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Vasculoprotective Effects of Apolipoprotein Mimetic Peptides: An Evolving Paradigm In Hdl Therapy (*Vascular Disease Prevention*, In Press.)

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Abstract

Anti-atherogenic effects of high density lipoprotein (HDL) and its major protein component apolipoprotein A-I (apoA-I) are principally thought to be due to their ability to mediate reverse cholesterol transport. These agents also possess anti-oxidant properties that prevent the oxidative modification of low density lipoprotein (LDL) and anti-inflammatory properties that include inhibition of endothelial cell adhesion molecule expression. Results of the Framingham study revealed that a reduction in HDL levels is an independent risk factor for coronary artery disease (CAD). Accordingly, there has been considerable interest in developing new therapies that specifically elevate HDL cholesterol. However, recent evidence suggests that increasing circulating HDL cholesterol levels alone is not sufficient as a mode of HDL therapy. Rather, therapeutic approaches that increase the functional properties of HDL may be superior to simply raising the levels of HDL *per se*. Our laboratory has pioneered the development of synthetic, apolipoprotein mimetic peptides which are structurally and functionally similar to apoA-I but possess unique structural homology to the lipid-associating domains of apoA-I. The apoA-I mimetic peptide 4F inhibits atherogenic lesion formation in murine models of atherosclerosis. This effect is related to the ability of 4F to induce the formation of pre-β HDL particles that are enriched in apoA-I and paraoxonase. 4F also possesses anti-inflammatory and anti-oxidant properties that are independent of its effect on HDL quality *per se*. Recent studies suggest that 4F stimulates the expression of the antioxidant enzymes heme oxygenase and superoxide dismutase and inhibits superoxide anion formation in blood vessels of diabetic, hypercholesterolemic and sickle cell disease mice. The goal of this review is to discuss HDL-dependent and -independent mechanisms by which apoA-I mimetic peptides reduce vascular injury in experimental animal models.

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Introduction

Since its inception over 50 years ago, the Framingham Heart Study has provided extensive insight into mechanisms underlying the development and progression of coronary artery disease (CAD) as well as associated risk factors. Early trial results clearly demonstrated a relationship between elevated serum cholesterol and CAD risk and cardiovascular (CV) death [1]. This relationship has been validated by clinical studies showing that HMG-CoA reductase inhibitors (statins) reduce CAD risk by approximately 25-30% [2-3]. The Framingham Study also provided insight into the relationship between HDL cholesterol levels and CAD risk [1]. Study participants with a "normal" LDL (100 mg/dl) and a high HDL (65-85 mg/dl) were found to be at low risk for a CV event [4]. In subjects with normal LDL, but reduced HDL, CAD risk significantly increased. Low serum HDL was thus identified as an independent risk factor for CAD [4]. This observation has fueled interest in the development of new therapies that specifically elevate HDL cholesterol [5].

Several commonly prescribed medications influence serum HDL levels by stimulating hepatic apolipoprotein (apo) A-I expression. Fibrates are agonists for peroxisome proliferator activated receptor-α. Lipid lowering effects of fibrates are due to their ability to stimulate expression of not only apoA-I, but also apoA-II and lipoprotein lipase, a mediator of cellular triglyceride uptake [6-8]. Niacin is an effective agent for raising HDL, resulting in a 20-35% increase in circulating levels of this lipoprotein particle [9]. Statins are used extensively in hyperlipidemic patients to lower LDL cholesterol. This class of drug is reported to increase HDL levels by 3-12% [10-11]. Similar to fibrates and niacin, statins exert a stimulatory effect on apoA-I synthesis, leading to an increase in HDL particle number [12]. Whether this modest increase in HDL in patients taking statins translates into a reduction in CAD risk is unclear. Analyses of 3 major randomized trials showed that a reduction in CAD event rates in patients taking pravastatin could not be ascribed to an increase in circulating HDL concentration *per se* [11].

