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Apolipoprotein E mimetic is more effective than apolipoprotein A-I mimetic in reducing lesion formation in older female apo E null mice

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Abstract

Objective—The apolipoprotein E mimetic peptide Ac-hE18A-NH₂, capable of reducing plasma cholesterol and possessing anti-inflammatory properties, was compared with the well-studied anti-atherogenic apoA-I mimetic peptide 4F for reducing lesion formation in female apoE null mice with already existing lesions.

Methods and Results—In initial experiments, Ac-hE18A-NH₂ was administered retro-orbitally two or three times weekly for 6 to 8 weeks, while peptide 4F was administered intraperitoneally every day for the same period. Age matched controls were injected with saline every day. At the end of the treatment period, plasma cholesterol levels of Ac-hE18A-NH₂ administered mice were significantly lower than in 4F and control mice. However, both 4F and Ac-hE18A-NH₂ showed reduced lesion areas in *en face* lesion analysis to a similar extent compared to the control group, while paraoxonase-1 (PON-1) activity was increased only in the Ac-hE18A-NH₂ group. In the third experiment, both peptides were administered at the same dose, frequency, and route of administration. The reduction in *en face* lesions with Ac-hE18A-NH₂ was significantly greater than the 4F and control groups, although lesions in 4F-treated mice were also significantly reduced compared with controls. Both peptide groups had significantly reduced plasma lipid hydroperoxides, but only the Ac-hE18A-NH₂ group had significantly reduced serum amyloid A levels. HDL and plasma inflammatory indices were significantly reduced in both peptide groups compared with controls.

Conclusions—Although both peptides had similar anti-inflammatory properties, Ac-hE18A-NH₂ was more effective in inhibiting lesions than 4F at the same dose, frequency, and route of administration, perhaps due to its cholesterol reducing properties.

Keywords

Atherosclerosis; cholesterol; peptides; oxidation; inflammation

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Disclosures

G. M. Anantharamaiah is a principal in Bruin Pharma, a startup biotechnology company.

Introduction

Major markers for atherosclerosis are increased plasma cholesterol levels (especially apolipoprotein (apo) B containing lipoproteins), low levels of high density lipoproteins (HDL), and inflammation due to increased levels of oxidized lipids [1, 2, 3]. Human apoA-I, the major protein component of HDL has been shown to inhibit atherosclerosis in several dyslipidemic animal models and in humans [4, 5]. On the other hand, apoE, the protein component of very low density lipoproteins (VLDL), inhibits atherosclerosis by enhancing the uptake of atherogenic lipoproteins by the liver and thus lowering plasma cholesterol levels [6, 7, 8]. ApoE has also been shown to regress lesion formation independently of plasma cholesterol levels [9]. These two proteins have been studied extensively in several laboratories.

Over the years, we and others have designed and studied synthetic peptide analogs that are much shorter than apolipoproteins A-I and E and do not possess sequence homology to either apoA-I or apoE but possess secondary structural motifs similar to these two proteins [10, 11, 12, 13, 14]. The structural motif that is common to both of these proteins is the presence of class A amphipathic helices that are characterized by the presence of a polar and a nonpolar face with positively charged residues at the polar-nonpolar interface and negatively charged residues at the center of the polar face [10]. Based on this motif, the first peptide designed and studied in detail was 18A with the sequence DWLKAFYDKVAEKLKEAF [15]. This peptide, despite being only 18 residues in length, mimicked several lipid-associating properties of apoA-I, which has 243 amino acids. Protection of N- and C-terminal ends with acetyl and NH₂ groups respectively produced Ac-18A-NH₂ (also referred to as 2F because of the presence of 2 phenylalanine (Phe) residues on the nonpolar face) with increased lipid associating ability [16]. Administration of this peptide in a dyslipidemic mouse model did not inhibit atherosclerosis [17]. However, when the nonpolar face of this class A amphipathic helical peptide was modified by substituting the existing aliphatic hydrophobic amino acids with the aromatic amino acid Phe, the resulting peptides 4F and 5F were able to inhibit atherosclerosis in dyslipidemic mouse models without changing plasma cholesterol levels [17, 18]. Class A amphipathic helical peptides, (especially 4F analogs) have been studied extensively by our and several other laboratories [19, 20, 21, 22].

