Apolipoprotein E Mimetic Peptide Dramatically Lowers Plasma Cholesterol and Restores Endothelial Function in Watanabe Heritable Hyperlipidemic Rabbits

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Background—These studies were designed to determine whether the dual-domain peptide with a class A amphipathic helix linked to the receptor-binding domain of apolipoprotein (apo) E (Ac-hE-18A-NH₂) possesses both antidyslipidemic and antiinflammatory properties.

Methods and Results—A single bolus (15 mg/kg IV) of Ac-hE-18A-NH₂ that contains LRKLRKRLLR (141- to 150-residue region of apo E) covalently linked to apo A-I mimetic peptide 18A not only reduced plasma cholesterol levels (baseline, 562±29.0 mg/dL versus 287.7±22.0 mg/dL at 18 hours, \(P<0.001\)) in the Watanabe heritable hyperlipidemic rabbit model but also significantly improved arterial endothelial function. This improvement was associated with a reduction in 2 markers of oxidative stress. First, the plasma lipid hydroperoxide content was reduced significantly, an effect associated with a 5-fold increase in HDL paraoxonase activity. Second, the formation of superoxide anion, a scavenger of nitric oxide, was also significantly reduced in arteries of these animals.

Conclusions—Because dyslipidemia and endothelial dysfunction are common features of the atherosclerotic disease process, this unique dual-domain peptide has ideal composite properties that ameliorate key contributory factors to atherosclerosis. (Circulation. 2005;111:3112-3118.)

Key Words: lipoproteins  • endothelium  • metabolism  • nitric oxide  • arteriosclerosis
restored endothelial function. The reduction of plasma cholesterol levels paralleled that of peptide clearance from plasma and was associated with a concomitant decrease in lipid hydroperoxides.

Methods

Peptide Synthesis

Peptides Ac-18A-NH₂, Ac-DWLKAFYDVKAELKAEF-NH₂, Ac-LRKLRKRLR-NH₂, and Ac-hE18A-NH₂ with the primary sequence Ac-LRKLRKRLR-DWLKAFYDVKAELKAEF-NH₂ were synthesized by the solid-phase method of peptide synthesis as described previously. The purity of synthetic peptides was established by analytical high-performance liquid chromatography and ion-spray mass spectrometry. The peptides Ac-18A-NH₂ and Ac-hE-18A-NH₂ were dialyzed against ammonium bicarbonate (100 mM) and then distilled water and 0.01N HCl and were lyophilized before use. Lyophilized peptides were dissolved in sterile 0.15 mol/L NaCl, and the pH was adjusted to 7.0. Ac-LRKLRKRLR-NH₂ was dissolved in 0.15 mol/L NaCl, and the pH was adjusted to 7.0. The peptide solutions were sterilized by membrane filtration before administration.

Animals

Male homozygous WHHL rabbits were purchased at 6 months of age from Covance Research Products (Denver, Pa). Baseline cholesterol values and lipoprotein cholesterol profiles ensured the hypercholesterolemic phenotype (ie, total plasma cholesterol levels >500 mg/dl) of these animals. Rabbits were fed a standard laboratory chow and water ad libitum. For vessel reactivity studies, normolipemic phenotype (ie, total plasma cholesterol levels 4.4 mmol/L butylated hydroxytoluene in methanol, was added to 100 L of the sample and incubated in the dark for 30 minutes. Absorbance was then measured at 560 nm. The test values were compared with a standard of cumene hydroperoxide to quantify the plasma hydroperoxide content.

In Vitro Assessment of Endothelial Function

Endothelium-dependent relaxation was monitored in isolated blood vessels of homozygous WHHL rabbits. Normolipidemic heterozygous WHHL rabbits were used as controls. Homozygous WHHL rabbits were randomized to receive either a single dose of Ac-hE-18A-NH₂ (15 mg/kg, n=5) or an equivalent volume of saline (n=5) by intravenous injection of the marginal ear vein. Blood samples were collected at times noted in Results and in the figures. Stock solutions of peptide were prepared at a concentration of ~5 mg/mL. Control injections were vehicle only (same volume of sterile saline).

