Published in final edited form as:

Pharmacol Ther. 2011 April ; 130(1): 83–91. doi:10.1016/j.pharmthera.2010.12.003.

Apolipoprotein Mimetic Peptides: Mechanisms of Action as Anti-atherogenic Agents

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Abstract

Apolipoprotein mimetic peptides are short synthetic peptides that share structural, as well as biological features of native apolipoproteins. The early positive clinical trials of intravenous preparations of apoA-I, the main protein component of high density lipoproteins (HDL), have stimulated great interest in the use of apolipoprotein mimetic peptides as possible therapeutic agents. Currently, there are a wide variety of apolipoprotein mimetic peptides at various stages of drug development. These peptides typically have been designed to either promote cholesterol efflux or act as anti-oxidants, but they usually exert other biological effects, such as anti-inflammatory and anti-thrombotic effects. Uncertainty about which of these biological properties is the most important for explaining their anti-atherogenic effect is a major unresolved question in the field. Structure-function studies relating the *in vitro* properties of these peptides to their ability to reduce atherosclerosis in animal models may uncover the best rationale for the design of these peptides and may lead to a better understanding of the mechanisms behind the atheroprotective effect of HDL.

Keywords

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

1. Introduction

Apolipoprotein (apo) mimetic peptides are short synthetic peptides that typically contain at least one amphipathic helix (Sethi, Amar et al. 2007; Getz, Wool et al. 2010; Navab, Shechter et al. 2010). They are often based on apoA-I, the most abundant protein on high density lipoproteins (HDL), but can also contain amino acid sequences and structural motifs shared by other apolipoproteins and hence are probably best referred to as apolipoprotein mimetic peptides. Although the first apolipoprotein mimetic peptides were first produced over 25 years ago as probes for understanding the structure of lipoproteins (Fukushima, Yokoyama et al. 1980; Anantharamaiah, Jones et al. 1985), there has been resurgence in the

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interest of these peptides, because of their potential therapeutic value for the treatment of cardiovascular disease.

In this review, we will first describe new research findings on the anti-atherogenic mechanisms of HDL and then the clinical trials involving the infusion of reconstituted HDL for the treatment of acute coronary syndrome. Next, we will review the main anti-atherogenic mechanisms that have been proposed for apolipoprotein mimetic peptides, namely facilitating reverse cholesterol transport and inhibiting inflammation and the oxidation of lipoproteins and how these various effects may be interrelated. Finally, we discuss how structure-function studies may not only provide a better rationale for the design of future apolipoprotein mimetic peptides for the treatment of cardiovascular disease, but may also help unravel which of the many biological properties of HDL may be the most critical for its anti-atherogenic effect.

1. HDL Mechanisms of Action

The first epidemiologic studies showing the inverse relationship between the cholesterol content of HDL (HDL-C) with cardiovascular disease were published over 50 years ago (Barr, Russ et al. 1951), but we still do not have a complete understanding of the biologic basis for this relationship. In various genetic animal models of either low or high HDL, it has been convincingly demonstrated that HDL is anti-atherogenic (Newton and Krause 2002) (Escola-Gil, Calpe-Berdiel et al. 2006), but these findings may not translate to humans because of the known limitations of animal models for atherosclerosis. In epidemiologic studies, low HDL-C is often associated with other cardiovascular risk factors, such as elevated low density lipoproteins (LDL) and obesity, but most studies have shown that HDL-C is an independent negative risk factor (Parolini, Marchesi et al. 2009). Measures of the functional properties of HDL may even correlate closer to the atheroprotective properties of HDL than its content (Movva and Rader 2008; Remaley AT 2008).

Reverse cholesterol transport (RCT) (Fig. 1), which is largely mediated by HDL, is a key pathway for maintaining overall cholesterol balance (Lund-Katz and Phillips 2010). Although the synthesis of cholesterol is exquisitely regulated by a wide variety of mechanisms, RCT appears to play a critical role in the process. For example, cells such as macrophages that uptake significant quantities of extracellular cholesterol by either the phagocytosis of cell membranes or by receptor mediated uptake of oxidized lipoproteins, appear to heavily depend on the RCT pathway for cellular cholesterol homeostasis. HDL initially promotes the RCT pathway by extracting or effluxing excess cellular cholesterol via ATP-binding cassette transporter 1 (ABCA1) and then transporting it to the liver for excretion into bile or reutilization. The ABCA1 transporter is expressed in both the liver and intestine and is responsible for the initial lipidation of apoA-I with phospholipid and cholesterol when it is secreted from hepatocytes or enterocytes (Nofer and Remaley 2005). ABCA1 is also expressed on cholesterol-loaded macrophages, and promotes the efflux of cholesterol to a phospholipid-rich but cholesterol poor species of HDL called pre-beta HDL. When ABCA1 is defective as in Tangier Disease (Nofer and Remaley 2005), there is an accumulation of cholesteryl esters in macrophages throughout the body, such as in the spleen, liver, and lymph nodes. In addition to ABCA1, there are several other proteins, namely ABCG1 and SR-BI that also contribute to cellular cholesterol efflux to more lipid rich forms of HDL (Lund-Katz and Phillips 2010). The relative degrees of importance of all these cholesterol efflux transporters is not known, but ABCA1 is likely to impact in all these processes, including the passive exchange of cholesterol from cells by aqueous diffusion (Lund-Katz and Phillips 2010), because mutations in ABCA1 affects the formation of all the different species of HDL.

