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Retention of α-helical structure by HDL mimetic peptide ATI-5261 upon extensive dilution represents an important determinant for stimulating ABCA1 cholesterol efflux with high efficiency

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ABSTRACT

ATI-5261 is a novel, single-helix peptide that stimulates cellular cholesterol efflux with high potency similar to native apolipoproteins on a molar basis. Presently we investigated structural features of the peptide that conferred cholesterol efflux activity. Analogs of ATI-5261 with amino acids arranged in reverse order or with individual arginine (R) to glutamine (Q) substitutions (i.e. R3Q, R14Q, or R23Q) stimulated ABCA1 dependent cholesterol efflux similar to ATI-5261. Consequently, neither the presence of specific positively charged residues nor their specific arrangement along the length of the peptide was necessary for mediating cholesterol efflux. Similarly, peptides composed of all D-amino acids stimulated cholesterol efflux efficiently, indicating a stereospecific component was not required for promotion of cholesterol efflux from macrophages. Removal of two or more positively charged residues (R3, 14 → Q and R3, 14, 23 → Q) however, greatly reduced the ability of ATI-5261 to mediate cellular cholesterol efflux. This was accompanied by a loss of α-helical structure upon dilution, indicating the secondary structure of individual peptide strands was important for stimulating cholesterol efflux. Surprisingly, peptides with removal of two or more positively charged residues retained the ability to bind phospholipid and adopt an α-helical structure. These data indicate that the propensity of a hydrophobic peptide to form an amphipathic α-helix is not sufficient to mediate cellular cholesterol efflux. Efficient stimulation of cholesterol efflux requires that ATI-5261 retain α-helical structure upon dilution.

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1. Introduction

High density lipoproteins (HDL) are thought to protect against the development of atherosclerosis [1,2]. This protection has been attributed, in part, to the ability of HDL to mediate reverse cholesterol transport [3–5]. Reverse cholesterol transport involves stimulation of cholesterol efflux from peripheral cells followed by the transport of cholesterol to the liver for elimination in feces. Several mechanisms of cholesterol efflux have been described, including aqueous diffusion and apolipoprotein (apo)-mediated processes [6–14]. The latter requires the membrane protein ABCA1 (i.e. ATP-binding cassette transporter A1), that transfers cellular cholesterol and phospholipid to extracellular, lipid-poor apos, such as apoA-I and E [15–22].

Recently, we demonstrated that the C-terminal (CT) lipid-binding domain of apoE was responsible for mediating cholesterol efflux via ABCA1 [18]. These studies revealed that a region of the apoE CT domain consisting of a hydrophobic segment linked to a class A α-helical motif was required for mediating cellular lipid efflux efficiently. This information was used to create a small peptide that mimics the activity of native apos to stimulate ABCA1 cholesterol efflux and reduce substantial atherosclerosis in mouse models [23]. The resulting peptide, ATI-5261, shares oligomeric properties of native apos and transitions from a self-associated form (i.e. tetramers) to low molecular weight species, including monomers of high α-helical content (~70%) in the absence of lipid [24]. The latter occurs upon extensive dilution over the active concentration range for mediating cellular cholesterol efflux, indicating peptide monomers with exposed hydrophobic surfaces are important for activity. Such a mechanism is supported by studies with crosslinking reagents that lock ATI-5261 in tetramer form and inhibit cholesterol efflux activity [25].

Presently, we utilized site-specific amino acid variants and peptidomimetics of ATI-5261 to investigate structural features of the peptide that conferred potent cholesterol efflux activity. No sequence- and/or stereo-specific requirements for mediating

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cholesterol efflux were observed with ATI-5261, consistent with previous studies of other apo mimetics [26–28]. Moreover, stimulation of cellular cholesterol efflux did not require a specific pattern of positively charged residues. However, extensive ablation of cationic character greatly reduced the α-helical content of ATI-5261 upon extensive dilution and this was associated with loss of cholesterol efflux activity. Reduction in α-helical content was not observed at high concentrations of peptides, i.e. in self-associated form, or in the presence of POPC or 50% TFE, indicating the loss of cationic residues did not disrupt the inherent nature of the peptide to form an amphipathic α-helix. These results indicate that potent stimulation of cholesterol efflux via ABCA1 is dependent on ATI-5261 retaining its α-helical structure upon extensive dilution.

2. Materials and methods

2.1. Peptides

Peptides were synthesized (Biosynthesis Inc., Lewisville, TX) using either all L- or all D-amino acids, capped with N-terminal acetyl and C-terminal amide groups, isolated by HPLC and used at a purity of >95%. Lyophilized peptides were dissolved in 10 mM phosphate buffered (pH = 7.4) saline (150 mM NaCl), referred to as PBS, or 10 mM phosphate (pH = 7.4). Peptide concentrations were determined by absorbance at 280 nm. All peptides used in these studies were highly soluble (up to 5 mg/ml concentrations normally tested) in PBS or phosphate buffer (pH = 7.4) and self-associated as tetramers, with no apparent signs of non-specific aggregation or precipitation determined upon visible inspection of solutions (over several months) or FPLC [24].

