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Anti-Inflammatory and Recycling Properties of an Apolipoprotein Mimetic Peptide, Ac-hE18A-NH₂

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Abstract

Apolipoprotein E (apoE) exerts prominent anti-inflammatory effects and undergoes recycling by target cells. We previously reported that the peptide, Ac-hE18A-NH₂, composed of the receptor binding domain (LRKLRKRLLR) of apoE covalently linked to the Class A amphipathic peptide 18A, dramatically lowers plasma cholesterol and lipid hydroperoxides and enhances paraoxonase activity in dyslipidemic animal models. The objective of this study was to determine whether this peptide, analogous to apoE, exerts anti-inflammatory effects and undergoes recycling under *in vitro* conditions. Pulse chase studies using [¹²⁵I]-Ac-hE18A-NH₂ in THP-1 derived macrophages and HepG2 cells showed greater amounts of intact peptide in the cells at later time points indicating recycling of the peptide. Ac-hE18A-NH₂ induced a 2.5 fold increase in pre β -HDL in the conditioned media of HepG2 cells. This effect persisted for three days after removal of the peptide from culture medium. Ac-hE18A-NH₂ also induced the secretion of cell-surface apoE from THP-1 macrophages. In addition, the peptide increased cholesterol efflux from THP-1 cells by an ABCA-1 independent mechanism. Moreover, Ac-hE18A-NH₂ inhibited LPS-induced vascular cell adhesion molecule-1 (VCAM-1) expression, and reduced monocyte adhesion in human umbilical vein endothelial cells (HUVECs). It also reduced the secretion of interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) from THP-1 macrophages even when administered post-LPS and abolished the 18-fold increase in LPS-induced mRNA levels for MCP-1 in THP-1 cells. Taken together, these results suggest that addition of the putative apoE receptor-domain to the Class A amphipathic peptide, 18A results in a peptide that, similar to apoE, recycles, thus enabling the potentiation and prolongation of its anti-atherogenic and anti-inflammatory effects. Such a peptide has great potential as a therapeutic agent in the management of atherosclerosis and other inflammatory diseases.

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Keywords

pre β HDL; VCAM-1; MCP-1; apoA-I; apoE-mimetic; recycling; macrophages; HepG2

1. Introduction

Apolipoprotein E (apoE) is expressed ubiquitously in all tissues and plays a major role in lipid transport and cell signaling processes (1,2). The importance of apoE in inhibiting atherosclerosis has been demonstrated in several animal models. Mice that overexpress apoE have lower plasma cholesterol levels compared to control mice that do not overexpress apoE (3), while deletion of the apoE gene in mice produces spontaneous atherosclerosis (4). Injection of apoE into cholesterol-fed rabbits also prevents the development of atherogenic lesions (5). ApoE also exerts vascular protective effects. While normal levels of apoE range from 5 to 8 mg/dl, a small amount of apoE (40 μ g/dl) is sufficient to maintain cholesterol homeostasis (6). Histochemical and biochemical studies have demonstrated the presence of apoE on the cell surface of hepatocytes (7) where it plays a role in lipoprotein processing, binding and uptake. Elegant studies from the laboratories of Swift (8,9) and Beisiegel (10) have established that apoE can be taken up by hepatoma cell lines, mouse hepatocytes, fibroblasts, and macrophages, and then recycled to the cell surface.

Both apoE and apoA-I, the major protein component of HDL, play key roles in lipoprotein metabolism and protect against atherosclerosis. The anti-atherogenic effects of HDL and apoA-I are attributed, in part, to their ability to efflux cellular cholesterol from peripheral tissues and transport it to the liver for excretion (11). HDL is a heterogeneous mixture of particles of different sizes, densities, and protein and lipid composition (12). ApoA-I is secreted from the liver as lipid-poor apoA-I (pre β -HDL), which is converted to mature α -HDL by the action of the plasma enzyme, lecithin cholesterol acyl transferase (LCAT). The small pre β -HDL fraction plays a major role in ABCA1-dependent Reverse Cholesterol Transport (RCT). ABCA1 exports cholesterol and phospholipids to lipid-poor apoA-I (pre β -HDL) whereas ABCG1 actively exports cholesterol to α -HDL (13). Both transporters play a key role in HDL-mediated cholesterol metabolism. In addition, HDL and apoA-I have anti-oxidative functions that prevent the oxidative modification of low density lipoproteins (LDL) (14,15) and exhibit anti-inflammatory properties (16). This leads to the inhibition of the expression of cellular adhesion molecules and monocyte chemotactic protein-1 (MCP-1). Although the anti-atherosclerotic properties of apoE can be attributed, in part, to its role in regulating cholesterol homeostasis, its vascular protective effects are independent of its cholesterol transport activity (2). ApoE is also linked to cholesterol efflux in macrophages, thus inhibiting foam cell formation (17).