Electrophoretic Mobility Assay

Total lipoproteins were isolated from the plasma of WHHL rabbits by density-gradient centrifugation. Plasma density was adjusted to 1.21 g/mL. Each sample was then centrifuged at 50,000 rpm in a Beckman Ti 50.2 rotor for 48 hours to isolate the total plasma lipoproteins. The top 500 L was collected; 300 L of this sample was chromatographed on 2 Superose 6 columns in series that were equilibrated and eluted with phosphate-buffered saline (PBS). Protein in the eluent was detected by spectrophotometry at 280 nm. Fractions (0.5 mL) were collected and radioactive counts measured. Fractions in the HDL size range were used to determine paraoxonase (PON) activity. Column-chromatographic elution volumes of individual lipoprotein species were identified by elution volumes of known human lipoproteins isolated by sequential density ultracentrifugation.

Column Cholesterol Lipoprotein Profiles

Plasma cholesterol lipoprotein profiles were measured with the CLiP method. In brief, 5 to 10 L of plasma was analyzed on a single Superose 6 (Pharmacia) column. Immediately after column chromatography, cholesterol reagent was introduced through a mixing tee, and the eluent-reactant mixture was passed into a postcolumn reaction coil. Cholesterol content of the eluent mixture was spectrophotometrically detected at 546 nm, and the data points were digitized. In some cases, fractions were collected to determine distribution of radioactive counts. Total cholesterol and triglyceride concentrations were determined enzymatically with commercially available reagents (Sigma Cholesterol 1000 and Thermo, respectively).

PON Assay

WHHL plasma samples were fractionated on Superoxone columns, and 2-L fractions from the HDL region were added to 200 L buffer (100 mMol/L Tris containing 2 mMol/L CaCl₂, pH 8.0) containing paraoxon (1 mmol/L O,O-diethyl-O-p-nitrophenylphosphate), and the rate of release of 4-nitrophenol was determined spectrophotometrically. The assay was performed in a 96-well plate, and readings were taken every 2 minutes at 405 nm. The quantity of 4-nitrophenol formed was calculated from the molar extinction coefficient of 71000 mol/L cm⁻¹. One unit of PON activity was defined as 1 nmol of 4-nitrophenol formed per minute.

Determination of Plasma Lipid Hydroperoxide Content

Lipid hydroperoxides were measured with the FOX2 colorimetric assay to determine their content in the plasma of WHHL rabbits. In brief, 900 L of the FOX reagent, containing 300 mMol/L FeSO₄, 7H₂O, 120 mMol/L xylene orange, 25 mMol/L H₂SO₄, and 4.4 mMol/L butylated hydroxytoluene in methanol, was added to 100 L of the sample and incubated in the dark for 30 minutes. Absorbance was then measured at 560 nm. The test values were compared with a standard of cumene hydroperoxide to quantify the plasma hydroperoxide content.
experiments, endothelium-independent relaxation was monitored by the addition of sodium nitroprusside (SNP, 10⁻⁶ to 3×10⁻⁶ mol/L). Effects of both agents on tension development were tested in 3 or 4 ring segments from each animal. Results were then averaged to yield a weighted mean for each animal.

