%.

Oxidation of LDL

The capacity of the peptides to inhibit LDL oxidation was assessed as described by
Kontush et al 7. In brief, freshly isolated LDL (final concentration 100 μg/ml) was
incubated at 25°C for the indicated periods of time with CuSO4 (final concentration
15 μMol/L) in the presence of the peptides or apoA-I (final concentration of 100
μg/ml) in the cells of a multi-cell spectrophotometer (Beckman, DU800) and
absorption was continually monitored at 234 nm. Rate of oxidation was calculated as
maximum absorbance divided to the length of the lag period according to the
published model of LDL oxidation 8. Each peptide was tested twice with different
batches of LDL, apoA-I and peptide 5A were included in each assay, inter-assay
variability was <15%.

Statistics

All experiments were reproduced at least 2-4 times. Unless otherwise indicated,
experimental groups consisted of quadruplicates; means ± SEM are presented.
Differences between groups were analyzed by ANOVA with t values modified by the
step down Bonferroni procedure; the differences considered statistically significant
when p<0.05. Correlations were calculated using Pearson Product Moment
Correlations or, when indicated, using Spearman Rank Order Correlations.

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**Supplementary Figure I. Dose-dependence of cholesterol efflux from THP-1 cells activated with LXR agonist**

Cellular cholesterol was labeled by incubation in serum-containing medium with \[^{[3]}\text{H}\]-cholesterol. Cells were then washed and incubated for 18 h at 37°C in serum-free medium in the presence of the LXR agonist TO-901317 (4 \(\mu\text{mol/L}\)). Cells were washed and incubated for another 4 h at 37°C in serum-free medium containing indicated concentrations of the peptides or lipid-free apoA-I. Cholesterol efflux was expressed as the proportion of \[^{[3]}\text{H}\]cholesterol transferred from cells to medium. Non-specific efflux (i.e. the efflux in the absence of an acceptor) was subtracted. Data from different experiments were normalized to the efflux to the peptide 5A, which was included in all experiments.
Supplementary Figure II. Dose-dependence of cholesterol efflux from THP-1 cells not activated with LXR agonist

Cellular cholesterol was labeled by incubation in serum-containing medium with [3H]-cholesterol. Cells were then washed and incubated for 18 h at 37°C in serum-free medium, washed and incubated for another 4 h at 37°C in serum-free medium containing indicated concentrations of the peptides or lipid-free apoA-I. Cholesterol efflux was expressed as the proportion of [3H]cholesterol transferred from cells to medium. Non-specific efflux (i.e. the efflux in the absence of an acceptor) was subtracted. Data from different experiments were normalized to the efflux to the peptide 5A, which was included in all experiments.
**Supplementary Figure III.** Dose-dependence of ABCA1-dependent cholesterol efflux from THP-1.

Data presented in this figure are a difference between values presented in Supplementary Figure I and Supplementary Figure II, calculated for each data point. When calculations gave negative values they were interpreted as “0” value.
Supplementary Figure IV: Contribution of ABCA1-dependent efflux
Data presented in this figure show efflux from activated and non-activated THP-1 cells (data taken from Supplementary Figure I and Supplementary Figure II) at peptide concentration 20 μg/ml
Supplementary Figure V. Specificity of cholesterol efflux from THP-1 cells.
Cellular cholesterol was labeled by incubation with $[^3]$H-cholesterol for 48 h in a CO$_2$ incubator. Cells were then washed and incubated for 18 h at 37°C in serum-free medium, and fixed or not fixed by incubation for 20 min with paraformaldehyde (4%). Cells washed and incubated for another 4 h at 37°C in serum-free medium containing 80 μg/ml of the peptides or lipid-free apoA-I. Cholesterol efflux was expressed as the proportion of $[^3]$H-cholesterol transferred from cells to medium. Non-specific efflux (i.e. the efflux in the absence of an acceptor) was subtracted.
**Supplementary Figure VI. Oxidation of LDL**

Freshly isolated LDL (final concentration 100 μg/ml) was incubated at 25°C for the indicated periods of time with CuSO$_4$ (final concentration 15 μMol/L) in the presence of the peptides or apoA-I (final concentration 100 μg/ml) in the cells of a multi-cell spectrophotometer continuously measuring absorption at 234 nm.