Published in final edited form as:

Biochem Biophys Res Commun. 2011 July 8; 410(3): 446–451. doi:10.1016/j.bbrc.2011.05.154.

Helix stabilization of amphipathic peptides by hydrocarbon stapling increases cholesterol efflux by the ABCA1 transporter

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Abstract

Apolipoprotein mimetic peptides are short amphipathic peptides that efflux cholesterol from cells by the ABCA1 transporter and are being investigated as therapeutic agents for cardiovascular disease. We examined the role of helix stabilization of these peptides in cholesterol efflux. A 23-amino acid long peptide (Ac-VLDSFKVSFLSALEEYTKKLNTQ-NH₂) based on the last helix of apoA-I (A10) was synthesized, as well as two variants, S1A10 and S2A10, in which the third and fourth and third and fifth turn of each peptide, respectively, were covalently joined by hydrocarbon staples. By CD spectroscopy, the stapled variants at 24°C were more helical in aqueous buffer than A10 (A10 17%, S1A10 62%, S2A10 97%). S1A10 and S2A10 unlike A10 were resistant to proteolysis by pepsin and chymotrypsin. S1A10 and S2A10 showed more than a 10-fold increase in cholesterol efflux by the ABCA1 transporter compared to A10. In summary, hydrocarbon stapling of amphipathic peptides increases their helicity, makes them resistant to proteolysis and enhances their ability to promote cholesterol efflux by the ABCA1 transporter, indicating that this peptide modification may be useful in the development of apolipoprotein mimetic peptides.

Keywords

Apolipoproteins; Peptides; Atherosclerosis; Cholesterol Efflux; High Density Lipoproteins

Introduction

Apolipoprotein mimetic peptides are being investigated as possible therapeutic agents for the treatment of cardiovascular diseases [1,2], as well as disorders associated with inflammation [1,3]. They reduce atherosclerosis in animal models and appear to be safe in early stage clinical trials [4,5,6]. Apolipoprotein mimetic peptides have similar biological properties as full length apolipoproteins, such as apoA-I, the main protein on High Density Lipoproteins (HDL). Weekly intravenous infusions of recombinant or purified apoA-I reconstituted with phospholipids for 4-5 weeks have been shown to reduce plaque volume in

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patients with acute coronary syndrome comparable to what has been achieved with several years of statin treatment [7,8]. A major limitation of the use of full length apoA-I is the cost to produce the large quantities that are needed for this type of treatment and hence the interest in the use of short synthetic mimetic peptides [1]. Another potential advantage of apolipoprotein mimetic peptides is that when they are synthesized with D-amino acids, such as the D4F peptide, they are resistant to proteolysis and can reduce atherosclerosis in animal models when given orally [9,10]. Clinical development of D4F, however, has been halted because of concerns related to long-term tissue accumulation [4].

ApoA-I and apolipoprotein mimetic peptides potentially have several different beneficial effects in preventing or reducing atherosclerosis [1], but the best understood and possibly the central mechanism behind many of their properties is based on their ability to increase the reverse cholesterol transport pathway [11,12]. Recently, it was shown that the ability of HDL in serum to efflux excess cholesterol from macrophages was, in fact, a better predictor of the atheroprotective effect of HDL than the cholesterol content of HDL [13], the current diagnostic test for assessing atheroprotection by HDL. An early step in this process involves the interaction of apoA-I with the ABCA1 transporter, which by a detergent-like extraction step removes cholesterol and phospholipid from cells and forms small nascent HDL [14]. Only proteins, such as apoA-I and other apolipoproteins that contain amphipathic alpha helices, can remove lipid from the plasma membrane microdomain created by the ABCA1 transporter [2,15].