After cholesterol is removed from cells by HDL, it is esterified by the enzyme lecithin:cholesterol acyltransferase (LCAT) (Rousset, Vaisman et al. 2009). The esterification of cholesterol on the surface of HDL and to a smaller degree on LDL causes the cholesteryl ester that form to partition into the core of lipoproteins, because of their increased hydrophobicity. This converts the discoidal shaped pre-beta HDL particles to a spherical alpha-migrating form of HDL, which is the most common HDL subfraction found in plasma. The esterification of cholesterol is also believed to drive the net efflux of cholesterol from cells (Czarnecka and Yokoyama 1996; Yokoyama 2000), because it traps cholesterol on lipoproteins and prevents its back exchange onto cells by passive exchange. Some of the cholesteryl esters formed by LCAT are transferred to LDL by the cholesteryl ester transfer protein (CETP), in exchange for triglyceride. Cholesterol is then returned to the liver when LDL is taken up by hepatocytes by the LDL receptor or when HDL interacts with hepatic SR-BI receptors. Interestingly, in several recent studies, the correlation between HDL-C and the cholesterol efflux potential of serum has been found to be relatively poor (Movva and Rader 2008; Remaley AT 2008; Sviridov, Hoang et al. 2008), which suggests that other parameters besides HDL-C, particularly functional assays of HDL may be a better way for assessing the cardiovascular protection by HDL.

In addition to its role in promoting the RCT pathway, HDL has also been shown, at least *in vitro*, to have many other possible beneficial atheroprotective properties (Table 1). Many of these other non-RCT effects of HDL were initially viewed as likely being indirect consequences of the effect of HDL on cells and or possibly related to its ability to remove cholesterol from cells, as will be discussed later. It is important to note, however, that HDL is now known to be more complex than originally envisioned. Besides apoA-I and the other more minor apolipoprotein components of HDL, recent proteomic analysis have revealed over 40 different proteins associated with HDL (Vaisar, Pennathur et al. 2007). These proteins have a wide variety of roles, such as in inflammation, proteolysis and complement activation, thus some of these non-RCT functions of HDL could be mediated by these other proteins. In addition to phospholipids, cholesterol and triglycerides, HDL can also efflux, transport and deliver potent bioactive signaling molecules, such as sphingosine-1-phosphate and oxysterols, which also can have a wide variety of cellular effects (Argraves and Argraves 2007; Terasaka, Wang et al. 2007; Yetukuri, Soderlund et al. 2010).

One of the most thoroughly examined non-RCT functions of HDL is its ability to act as an anti-oxidant. Paraonase is an enzyme transported by HDL and can neutralize oxidized lipids by hydrolysis (Goswami, Tayal et al. 2009). Over expression of paraonase decreases atherosclerosis in mouse models, whereas paraonase knockout mice have an increased propensity to develop atherosclerosis. HDL is also associated with glutathione peroxidase, PAF acetyl hydrolase and LCAT, which are other enzymes that may contribute to the anti-oxidant role of HDL. HDL also transports potent small molecule anti-oxidants, such as vitamin E and carotene. Furthermore, HDL can also sequester oxidized lipids from cells and LDL and can also reduce cell signaling events triggered by the exposure of cells to oxidized lipids (Navab, Imes et al. 1991). The relevance of these findings is supported by studies that have shown that assays that measure the anti-oxidant function of HDL often correlate better with the atheroprotective function of HDL than HDL-C (Navab, Reddy et al. 2009). Besides acting as a direct anti-oxidant and scavenging oxidized lipids, HDL by altering gene expression may also modulate the inflammatory response of cells to oxidized lipids or other pro-inflammatory stimuli (Hsieh, Schnickel et al. 2007; McGrath, Li et al. 2009; Tang, Liu et al. 2009).

2. HDL Infusion Therapy

Interest in developing drugs to raise and or improve HDL function stems from the relatively large residual disease burden that remains even after aggressive treatment with statins and other lipid lowering drugs (Hausenloy and Yellon 2008). Unfortunately, there has been limited success in developing such agents. Niacin is the only approved drug that substantially raises HDL-C, and there are several large ongoing clinical trials to definitively assess whether raising HDL with niacin can lower the incidence of cardiovascular events (Al-Mohaisen, Pun et al. 2010). Niacin, however, is under utilized, in large part, because of side effects related to flushing and from liver toxicity with earlier slow-release formulations (Guyton and Bays 2007).