Addition of apoE to VLDL enhances its uptake via the heparan sulfate proteoglycan pathway in the space of Disse [23] due to the clustering of positively charged arginine (Arg) residues in the N-terminal putative receptor binding region of apoE (residues 141–150). It has been shown that the lipid-associating C-terminal class A amphipathic helix must be present for the binding and uptake of atherogenic lipoproteins by hepatocytes [24]. Based on these observations that the dual-domain nature of apoE is important for its association with apoB-containing lipoproteins, we covalently linked the putative receptor binding domain (residues 141–150) from apoE to 18A. The resulting peptide, Ac-hE18A-NH₂, has been shown to enhance the uptake of atherogenic apoB-containing lipoproteins both *in vitro* and *in vivo* via the heparin sulfate proteoglycan (HSPG) pathway in dyslipidemic mouse and rabbit models [25, 26, 27, 28]. Recently we also described the anti-inflammatory and recycling properties of this peptide [29]. A single administration of Ac-hE18A-NH₂ has the ability to dramatically decrease plasma cholesterol in dyslipidemic mouse and rabbit models. However, the cholesterol levels return to the original levels within 24 hours. This peptide also inhibits atherosclerosis in apoE null mice [30]. A comparison of the efficacy of this peptide to the extensively studied class A amphipathic helical peptide 4F has not yet been reported. In the present paper, we have compared the ability of the apoE mimetic peptide Ac-hE18A-NH₂ and the apoA-I mimetic peptide 4F in decreasing the progression of atherosclerosis in apoE null mouse model with established aortic lesions. We have compared

the effect of prolonged administration of these two peptides on plasma cholesterol levels and the properties of lipoproteins that may be responsible for the beneficial properties of these two peptides.

Materials and Methods

2.1. Synthesis of peptides

Peptides Ac-hE18A-NH₂ with the amino acid sequence LRKLRKLLRDWLKAFYDKVAEKLKEAF and 4F with the amino acid sequence DWFKAFYDKVAEKFKEAF were synthesized using solid-phase synthesis as previously described [31].

2.2. Effect of intravenous administration of peptides on plasma cholesterol in apoE null mice

Female apoE null mice were purchased from Jackson Laboratories (Bar Harbor, MI). After acclimatization for several weeks they were randomized into three groups, control (receiving vehicle only), 4F, and Ac-hE18A-NH₂. Doses and periods of administration were as described in the results and figure captions. Blood was collected from the retro-orbital sinus under anesthesia at time points mentioned in the figures and cholesterol and triglyceride was measured manually using commercially available kits (Infinity Cholesterol Reagent, Thermo Scientific). All animal studies were performed using the protocols approved by the Institutional Animal Care and Use Committee of The University of Alabama at Birmingham.

Plasma turnover for intravenously administered [¹²⁵I]4F was performed in female apo E null mice as previously described [27]. The peptide was labeled using Iodogen tubes (Thermo Scientific Pierce) and the mixture was separated from free ¹²⁵I with a desalting column (BioRad; Econopak 10DG) which had been pre-equilibrated with the final desired buffer. Peptide was injected into fasted animals and blood samples were collected at 2, 5, 15, 30 and 45 minutes, and at 1, 2, 4, 6, and 24 hours. Each animal provided three blood samples, at 2 minutes and at two other time points, and at least three samples were collected (from different animals) at each time point. Data were calculated as percent 2 min cpm in order to correct for injection errors. Fitting was performed using GraphPad Prism 5.