In the absence of associated phospholipids, apolipoproteins do not as readily form amphipathic alpha helices [16,17]. This is particularly true for short synthetic amphipathic peptides, which largely form random coils when present in aqueous solutions, because water more effectively competes with the intermolecular hydrogen bonds that stabilize alpha helices. Whether this less conformational constrained state for apolipoproteins or their mimetic peptides is beneficial or detrimental in their interaction with the ABCA1 transporter in the cholesterol efflux process is not known. Recently, it was shown that the hydrocarbon stapling of short synthetic peptides markedly increases their ability to form helices and has been used to improve the immunogenicity of synthetic peptide vaccines when an antigenic epitope is present in an alpha helical region of an intact protein [18,19]. Because of the hydrophobicity of the hydrocarbon staple, these modified peptides also readily cross cell membranes, enabling their use for blocking intracellular protein-protein interactions [18,19]. Increased membrane permeability may also account for the overall improved oral availability of stapled peptides, including relatively long peptides containing multiple helices [20]. In addition, because most proteases preferentially degrade unfolded proteins, the increased helical structure of stapled peptides reduces their degradation in the digestive tract and may increase their half-life in the plasma compartment [20].

In this study, we investigate the biophysical properties of the last helix of apoA-I and two hydrocarbon stapled variants of this peptide. The last helix of apoA-I has been shown to be critical in the ability of the full length protein to promote cholesterol efflux, but when synthesized as a single helical peptide, it is unable to promote cholesterol efflux [21]. In this study, we, therefore, also examined the effect of hydrocarbon stapling of the last helix of apoA-I on cholesterol efflux from cells by the ABCA1 transporter, as well as by several other mechanisms.

Methods

Peptide Synthesis

A peptide based on the last helix of apoA-I (A10) (Ac-VLDSFKVSFLSALEEYTKKLNTQ-NH₂) and 2 stapled variants of this peptide, referred to as S1A10 and S2A10, were made by

solid phase synthesis, using standard Fmoc chemistry and the Fmoc-modified amino acid linkers ((R)-a-(7'-octanyl)Ala and (S)-a-(4'-pentenyl)Ala) (AnaSpec Inc.) The cross linking of the modified amino acid linkers was done by the olefin metathesis reaction with Bis(tricyclohexyl-phosphine)-benzylidene ruthenium (IV) dichloride as the catalyst [22]. Peptides were purified by reverse phase HPLC and were determined to be over 95% pure by LC-MS analysis.

CD Spectroscopy

Peptides (0.1 mg/mL) in sodium phosphate buffer (pH 7.4) were loaded into a quartz cuvette (d= 0.2 cm path length) and CD spectra from 185 to 240 nm were recorded on a Jasco J715 spectropolarimeter at 24°C. Data were normalized by calculating the mean residue ellipticity (Θ). Peptide thermostability was monitored at 220nm by increasing temperature from 10 to 90°C.

Peptide Proteolysis

Peptides (5 mg/ml) were diluted 10x with either 10% acetic acid (pH 2), containing pepsin (0.5 μ g/ml final) or in 10mM NH_4HCO_3 (pH 7), containing chymotrypsin (0.5 μ g/ml final). After incubation at 37°C, aliquots at various time points were removed and dried on a MALDI AnchorChip target at 45°C. MALDI standards (Bombesin, ACTH 1-17, ACTH 18-39, Somastatin 28, CHCA in 50% ACN, 0.1% TFA) and matrix were added sequentially and allowed to dry between additions. Samples were analyzed on a Bruker AutoFlex III (Bruker Daltonics) in positive ion reflectron mode. Relative protein concentrations were determined from areas under the curve, using the MALDI standards as calibrators.

Vesicle Solubilization Studies

Dimyristoyl phosphatidyl choline (DMPC) vesicles were prepared by resuspension of dried DMPC with PBS and vortexing for 5 min. Changes in light scattering upon peptide addition were monitored at 24°C every minute for 1 h at 660nm, with shaking in a Perkin Elmer plate reader.

Cholesterol Efflux Studies

Cholesterol efflux studies were performed, as described previously [23]. Control BHK cells and BHK cells stably transfected with either human ABCA1, ABCG1 or SR-BI were radiolabeled with [^3H] cholesterol for 24 h, washed and then treated with the indicated concentration of peptides for 18 h. Percent efflux was calculated by subtracting the radioactive counts in blank medium (alpha-minimal essential media plus 10ug/ml bovine serum albumin) from the radioactive counts in the presence of a peptide and then dividing the result by the sum of the radioactive counts in the medium plus the cell fraction.