Recently, a new HDL raising strategy, involving the intravenous infusion of reconstituted HDL in patients with acute coronary syndrome, has been described (Shah 2007; Remaley, Amar et al. 2008; Meyer, Nigam et al. 2009). The rationale for this type of treatment is that it has been shown in animal studies that even a single infusion of HDL can have significant benefits in reducing lipid content and reducing inflammation (Newton and Krause 2002). This rapid effect of HDL has also been demonstrated in human atherosclerotic plaques (Shaw, Bobik et al. 2008), which suggest that HDL treatment could be useful for rapidly stabilizing patients with acute coronary syndrome and thus reducing their high risk for a future myocardial infarction. Patients treated with HDL infusion therapy would likely be concurrently treated with statins and or other lipid lowering drugs, but it has been shown that statins may take as long as several years before they have beneficial effect on plaque size (Nissen 2005). Two human clinical trials, involving Intravascular Ultrasound assessment of plaques after HDL infusion therapy, have been performed. The first was a study of recombinant ApoA-I_{Milano} reconstituted with phospholipids, which was given to acute coronary syndrome patients once a week for 5 weeks (Nissen, Tsunoda et al. 2003). ApoA-I_{Milano}, a natural mutant of apoA-I, was used because the Cysteine substitution in ApoA-I_{Milano} has been shown to improve its anti-oxidant function (Chiesa and Sirtori 2003). In this study, plaque volume was reduced by approximately 4% after the 5-week treatment. Another clinical trial, in which reconstituted HDL was made from apoA-I purified from plasma, showed similar results in decreasing plaque size, although it did not reach statistical significance (Tardif, Gregoire et al. 2007). Although the changes in plaque size observed in these studies are relatively small, they are comparable to the reduction in plaque size seen with statin treatment after several years, (Nissen 2005). Based on animal studies and a human study involving the examination of plaque from peripheral vascular disease after a single HDL treatment (Shaw, Bobik et al. 2008), a short course of HDL treatment appears to reduce lipid infiltration and inflammation in plaques, which may stabilize it from acute rupture. It is also important to note that although several years of statin treatment are needed to reduce plaque size, there is evidence of reduced cardiovascular events after only 6 months of treatment (Nissen 2005), which suggests that the acute treatment of patients with acute coronary syndrome with apolipoprotein mimetic peptides may also be beneficial in reducing clinical events.

In addition to its possible use in acute coronary syndrome, HDL has also been shown, at least in animal models, to possibly have benefit in many other disease processes, such as injury from stroke (Lapergue, Moreno et al. 2010), myocardial infarction (Gomaraschi, Calabresi et al. 2008), renal ischemia (Shi and Wu 2008) pulmonary fibrosis (Kim, Lee et al. 2010) asthma (Yao, Fredriksson et al. 2010), sepsis (Shor, Wainstein et al. 2008) and peripheral vascular disease (Shaw, Bobik et al. 2008). In many of these studies, the benefit can be demonstrated within hours of the HDL infusion, which makes it more likely that the effect of HDL observed in these other disease processes is more likely related to the non-RCT functions of HDL, such as anti-oxidation and or anti-inflammation.

A major practical limitation of HDL Infusion therapy is the relatively large quantity of HDL that is needed for the treatment. In the two major clinical trials of HDL infusion therapy, the maximum doses ranged from 45 to 80 mg/kg, thus several grams of either recombinant or purified apoA-I are needed per treatment. This amount of protein may be cost prohibitive unless simpler and less expensive means are identified for its production. In addition, contamination of the recombinant apoA-I with endotoxins is a major concern due to the strong affinity of apoA-I for lipids and the contamination transmissible viruses is a concern for apoA-I purified from donated plasma. Both apoA-I preparations in the two human trials were also complexed with phospholipid, which makes the formulation of these drugs even more difficult and expensive. Although some of these issues may be resolved in the long-term, several research and pharmaceutical groups have developed short amphipathic peptide as possible alternatives to apoA-I for HDL therapy (Table 2). Besides being potentially easier and less costly to produce, it may be possible to design these peptides with different structural features than apoA-I to further improve their anti-atherogenic properties. It may be also possible to develop oral (Navab, Anantharamaiah et al. 2002) or subcutaneously available forms of these peptides, which may make it possible to use such agents for chronic therapy. Finally, these apolipoprotein mimetic peptides also have great scientific interest, as will be discussed later, because of their potential to lead to a better understanding of the various function of HDL.

3. Cholesterol Efflux by Apo Mimetic Peptides

The first apolipoprotein mimetic peptides that were shown to mediate cholesterol efflux from cells were based on either the natural helices of apoA-I or a peptide called 18A (Mendez, Anantharamaiah et al. 1994; Yancey, Bielicki et al. 1995) (Table 1), which has no significant primary amino acid homology to apoA-I but forms an amphipathic α -helix. The ends of short single helical peptides like 18A are often blocked by acetylation on the amino-terminal end and by amidation on the carboxy terminal end, which protects these peptides against proteolysis and stabilizes helix formation by enabling additional hydrogen bonding between (Mishra, Anantharamaiah et al. 2006; Mishra, Palgunachari et al. 2008). Using peptides that contain a mixture of D and L amino acids, which interferes with helix formation, it was shown that the presence of an amphipathic helix is necessary for these peptides to interact with the ABCA1 transporter and to remove phospholipid and cholesterol from cells (Remaley, Thomas et al. 2003; Van Lenten, Wagner et al. 2009).

The most common type of amphipathic helix found on apoA-I and many other apolipoproteins are type A helices (Segrest, Jones et al. 1992). A diagram of a type A helix based on the 18A peptide is shown in Fig. 2. Type A helices are characterized by having the surface area of the hydrophobic face approximately equal to the size of the hydrophilic face (Segrest, Jones et al. 1992; Labeur, Lins et al. 1997). The polarized distribution of the different amino acids between the hydrophilic and hydrophobic face is often quantified by a parameter called hydrophobic moment, which provide a convenient but incomplete measure of the lipid affinity of a helix (Phoenix and Harris 2002). At the junction between the two faces of the helix are often positive charged amino acids, such as lysine, which stabilizes the binding of the peptides with phospholipids by forming an ionic bond with the negative charge on the phospholipid head group (Khoo, Miller et al. 1990). The central region of the hydrophilic face of these helices typically contains negatively charged residues, such as glutamic or aspartic acid. Alignment of negative charges along the center of the hydrophilic face between 2 helices has been shown to be an important feature in the ability of these peptides to efflux cholesterol (Natarajan, Forte et al. 2004). The hydrophobic face of 18A contains 2 phenylalanine residues, which is one of the most hydrophobic amino acids and is the reason why this peptide is also sometimes called 2F, typically when the ends of the peptide are chemically blocked.