**Measurement of Vascular Superoxide Anion Production**

The formation of O₂⁻ was measured in the aortas of homozygous WHHL rabbits by coelenterazine-dependent chemiluminescence. The O₂⁻-dependent oxidation of coelenterazine results in the formation of a high-energy intermediate that emits light as it relaxes to the ground state. Aortas were excised from homozygous WHHL rabbits injected with either Ac-hE-18A-NH₂ (n=6) or saline (n=7). The luminal surface of the artery was exposed, and tissue segments (≈16 mm²) were cut and placed in cell-culture wells containing PBS. Basal O₂⁻ production was monitored with a luminometer (BMG Lab Technologies Inc). At the beginning of each experiment, coelenterazine was injected into each well to yield a final concentration of 10 μmol/L. O₂-dependent chemiluminescence increased with time and stabilized after ≈3 to 5 minutes. Photon emission was measured during this plateau phase. The specificity of the chemiluminescence signal for O₂⁻ production was verified in some experiments by addition of the superoxide dismutase mimetic tetrakis-(N-ethylpyridinium-2-yl) porphyrin (T2E, 100 μmol/L). T2E used in these experiments was a generous gift of Incara, Inc. The assay was calibrated by monitoring the chemiluminescence signal of known amounts of O₂⁻ generated by xanthine (50 μmol/L) and xanthine oxidase (0.1 to 0.5 μmol/L). Rates of O₂⁻ production associated with these xanthine/xanthine oxidase incubation conditions were determined spectrophotometrically by measuring the O₂-dependent reduction of ferricytochrome c. Chemiluminescence signals measured in isolated aortic tissues were then converted to rates of O₂⁻ formation and normalized to tissue weight. Superoxide chemiluminescence was determined by subtraction of the signal that was not inhibited by T2E.

**Statistics**

Differences between treatment groups were determined with 2-tailed t tests. In some cases, differences were assessed by 1-way ANOVA with post hoc testing (Student-Newman-Keuls test). Differences were considered significant at P<0.05.

**Results**

A single intravenous injection of Ac-hE-18A-NH₂ to WHHL rabbits produced a dose-dependent change in plasma cholesterol levels relative to the preinjection time point, reducing both VLDL and LDL contents (Figure 1A). At a dose of 2.5 mg/kg body weight, the mean cholesterol level at 18 hours compared with time 0 was 85.09±2.33% after injection (n=3); at 7.5 mg/kg, the level was 58.61±0.63% (n=2); and at 15 mg/kg, the level was 41.90±6.55% (n=3). The plasma cholesterol level reduction was accompanied by a change in agarose gel electrophoretic mobility of isolated LDL from the WHHL rabbits treated with peptide, demonstrating that the peptide associated with LDL and changed its surface charge and thus, its electrophoretic properties (Figure 1B). To determine whether the 2 domains could act independently of each other, a mixture of the 2 peptides (8.0 mg Ac-LRKLRKKRRLL-ΝH₂ and 10.5 mg Ac-18A-ΝH₂) was administered to WHHL rabbits (n=3). These 2 peptides, being Ac- and amide-protected at the ends, do not covalently associate with each other. This resulted in a slight decrease in plasma cholesterol levels (81.83±3.3% of that of original value), a value similar to what was observed with the lowest dose (2.5 mg/kg) of Ac-hE-18A-NH₂.

Plasma turnover of 125I–Ac-hE18A-ΝH₂ and the kinetics of plasma cholesterol reduction were determined in 3 male WHHL rabbits injected intravenously with radiolabeled peptide (7.5 mg/kg). Blood samples were taken at the time points indicated in Figure 2A. Data through 12 hours after injection were fitted to biexponential equations as previously described; use of the 24-hour time point caused the curve-fitting procedure to fail. The initial rapid phases of clearance for both peptide and cholesterol were similar, with the peptide having a t₁/₂ of 38.59 minutes and cholesterol having a t₁/₂ of 37.09 minutes. The second clearance phase for the peptide...
had a t1/2 of 17.03 hours, and cholesterol had a t1/2 of 48.91 hours. Furthermore, in a separate experiment, Ac-hE-18A-NH2 (7.5 mg/kg, n = 8) or saline (n = 3) was injected intravenously into male WHHL rabbits (Figure 2B). The mean plasma triglyceride level was reduced by 45% for up to 8 hours after peptide injection but returned close to but significantly lower than preinjection levels by 24 hours (Figure 2B), reflecting the putative association of the peptide with VLDL, the major carrier of plasma triglycerides. Because single-domain peptides showed minimal effects on plasma cholesterol levels, plasma turnover experiments were not performed with these peptides.

Analyses of plasma at different time intervals showed that peptide Ac-hE-18A-NH2 radioactivity was distributed not only in VLDL and LDL but also in the HDL region (Figure 3), despite low levels of HDL cholesterol (Figure 1A). PON activity in the HDL region dramatically increased after injection of the peptide (Figure 4). A 5-fold increase in PON activity was observed at 90 minutes. The activity subsequently returned to baseline by 12 hours (data not shown).