Results

The primary sequence of the last helix of apoA-I and the position of the hydrocarbon linkers in the two modified stapled peptides are shown as helical net plots in Figure 1. The last helix of apoA-I, which we refer to as A10, is a Type A amphipathic helix [24]. Approximately half of the helix contains hydrophobic amino acids, whereas the other half contains negatively charged residues in the central polar region, and positively charged residues positioned at the interface between the polar and hydrophobic sides of the helix [24]. To preserve the hydrophobic moment of the modified peptides, the hydrophobic hydrocarbon staples were placed near the center of the hydrophobic region. In case of the first stapled peptide, S1A10, a hydrocarbon linker was used to covalently bridge two adjacent helices in

the center (third and fourth turn) of the peptide. In the case of S2A10, a longer hydrocarbon linker was used for linking the third and fifth helical turn of the peptide.

Based on CD spectroscopy, the unstapled A10 peptide at 24°C was 17% helical in aqueous buffer (Fig. 1B), whereas S1A10 and S2A10 were 62% and 97% helical, respectively. By CD spectroscopy (Fig. 1C), the heat denaturation curve for S1A10 was parallel to A10, but S1A10 was more helical than A10 at all temperatures tested and even had some residual helical content at the highest temperature tested, which may possibly be within the cross linked region. The S2A10 peptide, with the longer linker, showed even less susceptibility to thermal denaturation. Helicity at 90°C was calculated to be 11%, 25%, and 48% for A10, S1A10 and S2A10, respectively.

Unfolded proteins are more readily digested by proteases [20], so we, therefore, tested the 3 peptides for their susceptibility to proteolysis by pepsin and chymotrypsin (Fig. 2), both of which have several potential cleavage sites within and outside the linker region (Fig. 1). After the protease digestions, a decrease in the peaks corresponding to the intact parent peptides were monitored by MALDI-TOF mass spectrometry. The A10 peptide was readily digested by both proteases, with less than 5% of intact peptide remaining after 30 minutes. In contrast, the stapled peptides were relatively resistant to proteolysis by pepsin and chymotrypsin, although S2A10 appeared to be less sensitive to chymotrypsin than S1A10. In addition to its increased helical structure, the replacement of the Y16 residue, a potential cleavage site, in S1A10 for the cross linker in the S2A10 peptide (Fig. 1), may have also contributed to its resistance to chymotrypsin. Both stapled peptides, however, were almost fully digested after longer incubation times, with only 0.4% and 0.6% remaining for S1A10 and 12.2%, and 3.8% remaining for S2A10 after digestion for 24 h with pepsin and chymotrypsin, respectively.

In Figure 3, we assessed the ability of the peptides to act like detergents in a phospholipid vesicle solubilization assay. The unstapled A10 peptide caused some initial solubilization of the DMPC vesicles, but at later time points, the turbidity of the solution increased most likely because it also promoted the fusion of the DMPC vesicles. In contrast, both stapled peptides readily dissolved the phospholipid vesicles, although S1A10 was more effective. The ability of the three peptides and the bi-helical 5A peptide [25] to promote cholesterol efflux was tested on BHK cells stably transfected with either human ABCA1, ABCG1 or the human SR-BI receptor. For non-transfected BHK control cells, none of the peptides were able to promote significant amounts of cholesterol efflux (Fig. 4A), indicating that they cannot remove cholesterol by a nonspecific detergent-like process unlike some other more hydrophobic apolipoprotein mimetic peptides [26]. Consistent with previous reports [27], A10 was ineffective in promoting cholesterol efflux by the ABCA1 transporter (Fig. 4A) or by any other mechanism (Fig. 4B-D). In contrast, after hydrocarbon stapling, S1A10 and S2A10 showed more than a 10-fold increase in cholesterol efflux (Fig. 4B). Furthermore, the stapled peptides showed greater cholesterol efflux, particularly at lower doses, than the much longer 5A bi-helical non-stapled peptide, which was previously designed for specifically effluxing cholesterol by the ABCA1 transporter and was shown to decrease atherosclerosis in mice [23].

Cholesterol efflux can also occur by other mechanisms, involving the ABCG1 transporter and the SR-BI receptor, as well as by passive exchange [28]. Non-ABCA1 cholesterol efflux usually depends on the presence of a cholesterol acceptor that contains phospholipid into which cholesterol can be solubilized [28]. Compared to A10 and 5A, the stapled peptides appeared to have some limited ability to stimulate cholesterol efflux by the ABCG1 transporter and the SR-BI receptor even without reconstitution with phospholipids (Fig. 4C and 4D).