One of the first peptides specifically designed to efflux cholesterol by the ABCA1 transporter was the 5A peptide (Sethi, Stonik et al. 2008) (Table 2). It has two helices, which are joined together with proline, an amino acid that is commonly found between the helices of apolipoproteins and causes a beta-turn between helices (von Eckardstein, Funke et al. 1989). The first helix is identical to 18A peptide helix and the second helix is a modified 18A peptide in which 5 hydrophobic residues were changed to alanine and hence its name 5A (Sethi, Stonik et al. 2008). Alanine is only slightly hydrophobic; therefore, the 5 amino acid substitutions decrease the hydrophobic moment of the second helix and hence its lipid affinity. These changes were made to the second helix in order to better mimic the arrangement of the amphipathic helices on apoA-I. Only the first and last helices of apoA-I have relatively high hydrophobic moments and lipid affinity (Lund-Katz, Liu et al. 2003). Lipid-free apoA-I forms a relatively tight helical bundle, with the hydrophobic faces of the different helices approximating each other except for the last helix, which is more solvent exposed (Lund-Katz, Liu et al. 2003). It is believed that the last helix is responsible for the initial interaction of apoA-I with membranes and that the central helices with less lipid affinity work in a cooperative manner, with the first and last helix, to remove lipid from a lipid microdomain created by ABCA1 by a detergent-like extraction process (Lund-Katz and Phillips 2010). Previously, it was found that two 18A helices linked by proline called 37pA removes both phospholipid and cholesterol from cells in an ABCA1-independent process (Remaley, Thomas et al. 2003). In other words, it was also able to remove lipid from membranes besides the lipid microdomains created by ABCA1, because of its high affinity for lipid and its ability to act as a detergent. Removal of lipid from cells by this non-ABCA1 dependent pathway was found to be cytotoxic to cells (Remaley, Thomas et al. 2003). Reducing the lipid affinity of the second helix, as in the 5A peptide, better mimicked the structure of apoA-I by pairing a high lipid affinity helix, 18A, with a lower affinity helix, which, like apoA-I, resulted in a peptide that was non-cytotoxic and specific for removing cholesterol from cells by only the ABCA1 transporter (Sethi, Stonik et al. 2008).

The 5A peptide can readily complex with phospholipids and forms a HDL-like structure (Amar, D'Souza et al. 2010). Interestingly, the complex of 5A with phosphatidyl choline had nearly the same capacity as the free 5A peptide for effluxing cholesterol by the ABCA1 transporter, although it had a higher K_m . This may occur because the 5A-phospholipid complex resembles pre-beta HDL, which has a similar ratio of protein to phospholipid and may like pre-beta HDL interact with the ABCA1 transporter. Similar to apoA-I, the addition of phospholipid to 5A stabilized the peptide against degradation when injected into mice. The 5A-phospholipid complex, unlike the free 5A peptide, was also found to be competent for removing cholesterol by the ABCG1 transporter (Amar, D'Souza et al. 2010). The 5A-phospholipid complex, when injected into mice raised HDL-C and increased the capacity of serum to promote cholesterol efflux. Furthermore, it was found that 5A-phospholipid complex increased the RCT pathway by increasing the flux of cholesterol from peripheral tissues into the plasma compartment, but no net increase in fecal sterols excretion was observed after a single dose. The 5A-phospholipid complex, however, was found to mobilize ^3H -cholesterol from radio labeled resident peritoneal macrophages and to increase fecal sterol excretion of the ^3H -cholesterol tracer. Intravenous administration of the 5A-phospholipid complex (30 mg/kg three times a week) for 8 weeks reduced atherosclerosis by approximately 30–50% in apoE null mice (Amar, D'Souza et al. 2010). These results are consistent with the cholesterol efflux ability of 5A for being responsible for its observed atheroprotection, but 5A in a rabbit collar model was also found to have potent anti-inflammatory properties (Tabet, Remaley et al. 2010). In this study, 5A, like apoA-I, reduced the expression of adhesion proteins on endothelial cells after external collar placement on the carotid and reduced the vascular infiltration of inflammatory cells and the production of reactive oxygen species (Tabet, Remaley et al. 2010). Interestingly, the ability of 5A to reduce reactive oxygen species (ROS) was found to be dependent upon the

expression of ABCA1. Peptides for increasing other facets of the RCT pathway, such as activating LCAT (ETC-642) (Sethi, Amar et al. 2007) and stimulating hepatic uptake of lipoproteins (E-2F) (Gupta H 2005), have also been described (Table 2), but there is no reports yet on their effect on atherosclerosis.