The structural motif common to both apoE and apoA-I is the amphipathic helix, a motif responsible for lipid binding and other biological properties (18). Based on the class A amphipathic helical motif present in apolipoproteins, our laboratory has designed several apolipoprotein mimetic peptides and studied their structure-function relationship (19). Ac-hE18A-NH₂, is a unique dual-domain peptide containing the receptor binding domain of apoE (residues 141-150, LRKLRKRLLR) covalently bound to the lipid associating 18A (DWLKAFYDKVAEKLKEAF) (20) and has the sequence Ac-LRKLRKRLLRDWLKAFYDKVAEKLKEAF-NH₂. We have shown that this peptide, similar to apoE but unlike 18A, binds LDL and VLDL and facilitates their uptake by HepG2 cells via a heparan sulfate proteoglycan (HSPG) mediated pathway (21). When administered to Watanabe Heritable Hyperlipidemic (WHHL) rabbits (22), apoE null mice, or C57Bl6 mice on a high fat diet (23), Ac-hE18A-NH₂ dramatically lowers plasma VLDL and LDL, without affecting HDL. These changes were not observed when either the N-terminal peptide, LRKLRKRLLR or the lipid associating, C-terminal peptide Ac-18A-NH₂ was administered

to C57Bl6 mice on a high fat diet. Ac-hE18A-NH₂ had different *in vivo* effects from its lipid associating component (18A), suggesting that the dual-domain peptide has unique properties and is not just the sum of its constituent peptides.

Since Ac-hE18A-NH₂ is structurally similar to apoE, we tested the hypothesis that the peptide mimics biological effects of apoE in THP-1 macrophages and HepG2 cells. Herein, we present data showing that Ac-hE18A-NH₂ inhibits inflammatory responses and induces a sustained reduction in cholesterol by a mechanism involving peptide recycling. It is proposed that peptide recycling potentiates and prolongs the beneficial effects of Ac-hE18A-NH₂.

2. Materials and Methods

(Additional details presented in Supplementary material)

Materials

All cell culture materials were purchased from Cellgro unless otherwise mentioned. Other chemicals were obtained from Sigma Chemical Co. Antibody to VCAM-1 was obtained from Santa Cruz and antibodies to apoA-I and Ac-hE18A-NH₂ were from Brookwood Biomedicals, AL. KLH conjugates of apoA-I and Ac-hE18A-NH₂ were injected in rabbits. The antibodies to apoA-I and Ac-hE18A-NH₂ were purified from sera of rabbits by affinity chromatography. Antibody to apoE was generated as per the procedure of Curry et al (24). Radiochemicals were from Amersham Pharmacia Biotech (Piscataway, NJ).

Cells

HepG2 cells and THP-1 monocytes were obtained from American Tissue Culture Collection (ATCC), USA. HepG2 cells were grown in MEM containing 10% FBS and antibiotics. THP-1 monocytes were grown in RPMI media (ATCC, USA) containing 10% FBS and antibiotics and differentiated into macrophages by the addition of phorbol myristate acetate (PMA).

Peptide Synthesis

Ac-hE18A-NH₂ was synthesized by the solid phase method using Fmoc chemistry, as previously described by us (21).

Radiolabeling of the peptide

The peptide was labeled with ¹²⁵I by the method of Bilheimer et al (25) as detailed in Datta et al (21).

Recycling of Ac-hE18A-NH₂

The protocol of Swift et al (8) was followed as detailed in the Supplement.

Metabolic labeling of cells with [³⁵S] Methionine/Cysteine

The fate of newly synthesized apoE and apoA-I was studied by labeling with [³⁵S] Met/Cys as detailed in (26).