The increase in HDL-associated PON was accompanied by a decrease in total plasma lipid hydroperoxide levels, with the maximum reduction occurring over a similar time course as the maximum increase in PON (Figure 4). Administration of a mixture of single-domain peptides (8.0 mg/kg Ac-LRKLRRKLLR-NH2 and 10.5 mg Ac-18A-NH2) to the WHHL rabbits did not show changes in PON activity (320 ± 20 U) at either time 0 or 90 minutes after injection. As expected, there was also no change in plasma lipid hydroperoxides (35 ± 5 μmol/L) before and 90 minutes after administration of the mixture of Ac-LRKLRRKLLR-NH2 and Ac-18A-NH2.

Endothelium-dependent and -independent relaxations were monitored in isolated aortic ring segments of homozygous WHHL rabbits 18 hours after treatment with either saline (n = 5) or Ac-hE-18A-NH2 (n = 5). As a control, we also monitored relaxation in ring segments of normolipidemic, saline-treated heterozygous WHHL rabbits (n = 6). The plasma cholesterol level in homozygous WHHL rabbits was not different at baseline (saline, 532.6 ± 12.9 mg/dL; Ac-hE-18A-NH2, 562.9 ± 29.0 mg/dL) but was significantly reduced in peptide-treated rabbits 18 hours after injection (287.7 ± 22.0 mg/dL, P < 0.001). At the same time point, plasma cholesterol was unchanged in saline-treated WHHL rabbits (523.9 ± 5.9 mg/dL). Ach-mediated relaxation was...
impaired in ring segments of homozygous WHHL rabbits (n, Figure 5A) compared with heterozygous WHHL controls (l, Figure 5A). Maximum Ach-induced relaxation in homozygous WHHL rabbits was 72±7% compared with 100±3% in normolipidemic heterozygous WHHL rabbits. Treatment with a single dose of Ac-hE-18A-NH₂ (15 mg/kg) restored endothelium-dependent relaxation in ring segments of homozygotes (p, Figure 5A) essentially to the same level (95±6%) as that seen in normolipidemic heterozygous WHHL rabbits (Figure 5A). Thus, administration of the peptide improved Ach-induced relaxation by 24%. Endothelium-independent relaxation, assessed by SNP addition, was not impaired in any of these groups of rabbits (Figure 5B).

The formation of superoxide anion, O₂⁻, a known scavenger of nitric oxide (NO) in atherosclerotic blood vessels, was assessed in aortas of homozygous WHHL rabbits 18 hours after injection with saline (n=7) or Ac-hE-18A-NH₂ (n=6, Figure 5C). The specificity of the chemiluminescent signal for O₂⁻ was confirmed by addition of a superoxide dismutase mimetic, T2E, as noted in Methods (Figure 5C). Aortas of rabbits injected with the peptide had significantly reduced rates of O₂⁻ production (~68% decrease).

**Discussion**

The WHHL rabbit is commonly used as a model of atherosclerosis. These animals lack a functional LDLR and display elevated plasma concentrations of LDL and total cholesterol. WHHL rabbits closely approximate conditions associated with familial hypercholesterolemia in humans. Furthermore, arteries of WHHL rabbits display impaired endothelial function and develop vascular lesions (40% of the aortic surface at 6 months of age) similar to those found in human arteries.