Recent efforts in the field of HDL therapeutics have focused on the development of cholesteryl ester transfer protein (CETP) inhibitors. CETP mediates the transfer of cholesteryl esters from HDL to VLDL, thereby reducing HDL levels. CETP inhibitors are thought to reduce atherogenicity by increasing the levels of HDL. Laboratory studies showed that introduction of the human CETP gene in mice, a species that is deficient in CETP protein, reduces HDL levels and stimulates the formation of fatty lesions in the arterial wall [13]. Conversely, diet-induced atherosclerosis in rabbits, a species that expresses high levels of CETP, could be reduced by the CETP inhibitor torcetrapib [14]. Studies in human subjects with low HDL showed that torcetrapib induced a prominent increase in serum HDL (61% increase) [15]. The ILLUMINATE trial was subsequently designed to test effects of torcetrapib on HDL and outcomes in high risk patients but was terminated early due to an unanticipated increase in mortality [16]. While the mechanism underlying this adverse effect of torcetrapib is unclear, it has been suggested that the CETP inhibitor may convert HDL from an anti-inflammatory particle to one that is dysfunctional and pro-atherogenic [16-17]. This raises the possibility that the function of HDL rather than its absolute serum level may be important.

As indicated by this discussion, multiple drug classes may exert beneficial effects on serum HDL concentration. Overall, the widespread use of these drugs as HDL-raising agents has been limited due to their modest stimulatory effect on the lipoprotein. It is also unclear whether these drugs improve the functional properties of HDL. The search for an ideal drug to raise HDL,

therefore, goes on. The observation that HDL may become dysfunctional in some disease states is an evolving paradigm, and it has been suggested that the anti-inflammatory status of HDL may be of greater predictive value for CAD risk than HDL levels *per se* [18]. In this review, we discuss recent therapeutic approaches for raising HDL that involve the application of apoA-I itself. Specifically, we discuss anti-atherogenic and vasculoprotective effects of apoA-I mimetic peptides, molecules that improve the anti-inflammatory properties of HDL.

Oxidative mechanisms in atherosclerosis

Vascular function is compromised in a number of cardiovascular conditions including stroke, hypertension and atherosclerosis. In atherosclerosis, blood vessels undergo marked changes in both structure (cellular infiltration/proliferation, lipid deposition) and function (vasospasm, impaired vasodilation) that may predispose for angina and myocardial infarction. Defects in lipoprotein metabolism and vascular reactivity are fundamental pathological responses to hypercholesterolemia, with extensive evidence suggesting that reactive oxygen species play an important role in the initiation and progression of these pathological responses [19-21].

Nitric oxide (NO) becomes modified in a hyperlipidemic environment via its interaction with superoxide anion radical ($O_2^{\cdot -}$), resulting in diminished physiological activity [20-21]. Superoxide is generated in both intracellular and extracellular compartments in response to activation of the pro-oxidant enzymes, including NADPH oxidase, and reacts with the more membrane-permeable and diffusible NO, yielding the potent oxidant peroxynitrite ($ONOO^-$) [22-24]. Clinically, excess production of $O_2^{\cdot -}$ and $ONOO^-$ is associated with: 1) inhibition of endothelium-dependent vasodilatation; 2) enhanced formation of lipid peroxidation products; 3) increased expression of adhesion molecules and chemokines; 4) increased incorporation of proinflammatory cells (neutrophils, macrophages) by the vessel wall; 5) vascular cell proliferation, and, 6) thrombogenesis [25-27]. Pharmacological therapy that targets a reduction in oxidant formation while also stimulating NO formation may effectively blunt or even reverse these atherogenic processes.

The adhesion and infiltration of leukocytes in the vessel wall is a critical component of tissue injury in atherosclerosis and results in increased vascular permeability, elevated activities of oxidative enzymes, endothelial dysfunction and foam cell formation [28-33]. Myeloperoxidase (MPO) is a heme protein synthesized in granules of neutrophils found in atherosclerotic lesions that catalyzes the addition of chloride to hydrogen peroxide (H_2O_2), resulting in the formation of the potent oxidant hypochlorous acid (HOCl) [34-35]. MPO has been identified as a CAD risk factor [36], and mechanistic links between the loss of bioavailable NO and MPO activity in the vasculature have been established [37]. MPO also catalyzes chlorination and nitration reactions in vascular cells [34,38-39]. Chlorinated and nitrated protein tyrosine residues have been detected in human atherosclerotic lesions, thus implicating MPO and HOCl as mediators of atherogenesis [38].