Analysis of apoE and apoA-I degradation by pulse chase

The experimental protocol detailed in (27) was followed.

Peptide-mediated Cholesterol Efflux from THP-1 monocyte-derived macrophages

Peptide-mediated cholesterol efflux was measured in THP-1-derived macrophages as detailed by Kritharides et al (28).

Inhibition of LPS-induced inflammatory responses

THP-1 monocytes were differentiated to macrophages by PMA treatment. The effect of Ac-hE18A-NH₂ and Ac-18A-NH₂ on LPS stimulation was examined in three ways: (1) Co-incubation of the peptide (50µg/ml) with LPS(1µg/ml) for 6h, (2) pre-LPS treatment, macrophages treated with Ac-hE18A-NH₂ or Ac-18A-NH₂ (50µg/ml) for 20h, washed and then treated with LPS (1µg/ml) for 6h and (3) post-LPS treatment; macrophages were treated with LPS (1µg/ml) for 6h, washed with PBS and treated with Ac-hE18A-NH₂ or Ac-18A-NH₂ (50µg/ml) for 20h. Media was collected after each treatment and analyzed for IL-6 and MCP-1 using ELISA kits from BD Biosciences, NJ, USA. The effect of Ac-hE18A-NH₂ on VCAM-1 expression by HUVECs was determined by Western blot analysis as described by us earlier (29).

3. Results

Ac-hE18A-NH₂ recycles in HepG2 cells and in THP-1 macrophages

To establish if the peptide is taken up by cells and released at a later time point (recycled), pulse-chase studies were carried out. THP-1 derived macrophages and HepG2 cells were incubated for 2h with [¹²⁵I]-Ac-hE18A-NH₂. Surface-bound peptide was removed by heparin treatment and the percent of peptide secreted into the medium at 0, 10, 60, 120, 360 and 1440 mins after peptide uptake by the cells was determined (Figure 1A). In THP-1 cells, in the first 60min, 40 ± 2.3% of the peptide was retained in the cell. However, at 120min, 56 ± 1.3% of the peptide was detected in the cell and at 360min, 61 ± 1.2% of the peptide was in the cell. After 24h, 30 ± 1.1% of the peptide was cell-associated while 70 ± 1% was in the medium. Such recycling was not observed with Ac-18A-NH₂. Analytical HPLC studies confirmed that 98% of peptide released into the medium was completely intact. The medium (or cell lysate) was resolved on a C-8 reverse phase HPLC column and eluted with a gradient of acetonitrile in water (0.1% TFA). 1ml fractions were collected and counted. The tubes containing 98% of the counts corresponded to tubes that eluted with the retention time of the peptide (Figure 1B). These results demonstrate that the peptide is recycled by THP-1 macrophages. Similar results were obtained with [¹²⁵I]-Ac-hE18A-NH₂ in HepG2 cells (Figure 1C), except that more Ac-hE18A-NH₂ (80-90%) is retained in the cell for at least 6h. 120min after the initiation of the chase, 75 ± 1.5% of the peptide was retained in the cell. However, at 240mins and 360mins, 80 ± 0.5% and 82 ± 0.8% of the peptide was detected in the cell, respectively. As in THP-1 cells, intact peptide was recovered in culture medium of HepG2 cells (similar to Figure 1B).

Ac-hE18A-NH₂ stimulates the formation of pre β -HDL in HepG2 cells in a time-dependent manner

To evaluate the effect of the peptide on apoA-I synthesis and secretion, HepG2 cells, grown to 80% confluency in 6-well plates, were treated with Ac-hE18A-NH₂ (20µg/ml). Incubation of HepG2 cells with Ac-hE18A-NH₂ for 18h caused a significant (2.5 fold) increase in apoA-I in the pre β -HDL form and a decrease in the α -HDL form compared to control cells (Figure 2A, **lane 2**). On the other hand, in the control cells, apoA-I was predominantly secreted in the α -HDL form in a time-dependent manner. After 3h of incubation with Ac-hE18A-NH₂, 99% of apoA-I in the medium was in the pre β -HDL region whereas in the control cells only 35% of apoA-I was in the pre β -HDL form. Further, after 20h of incubation, the peptide-treated conditioned medium contained 99% of apoA-I in the pre β -HDL form, while in control cells only 8% of apoA-I was in the pre β -HDL form and 92% was in the α -HDL form (Figure 2A). SDS-PAGE of the 18h conditioned medium (Figure 2B) showed no corresponding increase in apoA-I concentration suggesting that after an 18h incubation, the peptide treatment did not increase the synthesis and secretion of apoA-I. However, peptide treatment of HepG2 cells clearly modulated the subspeciation of HDL in favor of pre β -HDL formation (Fig. 2A).