Although we found that peptide Ac-hE-18A-NH₂ did not exert any effect on plasma cholesterol levels in LDLR-null mice, in the current study, we found that a single intravenous injection of Ac-hE-18A-NH₂ to homozygous WHHL rabbits rapidly reduced total plasma cholesterol, with the maximum effect occurring at ~6 hours. After 24 hours, plasma cholesterol levels were still significantly lower than at time 0 (Figure 2A). This response was characterized by a significant reduction in both VLDL and LDL content (Figure 1A). Administration of an equivalent dose of a mixture of single-domain peptides showed minimal changes in plasma cholesterol levels. Thus, Ac-hE-18A-NH₂ is a peptide with unique properties not observed in the individual domains. Analysis of lipoproteins by agarose electrophoresis also showed that cationic Ac-hE-18A-NH₂ associated with LDL and decreased its mobility owing to peptide association with LDL (Figure 1B). The accelerated clearance of VLDL and LDL in peptide Ac-hE-18A-NH₂–treated WHHL rabbits probably occurs through LDLR family members, because LDLR expression in WHHL rabbits is defective. In previous studies, we have shown that the peptide enhanced the uptake and degradation of atherogenic apo B–containing lipoproteins via a pathway that requires binding to cell surface proteoglycans (HSPGs). The current studies clearly show a physical interaction between Ac-hE-18A-NH₂ and LDL of WHHL rabbits (Figure 3). The results support our earlier observations that when the peptide is able to associate with apo B–containing lipoproteins, it induces a rapid reduction in plasma cholesterol levels via a high-capacity, low-affinity pathway involving HSPGs. Thus, the peptide Ac-hE-18A-NH₂ is able to dramatically reduce plasma cholesterol and apo B-containing lipoprotein levels in WHHL rabbits.

In addition, the peptide Ac-hE-18A-NH₂ also reduced plasma lipid hydroperoxide levels significantly when compared with those in control rabbits (Figure 4). This response was concomitant with an upregulation of PON activity, an HDL-associated enzyme that hydrolyzes oxidized phospholipids. Administration of a mixture of single-domain peptides (8.0 mg/kg Ac-LRKLRKLLR-NH₂ and 10.5 mg Ac-18A-NH₂) to WHHL rabbits did not show significant changes in lipid hydroperoxides or PON activity. Previous studies have demonstrated that D-4F, an apo A-I mimetic peptide, stimulates an increase in plasma HDL concentration and PON activity. We have also shown earlier that whereas D-4F is highly effective in inhibiting LDL-induced monocyte chemotaxis, Ac-18A-NH₂ is not. These and our earlier results with this peptide suggest that Ac-hE-18A-NH₂ not only reduces plasma cholesterol in dyslipidemic animal models but also exerts antiinflammatory/antioxidant effects through its ability to scavenge "seeding molecules," whereas the 2 domains by themselves are not effective, suggesting that combining LRKLRKLLR with the lipid-associating 18A produced a novel peptide with a highly effective dual function that combined dyslipidemic and antiinflammatory properties. Further studies are needed to understand the mechanism(s) by which the peptide exerts these effects.

The development of endothelial dysfunction is an early response to hypercholesterolemia and is now considered an independent clinical prognostic indicator in patients with or
without established coronary artery disease.\textsuperscript{21} In this regard, it has been shown that the statin-mediated lowering of total plasma cholesterol and LDL is associated with improved endothelial function in humans at high risk for cardiovascular diseases.\textsuperscript{22} Laboratory studies indicate that NO bioactivity may be impaired by different mechanisms. First, NO may be modified in a hyperlipidemic environment through its interaction with elevated levels of $O_2^-$, resulting in loss of NO function.\textsuperscript{16,17} Elevated plasma cholesterol may enhance $O_2^-$ formation by stimulating the expression of pro-oxidant enzymes such as NADPH oxidase and xanthine oxidase. Second, the hyperlipidemic environment may also diminish NO production itself by mediating the uncoupling of endothelial NO synthase (eNOS) from its substrate L-arginine.\textsuperscript{23} In support of these mechanisms, a recent report has shown that an apo A-I mimetic peptide both reduces LDL-mediated uncoupling of eNOS and reduces $O_2^-$ formation in cultured endothelial cells.\textsuperscript{23} Finally, lipid peroxyl radicals may also induce endothelial dysfunction by catalytically consuming NO.\textsuperscript{24}