2.3. Lesion quantification

, Blood samples were taken 24 hours after the final treatment under anesthesia by cardiac puncture and the aorta was prepared for quantification of total aortic lesion area. In brief, *en face* preparations of the entire aorta from the aortic arch to the iliac bifurcation were done [32]. The aorta was excised, cleaned, and opened longitudinally with extremely fine Vannas scissors, then pinned flat on a black wax surface. The aorta was then stained with Oil Red O and lesions were quantified by video capture under a stereo dissecting microscope. Lesion and total areas were determined using SigmaScan (Systat) and lesion area was expressed as a percentage of total area.

2.4. Paraoxonase, lipid hydroperoxides, and serum amyloid A

PON-1 activities were determined essentially as described by Gan and co-workers [33]. Briefly, arylesterase activity was measured by UV spectrophotometry in a 96-well plate format (BIO-TEK Synergy HT multi-detector microplate reader) using phenyl acetate (Sigma-Aldrich, St Louis, Mo) as substrate. Initial hydrolysis rates were determined at 270 nm in reaction mixtures composed of 2 mmol/L phenylacetate, 100 mmol/L Tris hydrochloride, pH 8, and 1 mmol/L calcium chloride at 25°C. An extinction coefficient (at 270 nm) of 1310 mol/l/cm was used for calculating units of arylesterase activity, which are

expressed as the amount of phenyl acetate hydrolyzed (PA units; $\mu\text{mol}/\text{min}$) by the enzyme. Organophosphatase activity was measured with 1.0 mM paraoxon (Sigma-Aldrich, St Louis, Mo) as a substrate. Activity was measured in 100 mM Tris/HCl buffer at pH 8.0 containing 1 mM calcium chloride at 25°C. The amount of p-nitrophenol was calculated from the molar extinction coefficients (412 nm) at pH 8.0 of 17,100 mol/l/cm, and was expressed as PON units (μmol product formed/min). Lactonase activity was determined as described by Draganov and co-workers [34]. Briefly, lactonase activity towards dihydrocoumarin (Sigma-Aldrich, St Louis, Mo) was measured at 270 nm, using an extinction coefficient of 1295 mol/l/cm. Activity was measured with 1 mM substrate in 100 mmol/L Tris hydrochloride, pH 8, and 1 mmol/L calcium chloride at 25°C and was expressed as DHC units ($\mu\text{mol}/\text{min}$).

Lipid hydroperoxides were measured using 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) (Invitrogen, Carlsbad, CA) as previously described [22, 35]. Serum amyloid A was measured using a commercially-available ELISA kit (Life Technologies Corp.).

2.5. Inflammatory indices

In a separate experiment, female apo E null mice were treated with peptides (100 $\mu\text{g}/\text{dose}$ retro-orbitally, 3 X weekly) and vehicle beginning at 14 weeks of age and continuing for 15 days. Plasma was collected 24 hours after the final treatment. HDL and plasma inflammatory indices were determined as previously described [36]. Briefly, three pools of plasma were prepared (two animals each) from each group. HDL was isolated from a portion of each pool by size-exclusion column chromatography. Control human LDL was prepared by ultracentrifugation of the plasma of a healthy volunteer. HDL from each pool, along with human LDL, was incubated with cultured human aortic endothelial cells. For the plasma inflammatory index, human LDL was not added to the plasma samples, but was used as a normalizing control. After 8 h, supernatants were collected and monocyte chemotactic activity was determined. Values for human LDL were normalized to 1.0, and HDL and plasma inflammatory indices were determined by dividing their values by the human LDL control.

2.6. Statistics

Groups were compared by one-way analysis of variance (ANOVA) when the data were normally distributed or one-way ANOVA on ranks when normality failed. Post-hoc analyses were by two-tailed Student's t-test. Groups were considered significantly different when $p < 0.05$.