Myeloperoxidase has also been co-localized with macrophages in human atherosclerotic lesions [40-41]. Recent evidence shows that MPO and HOCl modify both LDL and HDL under *in vivo* and *in vitro* conditions [42-43]. The apoB moiety of LDL normally plays an important role in the receptor-mediated endocytosis of the lipoprotein. Oxidative modification of apoB, however, promotes foam cell formation and the development of fatty lesions. Data suggest that reaction products of MPO increase the nitration of apoB tyrosyl residues of LDL [44]. Exposure of macrophages to this nitrated-LDL promotes a greater accumulation of cholesterol and cholesteryl esters compared to treatment of cells with unmodified LDL [44]. The oxidative tyrosylation of HDL by MPO converts it to a form that is resistant to metabolism by hepatic enzyme systems and/or receptor-mediated uptake. In this manner, MPO contributes to atherogenesis by inhibiting the hepatic processing of HDL and, thus, reverse cholesterol

transport [43]. The HOCl-mediated chlorination of apoA-I has also been shown to impair the ability of HDL to act as an acceptor for ATP-binding cassette transporter A1 (ABCA1)-mediated cholesterol efflux from macrophages [45].

Anti-atherogenic properties of HDL

LDL and very low-density lipoprotein (VLDL) are pro-inflammatory and pro-atherogenic particles, while HDL exerts opposing effects [46-47]. HDL and apoA-I also possess anti-oxidant properties that prevent the oxidative modification of LDL [48-49] and anti-inflammatory properties that include inhibition of endothelial cell adhesion molecule (VCAM, ICAM) expression [46]. ApoA-I comprises approximately 70% of total HDL protein and is thought to confer anti-atherogenic properties upon the lipoprotein particle. ApoA-I is synthesized and secreted from the liver as lipid-poor pre-β HDL particles. Pre-β HDL mediates a critical step in reverse cholesterol transport, a process by which cholesterol is removed from peripheral tissues and targeted to the liver for disposal. This is achieved via interaction of the particle with the ATP binding cassette transporter A-1 (ABCA-1) which mediates cholesterol efflux from target tissues. Lipid-poor HDL can subsequently be converted to a “mature” HDL particle via the action of lecithin-cholesterol acyltransferase (LCAT), an HDL-associated enzyme that converts the discoidal particle to a spherical particle containing cholesteryl ester in the core.

Anti-oxidant properties of HDL are attributed to apoA-I as well as the enzymes paraoxonase (PON) and platelet activating factor-acetyl hydrolase (PAF-AH) [50]. PON and PAF-AH hydrolyze oxidized phospholipids, thus reducing lipid peroxide content in LDL and VLDL [51-52]. The critical importance of PON is underscored by findings that HDL particles isolated from mice that overexpress the gene for PON are highly resistant to lipid hydroperoxide formation induced by copper [52]. Conversely, a reduction in PON activity is associated with dyslipidemia in LDL receptor-deficient mice and diabetic humans [53-54]. By virtue of its capacity to act as a sink for oxidized lipids, HDL reduces the atherogenicity of apoB-containing lipoproteins [50]. In addition to reducing inflammatory responses induced by oxidized LDL (oxLDL), HDL may also reduce binding of circulating monocytes to the vascular endothelium via inhibition of adhesion molecule expression [46].

Pro-inflammatory HDL

HDL is commonly viewed as the “good cholesterol” largely due to its ability to mediate cholesterol efflux from peripheral tissues. The proposal that HDL may become dysfunctional in some disease states is an emerging concept in the field of CV biology [55]. Atherosclerosis is characterized by the development of a systemic inflammatory response that influences HDL function [56]. Activation of an acute phase response produces changes in HDL that are characterized by the loss of apoA-I and PON and incorporation of acute phase proteins [56]. Remodeling of the HDL particle is associated with a reduction in reverse cholesterol transport, an increase in lipoprotein oxidation and stimulation of oxLDL-mediated inflammatory responses [56-58]. In patients who have undergone cardiac surgery, an acute phase response is induced that is associated with an increased incorporation of ceruloplasmin in HDL compared to HDL isolated from pre-surgical samples. Under *in vitro* conditions, this acute phase HDL lost its ability to inhibit LDL-induced chemokine formation, monocyte chemotaxis and lipid hydroperoxide formation in cultured endothelial cells [57].