In the current study, we found that the Ach-induced relaxation of arterial segments of homozygous WHHL rabbits was significantly impaired compared with those from normolipidemic heterozygous controls. In contrast, relaxation induced by the endothelium-independent vasodilator SNP was not different between groups, suggesting that impaired vascular function was caused by changes at the level of the endothelium. We found that a single intravenous injection of Ac-hE-18A-NH$_2$ significantly improved endothelial function (Figure 5A). This response was associated with a reduction in both vascular $O_2^-$ formation and plasma lipid hydroperoxide content. A dual mechanism of peptide action may explain the improvement in endothelial function in WHHL rabbits. First, cholesterol lowering coupled with increased PON activity will reduce lipid hydroperoxide content and thus, limit the direct scavenging of NO by peroxyl radicals. Second, decreased LDL should reduce $O_2^-$ formation by preventing the LDL-mediated uncoupling of eNOS.

Our results support the notion that the dual-domain synthetic peptide Ac-hE-18A-NH$_2$ may have therapeutic potential for reducing common pathological features of atherosclerosis. Recent reports on apo A-IMilano:lipid complex infusion into mice,\textsuperscript{25} rabbits,\textsuperscript{26} and humans\textsuperscript{27} have stimulated new interest in HDL-based therapies. The end points in the apo A-IMilano studies were very different from those reported here and included regression of coronary lesions in humans, as measured by intravascular ultrasound, and a reduction of stent-induced hyperplasia in porcine coronary arteries. The apo A-IMilano preparations are complicated and costly; adding

Figure 5. Endothelium-dependent and -independent relaxations of aortic ring segments and superoxide production. Vasodilator response of aortic ring segments from homozygous WHHL rabbits injected 18 hours previously with either saline (●, n=5) or hE-18A (▲, n=5, 7.5 mg/kg) was tested by cumulative addition of Ach or SNP. Heterozygous WHHL rabbits served as controls (●, n=6). A, Ach-mediated, endothelium-dependent relaxation was impaired in vessels of homozygous WHHL rabbits compared with heterozygous controls. Improvement in endothelial function induced by Ac-hE-18A-NH$_2$ is reflected by downward shift in dose-response curve for Ach. B, Endothelium-independent relaxation was monitored by cumulative addition of SNP. No difference in response to SNP was noted between groups, implicating endothelium as site of vessel dysfunction. Data are expressed as mean±SEM. *P<0.05 comparing saline-treated homozygous WHHL with peptide-treated animals and heterozygous WHHL controls by 1-way ANOVA and pairwise multiple comparisons with Student-Newman-Keuls method. C, In vitro measurement of superoxide anion formation in aortic ring segments of homozygous WHHL rabbits. Aortas were excised from homozygous WHHL rabbits injected with either saline (n=7) or Ac-hE-18A-NH$_2$ (7.5 mg/kg, n=6). Luminal surface of artery was exposed, and tissue segments (~10 to 20 mg) were cut and placed in PBS containing photochemical probe coelenterazine (10 μmol/L). Basal superoxide production was monitored with luminometer (BMG Lab Technologies Inc).
to this complexity is the production of a recombinant 28-kDa protein complexed to phospholipids. In this context, the present results indicate that a single administration of a 28-residue, amphipathic helical peptide that can be easily chemically synthesized in large quantities has effects on both plasma cholesterol reduction, similar to apo E, and antiinflammatory/antioxidant properties to improve endothelial relaxation, similar to apo A-I and HDL. Whether the Ac-hE-18A-NH₂ peptide can inhibit lesion progression or even cause regression, like apo A-Ilastrlipid complexes, is the subject of current and future studies. Nonetheless, the Ac-hE-18A-NH₂ dual-domain peptide has demonstrated that the beneficial properties of both apo A-I and apo E can be incorporated into a hybrid peptide. The implication then is that future designs may include even more targeted, beneficial properties into a single, perhaps even shorter, peptide. Thus, the ongoing investigation of minimized domain structure in apolipoproteins continues to be an ever-important asset to our understanding of the functions of apolipoproteins in atherogenesis and may yield new therapies in ameliorating cardiovascular diseases.

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Disclosure

G.M. Anantharamaiah is a principal in Bruin Pharma, LA, a start-up biotech company.

References


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