In addition to apoA-I, apolipoprotein mimetic peptides based on other apolipoproteins and proteins, such as apoE, apoJ, and serum amyloid A, have also been described (Sethi, Amar et al. 2007). ATI-5261, an apoE based peptide, has recently been shown to significantly reduce atherosclerosis in both LDLR-receptor null and Apo-E null mice when the free peptide (30 mg/kg) was injected interperitoneally (Bielicki, Zhang et al. 2010). This relatively small 26 amino acid long single helical peptide is based on the central region of the carboxy terminal helix of apoE. Several amino acid substitutions were made at various positions of the helix in order to increase its α -helicity and to align the negatively charged residues along its polar face, which has been shown to facilitate cholesterol efflux of apolipoprotein mimetic peptides (Natarajan, Forte et al. 2004; Bielicki, Zhang et al. 2010). Like 5A, ATI-5261 enhanced cholesterol efflux by the ABCA1 transporter, indicating that it is not necessary to have a bihelical peptide to get ABCA1-specific cholesterol efflux. Another apoE based peptide (E2-F), containing the LDL-receptor binding domain of apoE, which is rich in basic residues, linked to the 18A helix with a proline (Table 2) has also been described (Gupta H 2005). This peptide primarily binds to apoB-containing lipoproteins and was found to enhance the last step in RCT, the hepatic uptake of lipoprotein bound cholesterol. A similar apoE based peptide that just contains the LDL receptor binding motif of apoE (Table 2) was recently found to reduce cytokine production and inflammatory cell infiltration in a house dust mite model of murine asthma (Yao, Fredriksson et al. 2010), indicating that these peptides also have anti-inflammatory effects. Finally, because of the link between apoE isoforms and Alzheimer's disease, apoE based peptides for the treatment of Alzheimer's disease have also been developed and have shown positive effects in animal models (Minami, Cordova et al. 2010).

4. Non-RCT Functions of Apolipoprotein Mimetic Peptides

According to the response-to-retention theory of atherosclerosis (Tabas, Williams et al. 2007), the sub endothelial retention of LDL is a key initiating step in atherogenesis. The premise is that the retention of apoB-containing lipoproteins incites local biological responses that lead to a chronic and maladaptive macrophage and T cell inflammatory response, which promotes plaque formation. It has also been shown that oxidative modification of LDL enhances its uptake by the macrophages in the sub endothelial space and that HDL protects against oxidative LDL modification (Khoo, Miller et al. 1990). Besides promoting RCT, apolipoprotein mimetic peptides, such as 4F (Table 2), have also been investigated for their ability to prevent oxidation of LDL and or prevent one of its consequences, such as inflammation.

The 4F peptide, the most widely studied apolipoprotein mimetic peptide (Navab, Shechter et al. 2010), is commonly referred to as D4F when made with D-amino acids and L4F when made with the L-amino acids. It appears to be able to block several steps in the oxidative damage of lipoproteins and in the response of cells to oxidative stress. The 4F peptide is based on the 18A peptide, but unlike the 5A peptide in which alanine, a less hydrophobic amino acid was substituted in the hydrophobic face, and two phenylalanine (F) substitutions were made in the hydrophobic face of 4F. Because of its large non-polar ring, phenylalanine is one of the most hydrophobic amino acids, and with the 2 substitutions the 4F peptide has a total of 4 phenylalanine residues, and hence its name. Because it contains D-amino acids, D4F was found to be resistant to proteolysis and to be orally available (Navab, Anantharamaiah et al. 2002), although relatively low levels plasma levels were achieved

with oral administration (Navab, Anantharamaiah et al. 2005; Bloedon, Dunbar et al. 2008) when compared to intravenous or interperitoneal injection of the 5A (Amar, D'Souza et al. 2010) or ATI-5261 (Gupta H 2005) peptides.

The 4F peptide mimics several different aspects of HDL's biological properties and has been tested in several different animal models of diseases related to oxidation and inflammation and was the first apolipoprotein mimetic peptide shown to reduce atherosclerosis in an animal model (Navab, Anantharamaiah et al. 2002). The oral administration of D4F to LDL-receptor null mice on a Western diet markedly reduced aortic lesions, without affecting plasma or HDL cholesterol levels (Navab, Anantharamaiah et al. 2002). When given to Apo-E null mice, there was a marked reduction in lipid hydroperoxides, increased HDL anti-oxidant capacity and decreased atherosclerosis (Navab, Anantharamaiah et al. 2004). It also increased pre- β HDL, which was enriched in paraoxonase activity, and enhanced RCT from macrophages. When D4F and pravastatin were used in combination, in a mouse model of atherosclerosis, a dramatic increase in their effectiveness was observed (Navab, Anantharamaiah et al. 2005; Navab, Anantharamaiah et al. 2005). They were both administered at concentrations, in which they were relatively ineffective in reducing atherosclerosis, but when used together significant synergy was observed, leading to an increase in HDL-C, paraoxonase activity, and decreased lesion formation, and also plaque regression.

D4F has also been tested in several different animal models for diseases seemingly unrelated to atherosclerosis, but they all apparently share several common pathogenic pathways that D4F can favorably impact. In a mouse model of cognitive impairment, induced by feeding LDL-receptor null mice a Western diet, D4F in drinking water markedly improved cognitive function (Buga, Frank et al. 2006). D4F treatment also significantly reduced the amount of neuronal cells expressing MCP-1 and MIP-1 α , which are thought to interrupt synaptic transmission and cause cognitive decline. D4F was also investigated in an organ rejection model (Hsieh, Schnickel et al. 2007), because it was found to stimulate heme oxygenase-1 (HO-1) gene expression, an extracellular superoxide dismutase, which protects endothelial cells from oxidative stress. Daily interperitoneal injections of D4F were given to C57BL/6 mice after hearts transplantation for 24 days (Hsieh, Schnickel et al. 2007). It was found that donor hearts that received D4F treatment expressed higher levels of HO-1 and had less intimal lesions compared to a saline treated control group. In an animal model of scleroderma, due to mutations in fibrillin-1, D4F treatment improved flow mediated vasodilation by endothelial nitric oxide synthase (eNOS) (Weihrauch, Xu et al. 2007).