As noted previously, the MPO-derived oxidant hypochlorous acid degrades HDL function by oxidizing apoA-I [59]. This modification was associated with an impaired capacity of HDL to mediate reverse cholesterol transport [59]. Other *in vitro* studies show that elevated glucose decreases HDL-associated PON activity, suggesting a mechanism by which hyperglycemia reduces anti-oxidant capacity in the context of diabetes [60]. The composition of the HDL

particle also influences its functional properties. An increase in HDL triglyceride content is associated with the loss of apoA-I and an increase in the hepatic clearance of the lipoprotein [61]. Thus, hypertriglyceridemia may contribute to atherogenesis by lowering HDL levels and impairing reverse cholesterol transport. An increase in apoA-II expression relative to apoA-I is also associated with increased plasma VLDL and decreased HDL concentration [62]. Under these conditions, the anti-oxidant capacity of HDL is significantly reduced [62].

Additional support for the concept of "pro-inflammatory HDL" comes from the Framingham Study which showed that 40% of CAD events occurred in individuals with "normal" to "high" HDL levels. This observation suggested that functional properties of HDL, rather than absolute serum HDL concentration, may dictate its atheroprotective actions [63]. An HDL inflammatory index has recently been developed as a means to assess the functional quality of HDL [64]. This measurement is based on the *ex vivo* capacity of HDL to inhibit monocyte chemotactic activity in cultured endothelial cells [64]. Assessment of HDL isolated from atherosclerotic rabbits revealed an increase in the inflammatory index that positively correlated with circulating levels of the acute phase reactant serum amyloid A [64].

Reconstituted HDL as a therapeutic tool

HDL therapy, to date, has consisted of the use of molecules that are modified forms of apoA-I. In the presence of phospholipids, apoA-I forms discoidal HDL particles that incorporate cholesterol in the esterified form to yield mature, spherical HDL-like particles which possess cholesteryl ester in the core [65]. Clinical studies also show that infusion of apoA-I/phospholipid complexes in humans transiently increases HDL levels [66]. ApoA-I_{Milano} is a novel variant of apoA-I that was originally identified in carriers who were at low risk for development of CAD. ApoA-I_{Milano} is characterized by the substitution of cysteine for arginine at position 173 in the native apoA-I molecule. The molecule exists in the monomeric, homodimeric and heterodimeric form with apo A-II. The presence of the thiol group in apoA-I_{Milano} is thought to confer enhanced antioxidant activity which may account for the potent anti-atherogenic properties of apoA-I_{Milano} [67]. Laboratory studies show that apoA-I_{Milano} is more effective than wild type apoA-I in inhibiting lipoxygenase-induced lipid oxidation [67]. This antioxidant activity was dependent on the association of apoA-I_{Milano} with phospholipid to form HDL-like particles. Subsequent studies, performed in $apoE^{-/-}$ mice, showed that apoA-I_{Milano} reduces the lipid and macrophage content of arteries and prevents the progression of atherosclerosis [68]. ApoA-I_{Milano} has recently undergone clinical evaluation. In a small clinical study, patients with established CAD were randomized to receive either intravenous recombinant apoA-I_{Milano} (45 mg/kg) and phospholipid (45 mg/kg) or placebo weekly for a 5 week treatment period [69]. Coronary atheroma burden was measured at baseline and after 5 weeks by intra-vascular ultrasound (IVUS). Study results showed that atheroma volume was reduced by 4.2% in patients receiving apoA-I_{Milano} [69]. While these results show promise for apoA-I_{Milano} as a therapeutic agent, the study required intravenous administration of the drug and use of a relatively large amount of protein in the form of a protein-lipid complex [69]. The effect of phospholipid administration alone was not tested.

Structural characteristics of apoA-I

The amphipathic helix is a secondary structural motif found in many biologically active peptides and proteins. It is defined as an α-helix with opposing polar and nonpolar faces oriented along its long axis. These regions are hydrophilic and hydrophobic in nature, respectively. This sidedness of the helix forms a structure complementary to that of phospholipids, thus facilitating their interaction with phospholipids to form protein:lipid complexes [70]. The amphipathic α-helix is a common motif found in exchangeable apolipoproteins, peptide hormones, antibacterial peptides and other biologically active

peptides. It is categorized as 1 of 7 distinct classes: A, H, L, G, K, C and M [70]. Each class has a distinct charge, charge distribution and charge density. Class A amphipathic α-helixes, a common structural motif present in exchangeable apolipoproteins, are zwitterionic, and the positively charged amino acid residues cluster at the polar-nonpolar interface while the negatively charged residues are at the center of the polar face.