Results

Three experiments were performed, with differing frequencies and modes of administration. In the first experiment, peptide treatment was begun in 22 week old female apo E null mice and continued for 8 weeks. The both peptides were administered at a dose of 100 μg ; peptide 4F was administered daily intraperitoneally, while peptide Ac-hE18A-NH₂ was administered twice weekly retro-orbitally. 24 hours after the final treatment, plasma cholesterol in the Ac-hE18A-NH₂ group was significantly lower and PON-1 organophosphatase activity was significantly higher compared to both the saline and 4F groups (Figures 1A and B). The change in cholesterol is in agreement with the published results for these two peptides [18, 27, 28]. Aortic *en face* lesion area was significantly reduced in both the peptide groups compared with the saline group (Figure 1C).

In the second experiment, treatment was begun at 24 weeks of age and continued for 6 weeks; 4F treatment was again daily intraperitoneally, while Ac-hE18A-NH₂ was administered retro-orbitally three times a week. Again both peptides were administered at a

dose of 100 µg. Results were similar to the first experiment, in that the Ac-hE18A-NH₂ group again had significantly reduced plasma cholesterol (saline: 354±28; 4F: 336±11; Ac-hE18A-NH₂: 263±23 mg/dL, $p<0.05$ vs saline and 4F) and significantly greater PON-1 organophosphatase activity (saline: 1.190±0.062; 4F: 1.156±0.052; Ac-hE18A-NH₂: 1.462±0.062 PON units, $p<0.01$ Ac-hE18A-NH₂ vs saline and 4F) than the other two groups. Again, aortic *en face* lesion area was significantly reduced in both peptide groups compared with the saline group (saline: 14.70±1.56; 4F: 7.39±0.81; Ac-hE18A-NH₂: 6.35±1.06 % surface area; $p<0.001$ each peptide vs saline).

In the third experiment, treatment was begun at 24 weeks of age and continued for 6 weeks. In order to compare similar dosages and route and frequency of administration, both peptides were administered retro-orbitally three times a week at a dose of 100 µg. As in the previous experiments, plasma cholesterol was significantly reduced in the Ac-hE18A-NH₂ group compared with the other groups, as was plasma triglyceride (Figure 2A and B). Aortic *en face* lesion area was significantly reduced in both peptide groups compared with the control group (Figure 2D), but there was a significantly greater reduction in the Ac-hE18A-NH₂ group when compared with the 4F group. Unlike the previous experiments, the 4F group has significantly greater PON-1 organophosphatase activity, while the activity in the Ac-hE18A-NH₂ group was not different from the control group (Figure 2C). Similar results were found for PON-1 aryl esterase (phenyl acetate substrate) or lactonase (dihydrocoumarin as substrate) activity (data not shown). Lipid hydroperoxides were reduced to a similar extent in both peptide groups compared with the control group (Figure 3A), but serum amyloid A was significantly reduced only in the Ac-hE18A-NH₂ group compared with the control group (Figure 3B).

Turnover data have been reported for intraperitoneal 4F in apo E null mice [22] and intravenous Ac-hE18A-NH₂ in apo E null mice [27] and in Watanabe hypercholesterolemic (WHHL) rabbits [28]. The half-time of clearance for peritoneally-injected 4F was 2.01 h [22]. Clearance of Ac-hE18A-NH₂ was rapid and bi-phasic, with a half-time in mice of 0.027 h for the rapid phase and of 1.61 h for the slow phase [27]. In WHHL rabbits, intravenous Ac-hE18A-NH₂ was also rapid and bi-phasic, with a half-time of 0.64 h for the rapid phase and 17.03 h for the slow phase [28]. Turnover data reported here for intravenously-administered [¹²⁵I]4F in apo E null mice was best fitted to a single-phase model using data to 6 h, with a half-life of 1.08 h (95% confidence interval: 0.60 to 5.26h; $r^2=0.766$). When the 24 hour time point data were included, the half-life was 3.55 h (95% confidence interval 2.35 to 7.27 h; $r^2=0.89$).