To determine if the peptide was associated with α -HDL or pre- β HDL particles, HepG2 cells were treated with [125 I]-Ac-hE18A-NH₂. Agarose gel electrophoresis of the conditioned medium with and without peptide was carried out. The counts associated with the peptide were seen in the α -HDL region where very little apoA-I was seen.

The surface pressure exerted by the peptide Ac-hE18A-NH₂ at the egg PC-water interface is 45 dynes/cm, a value that is much higher than the surface pressure of 18A (30 dynes/cm) (21). Ac-18A-NH₂ (with a value of 35 dynes/cm surface pressure at the egg PC/water interface) is by itself capable of displacing apoA-I from HDL (30). However, because of its higher lipid affinity, Ac-hE18A-NH₂ is expected to displace apoA-I from HDL to a greater extent. To determine if Ac-hE18A-NH₂ is capable of displacing apoA-I from the α -HDL particle to form a lipid-poor apoA-I particle, HepG2 cells were grown in 6-well dishes to 80% confluency and fresh serum-free medium was added. After an overnight incubation, the medium was aspirated to a test tube and centrifuged to remove all floating cells. The cell-free conditioned medium was divided into eight tubes and the peptide was added to four tubes. Two of these tubes were incubated at 4°C and two at 37°C. Four other tubes (used as controls) were treated under similar conditions, but without Ac-hE18A-NH₂. After an overnight incubation, the media samples were subjected to agarose gel electrophoresis, transferred to nitrocellulose membranes and immunoblotted for apoA-I as described above. The lipid-poor apoA-I (pre- β HDL) particle was formed in presence of the peptide at both 4°C and 37°C even in the absence of cells (Figure 3). The fact that this was seen at 4°C and 37°C suggested that apoA-I from α -HDL was displaced by the peptide to form a lipid-poor pre- β HDL-like particle as described by us earlier with class A amphipathic helical peptides.

The formation of pre- β -HDL particles due to the displacement of apoA-I by Ac-hE18A-NH₂, and their continued presence over an extended period, suggests that the peptide is present in the medium. Since the peptide was shown to recycle (Fig. 1), we anticipated that displacement of apoA-I would be seen even after removal of the added peptide from the conditioned medium. Therefore, after an overnight incubation with Ac-hE18A-NH₂, the conditioned medium was removed, fresh serum-free medium without the peptide was added and the cells were incubated overnight. This was repeated for 3 consecutive days. During this period, the cells remained viable (as determined by trypan blue exclusion). The conditioned media samples from all three days were stored at 4°C and were analyzed on agarose gels for HDL subspeciation at the same time. Figure 4 (Panel A) shows the presence of pre- β -HDL in the conditioned medium from cells exposed to the peptide for 18h (lane 1), while there was a shift from pre- β -HDL to α -HDL in medium from control cells (lane 2). Panel B shows the α - and pre- β -HDL from conditioned medium on day 2 after the medium with (lane 3) or without peptide (lane 4) had been replaced with fresh medium without peptide in both treatment groups. Lane 3 shows 99% pre- β -HDL, similar to cells incubated with the peptide, even though the cells were treated only for 18h with Ac-hE18A-NH₂ after which the peptide containing medium was replaced with serum and peptide-free MEM. Lane 4 shows that both α - and pre- β -HDL are present in control cells similar to the distribution observed after 18h (lane 2). Thus, Ac-hE18A-NH₂ induced the formation of pre- β -HDL even after removal of the peptide from the incubation medium. These results suggest that Ac-hE18A-NH₂ enters the cell and is released into the medium, further corroborating our pulse chase data (Fig. 1A). These data further suggest that the peptide recycles in a fashion similar to that observed for apoE.