ApoA-I mimetic peptide design

In 1985, we designed a model peptide with the sequence, Asp-Trp-Leu-Lys-Ala-Phe-Tyr-Asp-Lys-Val-Ala-Glu-Lys-Leu-Lys-Glu-Ala-Phe, that, when folded into an α-helix, possessed characteristics of a class A amphipathic helix. This peptide was designated 18A due to the presence of 18 amino acids and a class A structural motif. Addition of 18A to a suspension of dimyristoyl phosphatidylcholine (DMPC) induced the formation of discoidal complexes that were structurally similar to those obtained by addition of apoA-I [71]. The ability of 18A-DMPC complexes to mediate cellular cholesterol efflux was assessed in cultured mouse fibroblasts and macrophages. This study showed that, when 18A was used as a cholesterol acceptor, the initial rate of cholesterol removal was similar to that of apoA-I [72]. In addition to forming small lipoprotein particles, 18A, like apoA-I, was able to activate the enzyme LCAT [73]. The model peptide 18A was thus able to mimic many of the properties of apoA-I. Refinements in peptide design showed that addition of an acetyl group at the amino terminus and an amide at the carboxyl terminus of 18A (Ac-18A-NH_2) increased the helicity of the peptide in solution and when associated with lipid [74]. By blocking N- and C-terminal ends of the peptide, the efficiency of Ac-18A-NH_2 for associating with phospholipids. Mediating cholesterol efflux and activating LCAT was significantly increased compared to the unblocked peptide 18A [74].

Detailed studies of the physical-chemical characteristics of class A peptides revealed that the hydrophobic region of the peptide was critical in determining its biological activity. This lead to the development of a family of apoA-I mimetic peptides that are structural variants of the basic 18A motif. The lipid binding ability of 18A is due to its hydrophobic face which contains 2 phenylalanine (F) residues. By systematically replacing existing nonpolar amino acids on 18A with F residues, we generated new peptides with increased hydrophobicity and lipid binding affinity (3F, 4F, 5F, 6F, 7F) [75]. There was a significant increase in the hydrophobicity between peptides 4F and 5F that was accompanied by increased ability to associate with phospholipids. Phospholipid binding with peptides 6F and 7F was reduced, suggesting a limit to increased hydrophobicity of these peptides to interact with lipid. Peptide 4F was more effective than 5F in inhibiting LDL-induced monocyte chemotactic activity, while the latter peptide showed the greatest capacity for LCAT activation [75]. It should be noted that while apo A-I does not solubilize 1-palmitoyl 2-oleoyl phosphatidylcholine (POPC) or egg phosphatidylcholine, the peptides 2F, 4F and 5F are able to solubilize these lipids to form discoidal HDL-like structures. These HDL-like structures have high capacity to efflux cellular cholesterol and also possess antioxidant and antiinflammatory properties [75].

ApoA-I mimetic peptides and atherogenic lesion formation in mice

The first *in vivo* demonstration that apoA-I mimetic peptides possess anti-atherosclerotic properties utilized the hydrophobic peptide 5F [76]. In this study, C57BL/6J mice were fed an atherogenic diet, and 5F was administered daily by intraperitoneal injection for 16 weeks. At the end of the treatment period, aortic atherosclerotic lesion area was significantly reduced in animals receiving 5F compared to hyperlipidemic controls receiving either saline or mouse apoA-I [76]. Surprisingly, total plasma cholesterol levels and lipoprotein profiles were not significantly different between the treated and control groups. In additional studies, HDL was isolated from 5F-treated mice for *ex vivo* analyses. Functional properties of isolated HDL

fractions were significantly different. Specifically, 5F treatment resulted in HDL that was more effective than HDL isolated from vehicle-treated controls in inhibiting LDL-associated lipid hydroperoxide formation and LDL-induced monocyte chemotaxis [76]. These results suggested that atheroprotective mechanisms of 5F action are not due to changes in lipoprotein profiles *per se*, but, rather, are mediated by an improvement in HDL quality and function and/or direct anti-inflammatory effects of the peptide itself [76].