In a separate experiment, HDL and plasma inflammatory indices were performed as described in Methods. Both peptides were similar in significantly reducing these indices compared with the control group, indicating that the peptides had similar abilities to reduce LDL-stimulated monocyte chemotaxis (Figures 4A and B).

Discussion

Previously we reported that chronic administration of peptide Ac-hE18A-NH₂ reduced plasma cholesterol and inhibited atherosclerotic lesion formation in apoE null mice [30]. This peptide was also effective in stimulating PON-1 activity, reducing lipid hydroperoxide levels, and exhibited recycling properties similar to apoE [28, 29]. In our earlier paper, we had compared the properties of Ac-hE18A-NH₂ to a peptide derived by attaching the residue 151–160 region of apoE to the class A amphipathic helical peptide (Ac-nhE18A-NH₂) and had shown that despite the peptide Ac-nhE18A-NH₂ being a class A amphipathic helical peptide, it did not exhibit the same atheroprotective properties [30]. In the present paper we compared the properties of a class A amphipathic helical peptide, 4F, well characterized for

its anti-atherogenic and anti-inflammatory properties. This peptide inhibited atherosclerosis without changing plasma cholesterol levels when administered to apoE null mice [18] or LDL-R null mice on Western diet.

Our hypothesis was that the apoE mimetic peptide, which possesses the antiinflammatory properties of 4F plus plasma cholesterol-lowering properties, would be more effective in inhibiting atherosclerosis. However, we wanted to directly compare these two peptides in older apoE null mice with established lesions.

In all of the three experiments, we observed that both peptides reduced progression of aortic atherosclerotic lesions in older female apoE null mice when administered for six to eight weeks. In the experiments where Ac-hE18A-NH₂ was administered at a dose of either half or less than half per week than 4F, this peptide was as effective as 4F in reducing the progression of atherosclerosis (Figure 1 and Results). In these experiments, peptide 4F did not reduce plasma cholesterol levels as determined at the termination point of the experiments, whereas peptide AchE18A-NH₂ significantly reduced plasma cholesterol levels (Figure 1 and Results). These experiments indicated that the peptide Ac-hE18A-NH₂ is at least as effective as 4F in its ability to reduce atherosclerosis.

However, since the dosages and routes of administrations for the two peptides were different, the previous experiments were not direct comparisons of the efficacy of the two peptides. Therefore, in the third experiment, we administered both peptides at the same dose, frequency, and route of administration. While both peptides were effective in reducing the aortic lesion area when compared with controls, the Ac-hE18A-NH₂ group had significantly less lesion area than the 4F group (Figure 2). At the termination point of the experiment, peptide AchE18A-NH₂ administration and not 4F lowered plasma cholesterol and triglyceride levels significantly. Although the peptides were not administered at equal molar concentrations, the molar dose for peptide 4F (MW=2310) was 1.55 greater than that for Ac-hE18A-NH₂ (MW=3577). Thus, a dose effect is not likely to be responsible for the differences observed. Likewise, turnover data demonstrate that plasma bioavailability is also less for Ac-hE18A-NH₂ than for 4F.

It has been shown that both peptides possess anti-oxidant and anti-inflammatory properties [28, 29, 37]. However, in the first two experiments, PON-1 activity was significantly increased only in the Ac-hE18A-NH₂ group. In the third experiment, paradoxically, PON-1 activity was increased in the 4F group but was not increased in the Ac-hE18A-NH₂ group when measured in whole plasma. The increase in the 4F group may have been due to the different route of administration (intravenous vs. intraperitoneal), but the lack of increase in the AchE18A-NH₂ group is not easily explained. Both peptides exhibited anti-oxidative activities as both significantly reduced plasma lipid hydroperoxide levels to a similar extent compared with controls (Figure 3). However, while levels of serum amyloid A, an acute phase reactant protein, were reduced in both peptide groups, this only reached significance in the Ac-hE18A-NH₂ group. In a separate experiment, both peptides exhibited similar significantly reduced HDL and plasma inflammatory indices (a measurement of LDL-induced monocyte chemotaxis) compared with the control group (Figure 4).