Ac-hE18A-NH₂ enhances the secretion of surface-bound apoE in THP-1 monocyte derived macrophages and HepG2 cells

Several amphipathic helical peptides have been shown to enhance the secretion of apoE from macrophages (31). Since apoE released from macrophages and hepatocytes plays an important role in lipoprotein metabolism, the effect of Ac-hE18A-NH₂ on apoE synthesis and secretion

was studied in THP-1 monocyte-derived macrophages and in HepG2 cells. Cells were incubated with and without Ac-hE18A-NH₂ (20 µg/ml) and [³⁵S]-labeled Met/Cys for 4h. [³⁵S]-labeled apoE was immunoprecipitated from the conditioned medium and from the cell lysate followed by SDS PAGE and autoradiography. Figure 5, **Panel A** shows a 4-fold increase in the newly synthesized apoE secreted by THP-1 monocyte-derived macrophages treated with Ac-hE18A-NH₂ (lane 2) compared to control cells (lane 1). A concomitant increase in apoE was observed in the control cell lysate (lane 4) compared to the peptide-treated cells (lane 5). Similar results were obtained in HepG2 cells (results not shown). The effect of the peptide on the intracellular stability of apoE was examined by pulse-chase experiments. ApoE was equally stable in control and peptide-treated cells; [³⁵S]-labeled apoE that disappeared from the cells was quantitatively recovered in the medium.

Secreted apoE can be of intracellular origin or from the small pool of cell-surface associated apoE. To determine the origin of the apoE secreted in the presence of the peptide, the experiment was carried out in the presence of heparin (32) which removes surface-bound apoE. Figure 5, **Panel B** shows a 2.6 fold increase in the amount of apoE in the medium of control cells treated with heparin (lane 3) compared to cells without heparin treatment (lane 1). On the other hand, cells treated with Ac-hE18A-NH₂ secreted a similar amount of apoE with or without heparin treatment (lanes 2 and 4). In this case, there was a 1.8 fold increase in the secretion of apoE in cells treated with peptide and heparin (lane 4) compared to control cells treated with heparin only (lanes 3). These results suggest that Ac-hE18A-NH₂ competes with cell surface apoE to release it to the medium.

To determine if Ac-hE18A-NH₂ also enhances secretion of newly synthesized apoE, mRNA levels for apoE were determined using real time PCR. There was no significant change in apoE mRNA levels in the peptide-treated cells compared with control cells (Figure 5, **Panel C**). The stability of apoE was not altered by the peptide as measured by ³⁵S pulse chase measurements.

Ac-hE18A-NH₂ stimulates cholesterol efflux in THP-1 monocyte-derived macrophages

Since pre-β-HDL enhances cholesterol efflux, we measured the effect of Ac-hE18A-NH₂ on cellular cholesterol efflux. Cholesterol efflux was measured in [³H]-cholesterol-loaded THP-1 macrophages in the presence of Ac-hE18A-NH₂ as an acceptor and was compared with controls (no added acceptor) and in the presence of HDL, a known enhancer of cholesterol efflux. Ac-hE18A-NH₂ enhanced cholesterol efflux in a concentration dependent manner (Figure 6). A stimulation of 10% compared to control was observed at 5 and 10 µg/ml peptide that was similar to that observed with HDL (10 µg/ml). At 50 µg/ml a maximum of 40% stimulation was observed. Unlike Ac-18A-NH₂, cholesterol efflux by Ac-hE18A-NH₂ was independent of ABCA1-mediated cholesterol efflux (data not shown).

Ac-hE18A-NH₂ inhibits LPS-induced inflammatory responses

We tested whether the peptide exerts similar anti-inflammatory effects as apoE. Specifically, we tested effects of Ac-hE18A-NH₂ on expression levels of the adhesion molecule VCAM-1 in HUVECs. Ac-hE18A-NH₂ significantly inhibited LPS-induced VCAM-1 expression in a concentration-dependent manner as determined by Western blot analysis (Figure 7A). Addition of 50 µg of the peptide also inhibited LPS-induced monocyte adhesion to HUVECs by 75%. Further, an 18-fold increase in LPS-mediated mRNA for MCP-1 was seen in THP-1 macrophages, which was attenuated by Ac-hE18A-NH₂ (results not shown).