In subsequent studies, the apoA-I mimetic peptide 4F has been used extensively to study atherogenic mechanisms *in vivo* and *in vitro*. This is due to the fact that 4F displays improved solubility properties and is more effective in reducing LDL-induced monocyte chemotactic activity than 5F [75]. Increased interest in 4F also derives from the observation that synthesis of the peptide using D-amino acids results in a molecule (D-4F) that is orally active. Oral treatment of LDL receptor null ($LDLR^{-/-}$) mice with D-4F showed that the peptide could be detected in the circulation, whereas 4F peptide synthesized from L-amino acids was absent [77]. D-4F reduced lesion formation in $LDLR^{-/-}$ mice, but, similar to 5F, did not induce appreciable changes in total plasma or HDL-cholesterol [77]. HDL isolated from D-4F-treated mice was more effective in inhibiting the *ex vivo* oxidation of LDL, demonstrating an improvement in functional properties of the lipoprotein. Other studies show that D-4F treatment in hyperlipidemic rabbits reduces arterial lesion formation. This response was associated with a reduction in the HDL inflammatory index but not total cholesterol or HDL cholesterol [64]. These data provide further evidence that the quality and function of HDL is a better predictor of lesion formation than HDL concentration, in agreement with the proposal by Ansell *et al* [63].

Subsequent studies showed that oral administration of D-4F to apo E-deficient mice resulted in the appearance of small cholesterol-containing particles in the plasma within 20 min [78]. These particles were characterized as pre-β HDL by non-denaturing gel electrophoresis and were found to be enriched in apoA-I and to have increased PON activity. D-4F administration was also associated with a reduction in the lipid hydroperoxide content of apoB-containing lipoproteins [78]. These results suggested that D-4F is able to induce formation of pre-β HDL, the smallest class of HDL particle with the greatest capacity for cholesteryl ester uptake from peripheral cells [78].

D-4F acts synergistically with statins to reduce arterial lesion formation. Oral administration of pravastatin and D-4F, at doses which independently have no effect on plaque reduction, produced a 79% decrease in lesion formation in $apoE^{-/-}$ mice that was characterized by a prominent reduction in macrophage content [79]. Combination therapy rendered HDL anti-inflammatory by virtue of an increase in apoA-I levels and PON activity. Co-administration of low dose D-4F and pravastatin in older $apoE^{-/-}$ mice also induced plaque regression [79]. This was the first study to report an overall increase in HDL cholesterol levels in response to 4F administration. In similar studies, D-4F and pravastatin was also found to render HDL isolated from cynomolgus monkeys anti-inflammatory [79].

ApoA-I mimetic peptides improve endothelium-dependent relaxation

Endothelium-dependent relaxation is impaired by hypercholesterolemia in both humans and experimental animals. This is manifested primarily by a loss of NO bioavailability with resulting changes in functional and structural properties of blood vessels. An increase in LDL may contribute to the development of endothelial dysfunction by uncoupling endothelial NO synthase (eNOS) activity [80]. NO production normally requires the transfer of electrons from co-factors to the heme prosthetic group of the enzyme. Association of heat shock protein 90 (hsp90) with eNOS stabilizes the enzyme, facilitates electron (e^-) transfer and augments NO production [80]. The principal mechanism underlying LDL-induced eNOS uncoupling is due

to inhibition of hsp90-eNOS complex formation. Under these conditions, O_2 may act as an e^- acceptor resulting in the generation of superoxide anion ($O_2^{\cdot -}$) rather than NO [80].

Data suggest that 4F modulates eNOS uncoupling *in vitro* and *in vivo*. Using bovine aortic endothelial cells (BAECs), Ou and colleagues showed that calcium ionophore-induced NO formation was reduced in cells that were pre-incubated with LDL [81]. This response was associated with an increase in $O_2^{\cdot -}$ formation. In contrast, pre-treatment of BAECs with LDL in the presence of 4F reversed these effects. Enhanced formation of NO in 4F-treated BAECs was associated with an increased interaction between hsp90 and eNOS [81].