The D4F peptide is also the only apolipoprotein mimetic peptide reported so far to undergo testing in human subjects (Bloedon, Dunbar et al. 2008). In a Phase I study of 50 patients with coronary heart disease or its risk equivalent, subjects were given different oral doses of D4F, with doses ranging between 30 to 500 mg/day (Bloedon, Dunbar et al. 2008). No apparent toxicity or side effects from the treatment were observed. Pharmacokinetic analysis showed that absorption of D4F was relatively fast, yielding a relatively low (approximately 5 ng/mL for the 500 mg/day dose) but dose-dependent plasma concentration. Although there was no effect on HDL-C or apoA-I levels from the D4F treatment, there was a significant drop in the HDL inflammatory index, which was determined with an *in vitro* assay that measured the ability of isolated HDL to prevent LDL oxidation (Navab, Anantharamaiah et al. 2002). Although no apparent toxicity was observed from this trial, there has been concern about whether long term use of D4F could result in the tissue accumulation of the peptide, because of its resistance to proteolysis. A Phase I clinical trial with a subcutaneous and intravenous preparation of L4F, which presumably would not have this potential problem, is now underway. It may also be possible to deliver L4F and other apolipoprotein mimetic peptides, if they are combined with niclosamide, an anti-helminthic drug used to treat

tapeworms, which was found to make L4F resistant to proteolysis during absorption (Navab, Ruchala et al. 2009).

D4F and its stereoisomer L4F, like other apolipoprotein mimetic peptides, most likely affect multiple pathways in cells, including RCT. D4F, however, compared to 5A or ATI-5261 does not appear to be as effective in promoting cholesterol efflux, at least specifically by the ABCA1 transporter. In a study comparing, single helical or bihelical peptides, it was found that *in vitro* the single helical L4F peptide did not readily bind and remodel HDL or promote cholesterol efflux as well as a bihelical version of the same peptide linked with proline, although the single helical L4F peptide may be better as an anti-oxidant (Getz, Wool et al. 2010) (Wool, Reardon et al. 2008). The ability of D4F and L4F to generate pre-beta HDL *in vivo*, however, may explain its ability to enhance RCT in animal models (Navab, Anantharamaiah et al. 2004). A relatively unique structural feature of D4F and L4F, which appears to account for much but probably not all of its properties, is its high lipid binding affinity. It was shown by varying the number of phenylalanine substitutions in the hydrophobic face, that the 4F peptide with 4 phenylalanine residues showed higher lipid affinity than those peptides with less phenylalanine residues (Datta, Chaddha et al. 2001; Getz, Wool et al. 2009; Van Lenten, Wagner et al. 2009). When it was compared to apoA-I by surface plasma resonance (Van Lenten, Wagner et al. 2008), D4F showed several fold higher affinity for oxidized lipids. Because of the role of oxidized lipids in triggering inflammation in cells, it has been proposed that this accounts for many of the anti-atherogenic functions of 4F (Navab, Shechter et al. 2010). Other anti-oxidant peptides have also been designed, such as those that contain free sulfhydryl groups and or histidines, which chelate heavy metals that promote oxidation (Table 2), (Nguyen, Jeong et al. 2006), but they have not yet been investigated in animal models.

5. Apolipoprotein Mimetic Peptides as Research Tools

In addition to their potential as therapeutic agents, apolipoprotein mimetic peptides can be used as a research tool for investigating fundamental questions related to the mechanisms of the anti-atherogenic activity of HDL. As described in Table 1, HDL has a long and growing list of possible anti-atherogenic activities. It is unknown, however, if there is a common pathway and or a shared component in the different anti-atherogenic activities of HDL. Our knowledge also remains fragmentary on whether the different anti-atherogenic functions of HDL are mechanistically independent from each other, or if one of its properties, such as cholesterol efflux, is causally linked to its other biological properties. One possible approach for solving this question is to create apolipoprotein mimetic peptides that mimic one but not the other activities of HDL.

At this time, the most plausible common mechanism behind the various anti-atherogenic activities of HDL is based on its ability to reduce the cholesterol content of a cell or a specific compartment of a cell. For example, Yvan-Charvet et al (Yvan-Charvet, Welch et al. 2008) demonstrated that reducing cholesterol efflux by knocking out ABCA1 and ABCG1 genes results in an increased expression of a number of pro-inflammatory factors in macrophages. These findings were reproduced by reducing or raising cellular cholesterol content, with cyclodextrin or cyclodextrin-cholesterol complex, respectively, thus ruling out the non-RCT effects of HDL in this process. Similar findings were also described for the anti-oxidant and anti-apoptotic function of HDL (Yvan-Charvet, Pagler et al. 2010) and for the role of HDL in suppressing proliferation of myeloid progenitor cells (Yvan-Charvet, Pagler et al. 2010). We have recently demonstrated that the ability of apoA-I and HDL to reduce the expression of a major monocyte adhesion molecule, CD11b, is also related to a reduction of cholesterol content of cells and to the abundance of lipid rafts, a plasma membrane domain where many cell signaling receptors are located and are known to be

affected by its cholesterol content (Murphy, Woollard et al. 2008). Similarly, the anti-platelet function of HDL was also shown to be related to its capacity to promote cholesterol efflux and to reduce abundance of lipid rafts (Calkin, Drew et al. 2009). Overall, these findings are consistent with several recent reports showing that cholesterol content regulates many functions of macrophages and other cell types (Koseki, Hirano et al. 2007; Zhu, Lee et al. 2008) and suggest that the reduction of cell cholesterol content and or disruption of lipid rafts may be mechanistically connected to many of the non- RCT functions of HDL.