The effect of Ac-hE18A-NH₂ on LPS-mediated induction of the inflammatory cytokine, IL-6 and chemokine MCP-1 was examined in THP-1 macrophages in three different ways: (1) Co-incubation of the peptide with LPS for 6h (2) pre-incubation (20h) of the macrophages with the peptide prior to stimulation of cells with LPS (6h) and (3) post-incubation with peptide

after stimulation with LPS (6h) and removal. Co-incubation and pre-incubation resulted in significant inhibition of both IL-6 (5-fold) and MCP-1 (3-fold) secretion (Figure 7B). Incubation (20h) of THP-1 macrophages with the peptide after LPS treatment (6h) and removal also significantly inhibited both IL-6 (2-fold) and MCP-1 (4-fold) secretion (Figure 7C). Although MCP-1 was inhibited similarly under both conditions, IL-6 inhibition was less when the peptide was administered post-LPS compared to pre-LPS treatment. Ac-18A-NH₂ inhibits LPS induced IL-6 and MCP-1 only when co-incubated with LPS. It had no effect in the pre- and post-incubation treatment groups.

4. Discussion

Apolipoprotein mimetic peptides represent a promising new strategy to not only raise HDL levels but also to improve its functional properties (22,33). ApoA-I mimetic peptides have been shown to possess anti-oxidant and anti-inflammatory activities (19) and promote cholesterol efflux (34) *in vitro* and *in vivo* (35). Ac-hE18A-NH₂, similar to apoE, enhances the uptake and degradation of plasma apoB-containing lipoproteins, leading to reduced plasma cholesterol levels. In Watanabe Heritable Hyperlipidemic (WHHL) rabbits, the peptide lowered apoB-containing cholesterol levels, without changing HDL levels (23); in dyslipidemic mice, it improved HDL function by increasing plasma PON-1 activity, resulting in enhanced clearance of lipid hydroperoxides (22). The present investigation shows that, in addition to the above properties, Ac-hE18A-NH₂ possesses highly unexpected antiatherogenic properties. First, the peptide enhances the secretion of pre β -HDL and apoE. Second, by virtue of its ability to recycle, protective, apoE-like properties are prolonged. These properties underscore the potential therapeutic benefit of this peptide to inhibit atherosclerosis and other lipid-related inflammatory diseases.

The most interesting observation in this study was the ability of Ac-hE18A-NH₂ to undergo recycling in macrophages and HepG2 cells (9). This was demonstrated by the reappearance of [¹²⁵I]-labeled peptide at the cell surface and by its stimulatory effect on pre β -HDL formation even after the removal of the peptide from the medium. ApoE recycling in hepatocytes and macrophages is associated with cholesterol efflux and HDL internalization (36). Our studies show that the recycling of Ac-hE18A-NH₂ is also associated with the formation of pre β -HDL particles which could be involved in increased cholesterol efflux. This peptide has significantly increased phospholipid affinity (21) and thus forms phospholipid-rich particles, which, in turn, are efficient mediators of cellular cholesterol efflux, by a mechanism that is ABCA1-independent (37). These data suggest that recycling of Ac-hE18A-NH₂ is linked to cellular cholesterol removal but is not mediated by ABCA-1, similar to apoE recycling in CHO cells (38). A small molecule such as Ac-hE18A-NH₂ that would mimic the lipid associating and HSPG binding properties of apoE could compete for cell surface apoE.

ApoE has been shown to reduce plasma cholesterol *via* the binding of the apoE-containing particles to HSPG (5). In addition, apoE also exhibits anti-inflammatory properties. Secretion of trace amounts of apoE by macrophages has been shown to inhibit lesion formation in apoE null mice (39). Cell-derived apoE also inhibits the expression of VCAM-1 in HUVECs (40). The effect of macrophage-derived apoE on cholesterol efflux and its other pleiotropic effects (41,42) may collectively contribute to the atheroprotective properties of this peptide. Ac-hE18A-NH₂ induces the secretion of apoE and also enhances the ability to efflux cholesterol from THP-1-derived macrophages. Endogenously secreted apoE has been shown to stimulate sterol efflux by an ABCA1-independent pathway in mouse fibroblast cells (43). The observed effect of Ac-hE18A-NH₂ could be due to the combined effluxing capacity of the secreted apoE and that of the peptide.