Discussion

The main findings from this study are that hydrocarbon stapling of amphipathic peptides increases their alpha helical structure, reduces their susceptibility to proteolysis and increases their ability to promote cholesterol efflux by the ABCA1 transporter. When in a random coil, the chiral carbon of amino acids are typically more than 5 angstroms apart, but when peptides form alpha helices there are approximately 3.5 residues per turn, with a mean distance of 1.5 angstroms between each chiral carbon [18,24]. By constraining the distance between the amino acids with the hydrocarbon linkers, it promoted helix formation (Fig. 2), which is consistent with several recent reports of other stapled peptides [19]. We are unaware, however, of any previous attempts to specifically stabilize amphipathic peptides with hydrocarbon linkers. Because the hydrocarbon linker is composed of only carbon and hydrogen groups and thus is hydrophobic, we placed the linker in the hydrophobic region of the last helix of apoA-I. The number of carbon hydrogen bonds in the linkers are similar to the number of carbon hydrogen bonds in the side chains of the replaced amino acids, so the hydrophobic moment of the stapled peptides would be expected to be similar to the parent peptide. In the case of S2A10, however, the replacement of Y16, which is only partially hydrophobic, with the hydrocarbon linker may have further increased its hydrophobic moment and helicity.

Although there are several pepsin and chymotrypsin sites outside of the linker region of the stapled peptides (Fig. 1), the whole peptide appeared to be relatively resistant to proteolysis (Fig. 2). This suggests that the hydrocarbon staple may not only prevent proteolysis of the stapled region because of steric hindrance from the linker but also outside of the cross linker region, most likely because helix formation of the peptide prevents its entry into the active site of protease [22]. The alignment of the hydrogen bonds within the linker region most likely promotes overall helix formation by the propagation of hydrogen bonds to residues before and after the linker region. Because pepsin is one of the principal proteases in the stomach and chymotrypsin in the small intestine, the resistance of stapled peptides to proteolysis suggests that they could possibly be orally available. Recently, a therapeutic stapled peptide used for the intravenous treatment of HIV was found to be partially orally available even though it is about twice the size of S1A10 and S2A10 [20]. Like S1A10 and S2A10, the amino and carboxy terminal ends of the HIV therapeutic peptide were blocked with acetyl and amino groups, respectively, which would also prevent these peptides from being degraded by exopeptidases. Stapled peptides could also be co-administered with agents, such as niclosamide, which have been shown to further prevent proteolysis and enhance absorption of amphipathic peptides [29]. The apolipoprotein mimetic peptide, D4F, which is made with D-amino acids, is almost completely resistant to proteolysis and has been used as an oral agent in animal and human studies [4,5]. The hydrocarbon stapled peptides, in this study, do not appear to be as resistant to proteolysis as D4F and are mostly degraded by 24 hours. Based on this result, stapled peptides are, therefore, less likely than D4F to accumulate in tissues with long term use and possibly cause toxicity [4].

When in the lipid-free state, apoA-I is only about 48% helical but when associated with lipids, it is over 67% helical [31]. When lipid-free apoA-I or short amphipathic peptides are dissolved in aqueous solution with a high dielectric constant, water molecules compete with inter-helical hydrogen bonds, thus reducing their ability to form helical structures. It is in this relatively disordered state, however, that apoA-I interacts with the ABCA1 transporter and initiates cholesterol efflux. When apoA-I is bound to HDL and is fully lipidated, it loses its ability to interact with the ABCA1 transporter and instead promotes cholesterol efflux by acting as an acceptor for cholesterol that is released from cells by either ABCG1, SR-BI or by aqueous diffusion [28]. The fact that the stapled peptides showed a marked increase in cholesterol efflux from ABCA1 transfected cells (Fig. 4), indicates that even in the lipid-free