We have recently tested this hypothesis by using a library of apolipoprotein mimetic peptides with wide variety of structures, which were tested *in vitro* for not only specific and non-specific cellular cholesterol efflux but also for various anti-oxidant and anti-inflammatory activities (D'Souza, Stonik et al. 2010). If the common shared pathway hypothesis of HDL is correct, we expected that the peptides most effective in cholesterol efflux assay would be also most effective in the other anti-atherogenic activities and that there would be a relatively close correlation between the different biological properties of the peptides. Although a few peptides showed overall good activity for all the different *in vitro* assays, the peptides with the highest capacity to support both specific and non-specific specific cholesterol efflux were only modestly active in the anti-inflammatory and anti-oxidation assays. Conversely, some of the peptides with the highest anti-inflammatory and anti-oxidant capacities had only modest capacity for cholesterol efflux. Analysis of structure-function relationships of the peptides suggested that the structural features favoring one anti-atherogenic function of HDL are either neutral or sometimes detrimental for another; thus, there was a limited correlation between the different *in vitro* activities of the peptides. In addition, it was found that the dose response relationship significantly differed for the different *in vitro* assays. Cholesterol efflux from cells reached saturation at relatively low concentrations of the peptides, while the anti-inflammatory effect of the peptides required almost a 40-fold higher concentration for maximum effect (D'Souza, Stonik et al. 2010). These findings strongly suggest that the relationships between different anti-atherogenic functions of HDL, tested at least in this study, may be more complex than just the removal of cholesterol and that the underlying mechanisms for these functions may also be different. This could offer a "biological rationale" of apoA-I being composed of a series of amphipathic helices with different lipid affinities and structures, which may be necessary for its multiple roles. Recent findings related to the role of ABCA1 in suppressing inflammation through activation of the transcription factor STAT3, which appear to act independent of cholesterol efflux, may also help explain how the cholesterol efflux capacity of peptides may be related but not closely linked to its other non-RCT properties (Tang, Liu et al. 2009).

The possibility of "dissociating" the different anti-atherogenic functions of HDL with synthetic apolipoprotein mimetic peptides also presents another interesting opportunity. Creating synthetic peptides that *in vitro* primarily display only cholesterol efflux capacity or only anti-inflammatory or anti-oxidant activity, and then testing these peptides in animal models of atherosclerosis may show which activity and or their combination is the most critical for preventing atherosclerosis. Such information would enable a rationale drug design of apo mimetic peptides, as well as perhaps other forms of anti-atherosclerosis therapy, such as chronic versus acute treatments for early prevention of disease versus advanced atherosclerosis.

Even without creating peptides specific for one function, apolipoprotein mimetic peptides will likely in other ways lead to a better understanding of atherogenesis. For example, the 5A peptide was both atheroprotective and elevated HDL-C level (Amar, D'Souza et al. 2010), whereas 4F was capable of reducing the development of atherosclerosis in diabetic (Morgantini, Imaizumi et al. 2010) and LDL-receptor null mice without significantly

increasing HDL-C. This suggests that the athero-protective effect of 4F may be relatively independent of cholesterol efflux. In support of this model, the prevention of platelet aggregation by 4F was recently linked to the specific removal of oxidized lipids rather than decreasing of platelet cholesterol content (Buga, Navab et al. 2010). Apolipoprotein mimetic peptides have also been used as probes for investigating the organization of cell membranes (Epand, Epand et al. 2004; Handattu, Garber et al. 2007), as well as for investigating the molecular events related to cholesterol efflux (Tang, Vaughan et al. 2006). Apolipoprotein mimetic peptides have also been used to investigate another important question related to the mechanisms of atheroprotection: whether HDL prevents newly developing atherosclerosis or is capable of reducing already established atherosclerotic plaque. Using this approach, it has been shown in a mouse venous graft model that 4F was capable of preventing plaque formation but not for reducing existing atherosclerosis (Li, Chyu et al. 2004).

Finally, the use of apolipoprotein mimetic peptides as research tools is not limited to atherosclerosis. The anti-inflammatory properties of apolipoprotein mimetic peptides were used to investigate aspects of inflammation that were unrelated to atherosclerosis, such as infection induced inflammation (Van Lenten, Wagner et al. 2004; Van Lenten, Wagner et al. 2009). Apolipoprotein mimetic peptides have also been used as probes for understanding insulin resistance (Peterson, Kim et al. 2009), vascular reactivity (Kruger, Peterson et al. 2005), septic shock (Dai, Datta et al. 2010), kidney failure (Vaziri, Bai et al. 2010), transplant rejection (Hsieh, Schnickel et al. 2007) and even as carriers for MRI contrast agents (Cormode, Briley-Saebo et al. 2008).

6. Summary and Conclusions

Apolipoprotein mimetic peptides are promising new agents for the treatment of cardiovascular disease. Their effectiveness in animal models of atherosclerosis, as well as in several other diseases, suggests that they may work through several different mechanisms, but the best understood ones are related to their ability to promote cholesterol efflux from cells and to act as anti-oxidants. Structure-function studies of apolipoprotein mimetic peptides will likely lead to a better understanding on how to design such peptides for maximum efficacy. In addition, research on apolipoprotein mimetic peptide may also help us in our decades-long quest to unravel all the complex anti-atherogenic properties of HDL. Finally, Phase I trials on these peptides have only just begun, in the past year, and considerable more work still needs to be done in developing apolipoprotein mimetic peptides into a therapy for cardiovascular disease.