One significant difference between the peptides is that peptide Ac-hE18A-NH₂ must be administered intravenously, while peptide 4F can be administered through several routes. Further research toward enabling more convenient modes of administration of Ac-hE18A-NH₂ is underway.

Conclusions

At a similar dosage, frequency, and route of administration, Ac-hE18A-NH₂ is more effective than 4F in reducing atherosclerotic aortic lesions in older apo E null mice. Although both peptides exhibited similar anti-inflammatory and anti-oxidative properties, the cholesterol-reducing property of Ac-hE18A-NH₂ may be responsible for the greater atheroprotection.

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Highlights

- At a similar dosage, frequency, and route of administration, the apoE mimetic peptide Ac-hE18A-NH₂ is more effective than the apoA-I mimetic peptide 4F in reducing atherosclerotic aortic lesions in older apo E null mice.
- Both peptides exhibited similar anti-inflammatory and anti-oxidative properties.
- The cholesterol-reducing property of Ac-hE18A-NH₂ may be responsible for the greater atheroprotection.

A

B

Figure 1. Cholesterol, PON-1, and aortic lesion area with daily 4F and 3X weekly AchE18A-NH₂ treatment

Female apo E null mice were administered vehicle (saline), peptide 4F (daily intraperitoneally, 100 µg/dose), or peptide Ac-hE18A-NH₂ (hE18A; twice weekly retroorbitally, 100 µg/dose) beginning at 22 weeks of age and continuing for 8 weeks. Samples were collected 24 hours after the final treatment. **A:** Plasma cholesterol levels and **B:** PON-1 organophosphatase activity at the end of the treatment period and expressed as PON units (µmol product formed/min). **C:** Aortic *en face* lesion area. †p<0.05 vs saline, ‡p<0.01 vs saline, §p<0.001 vs saline, *p<0.05 vs 4F, **p<0.01 vs 4F; one way ANOVA.

A

B

C

D

Figure 2. Cholesterol, triglyceride, PON-1, and aortic lesion area with equal dosage, frequency, and route of administration

Female apo E null mice were administered vehicle (saline), peptide 4F (3 times weekly retro-orbitally, 100 µg/dose), or peptide Ac-hE18A-NH₂ (hE18A; 3 times weekly retro-orbitally, 100 µg/dose) beginning at 24 weeks of age and continuing for 6 weeks. Samples were collected 24 h after the final treatment. **A:** Plasma cholesterol levels, **B:** triglyceride levels, and **C:** PON-1 organophosphatase activity at the end of the treatment period and expressed as PON units (µmol product formed/min). **D:** Aortic *en face* lesion area. ‡p<0.01 vs saline, §p<0.001 vs saline, *0.05 vs 4F, **p<0.01 vs 4F, #p<0.001 vs 4F; one way ANOVA.

A

B

Figure 3. Plasma lipid hydroperoxide and serum amyloid A (SAA) levels

Plasma was taken from mice in Figure 2 24 hours after the final treatment and analyzed for **A**: lipid hydroperoxide and **B**: serum amyloid A (SAA) content. † $p < 0.05$ vs saline, ‡ $p < 0.01$ vs saline; one way ANOVA.

A

B

Figure 4. HDL and plasma inflammatory indices

Female apo E null mice were administered vehicle (saline), peptide 4F (3 times weekly retro-orbitally, 100 µg/dose), or peptide Ac-hE18ANH₂ (hE18A; 3 times weekly retro-orbitally, 100 µg/dose) beginning at 14 weeks of age and continuing for 15 days. Plasma was collected 24 hours after the final dose, and three pools were prepared from each group (two mice per pool). Inflammatory indices were determined as described in Methods. **A:** HDL inflammatory index. **B:** Plasma inflammatory index. †p<0.05 vs saline, §p<0.001 vs saline.