These studies have been extended under *in vivo* conditions by examining effects of 4F on endothelial dysfunction in LDL receptor-null ($LDLR^{-/-}$) and sickle cell disease (SCD) mice [82]. Endothelial dysfunction in $LDLR^{-/-}$ mice is associated with LDL-induced eNOS uncoupling with concomitant $O_2^{\cdot -}$ generation. Chronic treatment with 4F resulted in a significant improvement in the vasodilatory response of small arterioles from $LDLR^{-/-}$ mice compared to animals receiving vehicle [82]. As in the cell culture studies, the improvement in endothelium-dependent relaxation was associated with a decrease in vascular $O_2^{\cdot -}$ formation. In contrast to $LDLR^{-/-}$ mice, endothelial dysfunction in SCD mice is associated with an increase in the circulating concentration of the pro-oxidant enzyme xanthine oxidase (XO) [82]. Circulating XO binds to the vascular endothelium and, in the presence of xanthine substrate, reduces NO bioactivity by generating $O_2^{\cdot -}$ [23]. Similar to $LDLR^{-/-}$ mice, 4F treatment improved endothelial function in small arteries of SCD mice [82]. The authors proposed that the improved endothelial function in $LDLR^{-/-}$ mice was related to the ability of 4F to reduce the atherogenicity and pro-inflammatory properties of LDL. In the case of SCD mice, it was suggested that 4F may reduce oxidative stress in the vessel wall and prevent the formation of inflammatory lipid mediators. A common mechanism, therefore, to explain vasoprotective effects of 4F in these 2 distinct models may lie in the ability of the peptide to prevent the action and/or formation of inhibitory lipid metabolites [82].

Further evidence in support of a protective effect of 4F on endothelium-dependent relaxation comes from studies in tight skin mice [83]. The tight skin mouse ($Tsk^{-/+}$) is a commonly used model for systemic sclerosis (SSc), an autoimmune, connective tissue disorder in humans. Increased oxidant stress and endothelial dysfunction characterize the disorder in both humans and mice. Acetylcholine and flow-induced vasodilation were significantly impaired in arteries of $Tsk^{-/+}$ mice but were reversed by 4F treatment [83]. Auto-antibodies to oxidized phosphatidylcholine were also reduced by 4F treatment suggesting that inflammatory lipid metabolites play a role in endothelial dysfunction in this model [83].

ApoA-I mimetic peptides modulate antioxidant enzyme expression in diabetic rats

Antioxidant effects of D-4F have been tested in streptozotocin (STZ)-treated, diabetic rats. Endothelial dysfunction in STZ rats is associated with vascular $O_2^{\cdot -}$ formation and an increase in the number of circulating endothelial cells [84]. 4F treatment improved vascular relaxation in STZ rats by reducing $O_2^{\cdot -}$ formation and by preventing endothelial cell sloughing into the circulation. The improvement in endothelial integrity in 4F-treated STZ rats was due to an increase in the number of endothelial progenitor cells (EPCs) and expression of the endothelial cell marker $CD31^+$ [84]. Protective effects of 4F on vascular function and endothelial integrity were associated with an increase in the expression of the antioxidant enzymes heme oxygenase-1 (HO-1) and extracellular superoxide dismutase (EC-SOD) [84-85]. Subsequent studies in type 2 diabetic *ob/ob* mice showed that the 4F-mediated upregulation of HO-1 was associated with improved insulin sensitivity and glycemic control. 4F also increased serum adiponectin levels, reduced abdominal fat content and attenuated weight gain in *ob/ob* mice.

These exciting results suggest that, in addition to activating antioxidant enzyme expression, 4F treatment may activate metabolic pathways leading to an improvement in glucose disposal [86].

ApoA-I mimetics attenuate vascular remodeling in transplantation models

Cardiac allograft vasculopathy (CAV) is a major cause of organ rejection and is characterized by vascular lymphocyte infiltration and intimal lesion formation. In recent studies, CAV was assessed in donor hearts transplanted in C57BL/6 mice that were treated with 4F or saline [87]. 4F treatment resulted in a significant reduction in intimal lesion size and a decrease in lymphocyte infiltration 24 days after transplantation. These protective effects were associated with induction of HO-1 in donor hearts and were partially blocked by an HO-1 inhibitor [87]. *In vitro* studies also showed that 4F reduced T-lymphocyte proliferation and cytokine production by a mechanism that was HO-1 independent. It was concluded that 4F exerts dual protective effects. First, it reduces CAV by a mechanism that is HO-1-dependent. Second, it inhibits lymphocyte proliferation and cytokine formation in transplanted hearts by an alternate mechanism [87].

D-4F administration has also been shown to reduce atherosclerotic lesion formation in bypassed vein grafts. In these studies, a segment of inferior vena cava was grafted onto the right carotid artery of apoE-/- mice [88]. Lesion development in vein grafts was assessed 4 weeks later in mice that were treated with D-4F or saline vehicle. Native lesions in the aortic sinus served as a control. In 4F-treated animals, graft lesion size, plaque lipid content and macrophage immunoreactivity were reduced [88]. In contrast, native aortic lesions were not influenced by 4F treatment. These results suggested that 4F treatment was effective in reducing evolving atherosclerotic lesions but had less effect in reducing those already established [88].