Abbreviations

apo	Apolipoprotein
HDL	High density lipoproteins
LDL	Low density lipoprotein
LCAT	Lecithin: cholesterol acyltransferase
CETP	Cholesteryl ester transfer protein
RCT	Reverse cholesterol transport

Acknowledgments

Research by David O. Osei-Hwedieh, Marcelo Amar, and Alan T. Remaley are supported by intramural funds from the National Heart, Lung and Blood Institute of the NIH.

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Figure 1. Diagram of RCT pathway

The pathway begins with the biogenesis of HDL, which occurs in the liver and to a lesser degree in the intestine when lipid is added to apoA-I by the ABCA1 transporter to form pre-beta HDL. Pre-beta HDL can acquire additional cholesterol and phospholipid from peripheral cells by ABCA1, as well as by the ABCG1 transporter. Cholesterol effluxed from cells is esterified by LCAT and is transferred to LDL by CETP. Cholesterol is delivered back to the liver either by the SR-BI receptor or the LDL-receptor.

Figure 2. Diagram of structure of 18A peptide

(A) Helical Wheel plot of 18A peptide. Position of amino acid residues are shown in a circular orientation, as if the helix was viewed from one end. (B) Helical Net plot of 18A peptide. Position of amino acid residues are shown for a longitudinal view of the helix where the polar face was divided into two outer sections, with the central region illustrating the hydrophobic face. Hydrophobic residues are shown in bold circles.

Table 1**Non-RCT Functions of HDL**

Function	Key Findings	References
Anti-oxidant	Prevents LDL oxidation in cell-free systems and in the artery wall	(Navab, Ananthramaiah et al. 2004)
Anti-inflammatory	HDL inhibited LDL-induced monocyte chemotactic activity (MCA) in human artery wall	(Navab, Imes et al. 1991)
Anti-thrombotic	Promotes the production of the atheroprotective signaling molecule nitric oxide (NO)	(Mineo, Deguchi et al. 2006)
Enhanced NO production for enhanced Endothelial Integrity	Up regulates endothelial NO synthase (eNOS) expression	(Mineo, Deguchi et al. 2006)
Reduces risk of diabetic cardiomyopathy	Lowered activated stress-induced phosphorylated p38 mitogen-activated protein kinase (MAPK) Increased antioxidant enzyme SOD	(Van Linthout, Spillmann et al. 2008)
Oxysterol Efflux	HDL promotes oxysterol efflux from cells	(Terasaka, Wang et al. 2007)
Anti-apoptosis	Protects against oxLDL induced apoptosis in an ABCG1 dependent manner	(Terasaka, Wang et al. 2007)
Anti-platelet Activity	Inhibited platelet activity induced by collagen	(Lerch, Spycher et al. 1998)
Increases Fibrinolysis	Enhances fibrinolysis by inhibiting plasminogen activator inhibitor-I (PAI-I) and promoting tissue plasminogen activator (t-PA)	(Mineo, Deguchi et al. 2006) (Norata, Banfi et al. 2004)
Down regulation of Adhesion molecule Activity	Significantly reduced vascular cell adhesion molecule-1 (VCAM-1) promoter activity	(McGrath, Li et al. 2009)

Table 2**Apo Mimetic Peptides: Key Findings and Development**

Peptide	Primary Sequence	Key Findings	Development phase	References
2F (18A)	Ac-DWLKAFYDKVAEKLKEAF-NH ₂	Failed to inhibit diet-induced atherosclerosis in mice	Under research	Wool et al, 2008 (Wool, Reardon et al. 2008)
4F	Ac-DWFKAFYDKVAEKFEAF-NH ₂	Orally available and reduced atherosclerotic lesions in LDL receptor and apoE null mice.	Phase I trial Novartis	Navabet al, 2009 (Navab, Ruchala et al. 2009)
5A	DWLKAFYDKVAEKLKEAF-P-DWAKAAYDKAAEKAKEAA	Promoted cholesterol efflux by the ABCA1, ABCG1 transporters and athero protective in apoE null mice	Preclinical KineMed Inc.	Amar et al, 2010 (Amar, D'Souza et al. 2010)
ETC-642	PVLDLFRELLNELLEALKQKLK	Activates LCAT	Phase I trial Pfizer	Dasseux, 2007 (Dassuex J-L 2007)
ATI-5261	Ac-EVRSKLEEWFAAFREFAEEFLARLKSNH ₂	Promoted ABCA1 dependent cholesterol efflux and atheroprotective in LDL receptor and apoE null mice	Preclinical Roche	Bielicki, 2010 (Bielicki, Zhang et al. 2010)
apoE(130–149)	Ac-TEELRVRLASHLRKLRKRL- NH ₂	Inhibited eosinophilic airway inflammation, IgE production and cytokine release, improved pulmonary function	Under Research	Yao et al, 2010 (Yao, Fredriksson et al. 2010)
E-2F	Ac-LRKLRKRLR-P-DWLKAFYDKVAEKLKEAF-NH ₂	Promotes hepatic uptake VLDL and LDL	Under research	Gupta et al, 2005 (Gupta H 2005)