2.2. CD spectroscopy

Circular dichroism (CD) spectroscopy was carried out on a Jasco 810 spectropolarimeter at 25 °C, using lipid-free peptides in 10 mM sodium phosphate buffer (pH = 7.4) and averaging 4 scans (20 nm/min/scan) per run as described [24]. Samples were diluted in this same buffer to assess the α-helical content of peptides at various concentrations. Where indicated, peptides were prepared in 10 mM phosphate (pH = 7.4) plus 50% TFE or complexed with POPC to form small 7–8 nm sized particles to assess the ability of specific variants to adopt an α-helical structure [23].

2.3. Cell-culture and measurement of cholesterol efflux activity

J774 mouse macrophages were plated onto 24-well culture plates and labeled with [^{3}H] cholesterol (1 μCi/ml) in RPMI-1640 with 1% FBS for 48 h. A cAMP analogue (cpt-cAMP) was added (0.3 mM final concentration) to upregulate ABCA1 expression. Cells were extensively rinsed with serum-free RPMI-1640 medium followed by an extended rinse (2 h) with RPMI-1640 containing 0.2% bovine serum albumin (BSA). Lipid-free peptides were added to cells in serum-free RPMI-1640 medium to initiate cholesterol efflux. The amount of [^{3}H] cholesterol appearing in the medium was expressed as a percentage of the radioactivity initially present in cells at time zero [6,7,17,18]. The background release of [^{3}H] cholesterol to serum-free medium alone was subtracted from the values obtained with lipid-free peptides.

2.4. Lipid binding

A turbid solution of dimyristoylphosphatidylcholine (DMPC) was used to assess the capacity of peptides to solubilize phospholipid [17]. The DMPC was used at a final concentration of 0.16 mg/ml PBS (pH = 7.4). The mass ratio of DMPC to peptides was 2:1 or 4:1. The absorbance (400 nm) of samples was monitored continuously over 20 min at 25 °C.

2.5. Statistics

Where appropriate, data were expressed as means ± SD of at least 3 independent experiments and statistical analyses performed using Student's *t*-test.

3. Results

Sequence variants of ATI-5261 were used in the present study to identify features of the peptide that conferred cholesterol efflux activity. Reversing the sequence of ATI-5261 altered the specific positions of positively charged residues, but overall class A structure and amphipathic nature were retained (Fig. 1A). Despite the altered pattern of charged residues, the reverse sequence form of ATI-5261 stimulated cholesterol efflux in an ABCA1 dependent manner (Fig. 1B). Maximum levels of cholesterol efflux were achieved using 3 μg peptide/ml, indicating the reverse sequence form of ATI-5261 was a potent stimulator of cholesterol efflux (Fig. 1C, top right) like ATI-5261 [23]. Similarly analogs of ATI-5261 composed of all D-amino acids stimulated cholesterol efflux similar to the L-amino acid form of ATI-5261 (Fig. 1C, bottom vs. top panels), indicating there was no stereospecific requirement for mediating cholesterol efflux via ABCA1.

To further explore the contribution of specific charged residues to cholesterol efflux, R → Q variants of ATI-5261 were designed to ablate cationic character (Fig. 2A). Use of single R → Q substitutions at specific positions along the length of ATI-5261, i.e. R3Q, R14Q, or R23Q, did not alter cholesterol efflux activity compared to the parent ATI-5261 peptide (Fig. 2B). High-levels of cholesterol efflux were observed using 3 μg/ml peptides, consistent with the behavior of ATI-5261. This was verified using several of the single R → Q variants, which stimulated cholesterol efflux in a highly efficient manner similar to ATI-5261 (Km = 0.73, 0.95, 0.94 μg/ml, for R14Q, R23Q, and ATI-5261 respectively).

In contrast to the results obtained with the single R → Q substitutions, peptides with double (R3, 14 → Q) or triple (R3, 14, 23 → Q) glutamine substitutions had greatly reduced cholesterol efflux activity (Fig. 2). Reductions in absolute levels of ABCA1-dependent cholesterol efflux (Fig. 2C) as well as efflux efficiency (Fig. 2D) were observed with loss of two or more positively charged residues. The latter was associated with a marked decrease in the ability of the R14, 23 → Q and R3, 14, 23 → Q variants to mediate cholesterol efflux over the active concentration range (<10 μg/ml) characteristic of ATI-5261. This produced an increase in the Km values for cholesterol efflux activity of the double and triple R → Q variants, indicative of poor efflux efficiency (Fig. 2D). Interestingly, the ability of R → Q peptides to bind purified phospholipid was increased (Table 1 and Fig. 3). Nearly all the R → Q variants tested displayed a tendency for increased ability to clear turbid solutions of DMPC, with more dramatic effects seen using the double and triple R → Q variants (Fig. 3A and B). Similar results with peptides were obtained using the acidic phospholipid DMPG in clearance assays (data not shown). Therefore, the increased lipid-binding affinity of the R → Q peptides appeared to be unrelated to charge effects. Use of the R → Q substitutions did however increase the hydrophobicity of peptides (Table 1), which may have facilitated lipid binding.

The α-helical content of the R → Q variants was largely unaffected by removal of positively charged residues (Tables 1 and 2), i.e. at relatively high concentrations (>0.1 mg/ml, i.e. 31 μM) of peptide that far exceed those required for mediating cholesterol efflux [23–25]. Thus, peptides with single or multiple