New directions in apolipoprotein mimetic peptide design

An emerging area in the field of HDL therapy is the development of peptides with apoE functional properties. ApoE is associated with VLDL and HDL. It contains a lipid associating domain and a globular domain containing the LDL receptor binding site [89]. The presence of the LDL receptor domain facilitates the hepatic clearance of cholesteryl esters, resulting in a significant decrease in plasma total cholesterol [90]. A peptide encoding an arginine-rich region (residues 141-150: LRKLRKRLLR) of the putative LDL receptor binding sequence, linked to 18A, has been synthesized [89,91]. The peptide was acetylated and amidated at the C- and N-termini respectively to yield the stabilized peptide Ac-hE[141-150]18A-NH_2 (Ac-hE18A-NH_2). Ac-hE18A-NH_2 associates with LDL and VLDL and targets these lipoproteins to hepatocytes for clearance via binding to heparan sulfate proteoglycans (HSPG) [89,91]. This effect is similar to increasing the addition of apo E to the surface of VLDL which redirects VLDL to HSPG for enhanced uptake by hepatocytes. This peptide dramatically reduces plasma cholesterol in apo E-knockout mice [91]. Similar to apoA-I mimetic peptides, Ac-hE18A-NH_2 is able to bind to phospholipids with high affinity but has the additional advantage of facilitating cholesterol clearance due to the presence of the LDL receptor binding domain. We previously reported that a single injection of Ac-hE18A-NH_2 to hyperlipidemic Watanabe rabbits significantly reduces plasma cholesterol, an effect that was maintained for up to 3 days [92]. Fractionation of plasma samples after 24 h confirmed a significant lowering of VLDL and LDL. Ac-hE18A-NH_2 also reduced superoxide formation and improved endothelial function in isolated blood vessels of these animals [92].

Other HDL-modifying peptides that are currently under study include a class G* amphipathic helix corresponding to amino acids at positions 113-122 of apoJ and small tetrapeptides (KRES and FREL) that do not form helices but interact with HDL [93]. The apoJ peptide exerts anti-

atherogenic and anti-inflammatory effects by reducing lipoprotein-associated lipid hydroperoxides and by enhancing the activity of PON [94]. The 4 amino acid peptide KRES exerts similar effects on HDL quality but also increases HDL levels in apoE$^{-/-}$ mice [94]. Despite these effects on HDL, KRES does not induce pre-β HDL formation or directly stimulate cholesterol efflux from macrophages [94]. This is in contrast to the known effects of apoA-I mimetic peptides such as D-4F. Ongoing studies are defining mechanisms of small peptide action.

Conclusions

Apolipoprotein mimetic peptides are an evolving class of drugs with novel modulatory effects on plasma lipoproteins. Animal studies show that apoA-I peptide mimetics, such as 4F, do not directly alter plasma total cholesterol *per se* but improve HDL anti-atherogenic function by increasing the formation of HDL-like (pre-β HDL) particles with elevated PON activity. These responses are linked to a reduction in the lipid hydroperoxide content in apo B-containing lipoproteins and stimulation of cholesterol efflux from foam cells. Indeed, recent studies show that the binding affinity of 4F with oxidized products of 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphatidylcholine is 4-6 orders of magnitude higher than that of apoA-I itself [95]. Vasoprotective effects of apolipoprotein mimetics also include an increase in the expression of HO-1 and EC-SOD. Antioxidant enzyme induction decreases oxidant stress and the formation of pro-inflammatory lipid peroxides, while improving NO bioavailability and endothelial cell function. Whether the induction of HO-1 and EC-SOD is a direct effect of 4F or is related to improvement in HDL function is currently unknown.

A preliminary evaluation of D-4F in high-risk CAD patients has recently been completed. Subjects were randomized to receive a single dose (30-500mg) of D-4F orally or placebo [66]. Results show that unformulated D-4F has a low bioavailability but is safe and well-tolerated. Perhaps, the most important finding of this study was the observation that D-4F treatment reduced the HDL inflammatory index in high risk subjects [96]. The next step in the clinical evaluation of 4F will be to test whether multiple oral doses of D-4F improve HDL function in CAD patients.

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