state, the ability of a peptide to form an amphipathic helix enhances cholesterol efflux by the ABCA1 transporter. It may be that amphipathic helices are necessary for the initial interaction of apolipoproteins and their mimetic peptides with the lipid microdomain created by the ABCA1 transporter [28]. This result is also consistent with the findings that structural changes that enhance the helicity of apolipoprotein mimetic peptides usually increase their cholesterol efflux ability [30,31]. This is often accomplished by making peptides longer, such as in the case of the 5A peptide [25], but as shown in this study, it is also possible to accomplish the same goal with relatively short peptides, by using hydrocarbon linkers. There is also evidence that the ABCA1 transporter, along with apoA-I, is internalized by cells and that this event may be important in the cholesterol efflux process [32]. The stapling of peptides with hydrocarbon linkers has been shown to enhance the cellular uptake of small synthetic peptides [18,19], so this may have also contributed to the improvement observed in ABCA1-dependent cholesterol efflux by the stapled peptides. Interestingly, the lipid-free stapled S1A10 and S2A10 peptides were also able to remove a limited amount of cholesterol from cells transfected with the ABCG1 transporter and the SR-BI receptor (Fig. 4). Like other amphipathic peptides, it is likely that the stapled peptides form oligomers when dissolved in aqueous buffers [33]. If so, an oligomer of the stapled peptides containing hydrocarbon linkers in an inner core may possibly serve as a sink for the solubilization of cholesterol, in a manner similar to how the acyl chains of phospholipids on lipidated apolipoproteins can absorb and solubilize cholesterol effluxed by the ABCG1 transporter and the SR-BI receptor or by passive exchange [28].

In summary, the hydrocarbon stapling of apolipoprotein mimetic peptides based on the last helix of apoA-I was found to increase peptide helicity and to promote cholesterol efflux by the ABCA1 transporter. In addition, this modification made the peptides partially resistant to proteolysis. These findings suggest that the hydrocarbon stapling of amphipathic helical peptides may be useful in the development of oral therapeutic peptides for the treatment of atherosclerosis.

Acknowledgments

Research was supported by intramural NHLBI research funds from the National Institutes of Health.

Abbreviations

(apo)	Apolipoprotein
(HDL)	High density lipoproteins
(A10)	Helix 10 of apoA-I
(S1A10)	short stapled A10 peptide
(S2A10)	long stapled A10 peptide
(CD)	circular dichroism

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Figure 1. Primary sequence and biophysical characterization of peptides

Panel A. Helical net plot of A10, S1A10 and S2A10 and position of linkers. X1= (S)-a-(4'-pentenyl)Ala; X2=(R)-a-(7'-octanyl)Ala. **Panel B.** CD-spectroscopy of peptides at 24°C (0.1 mg/mL). **Panel C.** CD-spectroscopy of heat denaturation studies of peptides monitored at a residual ellipticity $[q]$ of 222 nm. (dotted line) A10, (solid line) S1A10, (dashed line) S2A10.

Figure 2. Peptide susceptibility to proteolysis

Peptides (A10 (○), S1A10 (●), S2A10 (□)) at a final concentration of 500 µg/ml were incubated for the indicated times with either pepsin (0.5 µg/ml) in 10% acetic acid buffer (**Panel A**) or with chymotrypsin (0.5 µg/ml) in 10 mM NH₄HCO₃ buffer (**Panel B**) and loss of intact peptide was monitored by MALDI-TOF MS. Results are expressed as normalized area under the curve measurements relative to three internal standards.

Figure 3. DMPC vesicle solubilization by peptides

Peptides A10, S1A10 and S2A10 at a final concentration of 33 $\mu\text{g/mL}$ or phosphate buffer saline (PBS) was incubated with DMPC vesicles (200 $\mu\text{g/mL}$) and monitored for turbidity at 1 min intervals.

Figure 4. Cholesterol efflux by peptides

Peptides [A10($\circ$), S1A10 ($\bullet$), S2A10 ($\square$), 5A($\blacksquare$)] at the indicated concentrations were incubated with BHK control cells (**Panel A**), ABCA-1 transfected BHK cells (**Panel B**), ABCG-1 transfected BHK cells (**Panel C**), and SR-B1 transfected BHK cells (**Panel D**) and monitored for cholesterol efflux. Percent of cholesterol efflux over 18 h is expressed as mean $\pm$ SD of triplicate determinations.