ApoE has been shown to inhibit IL-1 β -induced inflammatory responses (44). The observed inhibition of LPS-induced VCAM-1, IL-6 and MCP-1 expression by Ac-hE18A-NH₂ in HUVECs and THP-1 macrophages may be related to the ability of the peptide to neutralize LPS. Ac-hE18A-NH₂ and Ac-18A-NH₂ are amphipathic peptides and bind to the lipid A component of LPS. The positive charges at the N-terminus of Ac-hE18A-NH₂ also bind to the negatively charged headgroup of LPS, similar to polymyxin B. We have shown that Ac-hE18A-NH₂ inhibits LAL activity of LPS unlike Ac-18A-NH₂ (unpublished data). Pre-incubation of cells with the peptide also renders cells resistant to LPS-induced IL-6 and MCP-1 upregulation, suggesting that Ac-hE18A-NH₂ that is recycled can attenuate the effects of LPS. However, results with post-LPS incubation (Figure 7C), suggest that Ac-hE18A-NH₂ could either act directly on cells or exert its anti-inflammatory effects via the peptide-induced secretion of cell-surface apoE and pre β -HDL formation. While Ac-hE18A-NH₂ seems to inhibit LPS effects by either binding to LPS or exerting direct effects on the cell, Ac-18A-NH₂ appears to act by binding to LPS.

The results of the current studies show that Ac-hE18A-NH₂, an apoE-mimetic peptide, that is highly efficient in mediating the uptake of apoB-containing particles, is also anti-inflammatory. Its ability to recycle (similar to apoE), results in the generation of pre β -HDL for an extended period. Furthermore, the peptide-mediated secretion of apoE can contribute to its anti-inflammatory property. Ac-hE18A-NH₂, by virtue of its ability to enhance the uptake of atherogenic lipoproteins, its anti-inflammatory properties and its ability to recycle, has the potential to provide new therapeutic strategies for the treatment of cardiovascular and other inflammatory diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1B

Figure 1. Recycling of the peptide, Ac-hE18A-NH₂

(A) Pulse chase of [¹²⁵I]-Ac-hE18A-NH₂ in THP-1 derived macrophages. The experimental details are given in Materials and Methods. The experiment was repeated three times with N=6 each time. Black: media, grey: cell. (B) HPLC profile of conditioned medium from HepG2 cells at the 24h time point. The ¹²⁵I counts of the labeled peptide (----) overlap with the retention time of the cold peptide (—) (C) The peptide Ac-hE18A-NH₂ recycles in HepG2 cells. Pulse chase of [¹²⁵I]-Ac-hE18A-NH₂ in HepG2 cells. The experimental details are given in Materials and Methods. The experiment was repeated three times with N=6 each time. Black: media, grey: cell.

Figure 2. A: Ac-hE18A-NH₂ promotes the secretion of preβ-HDL in HepG2 cells

HepG2 cells were incubated overnight with serum-free MEM containing Ac-hE18A-NH₂ (20μg/ml). The conditioned medium was subjected to agarose electrophoresis and proteins were immunoblotted for apoA-I. Control medium (no Ac-hE18A-NH₂, lane 1) contained 99% of apoA-I in the α-HDL form while the medium from peptide treated cells (lane 2) contained 98% of apoA-I in the preβ-HDL form. **B:** Ac-hE18A-NH₂ does not alter the total mass of apoA-I secreted into the conditioned medium of HepG2 cells. HepG2 cells were incubated with the peptide (20μg/ml) overnight and the conditioned medium was subjected to SDS PAGE. Proteins were transferred to a nitrocellulose membrane and immunoblotted for apoA-I.

Molecular weight marker (lane 1), control conditioned medium (lane 2) and Ac-hE18A-NH₂ treated (lane 3).

Figure 3. Ac-hE18A-NH₂ displaces apoA-I from α -HDL particles

Media from HepG2 cells (at 90% confluence) was taken, centrifuged to remove any remaining cells, and incubated O/N at (Panel A) 4°C and (Panel B) 37°C with and without Ac-hE18A-NH₂ (20 μ g/ml). 10 μ l samples from each of these treatments were run on an agarose gel, transferred to a nitrocellulose membrane and probed for apoA-I with an antibody to human apoA-I. Control samples (lanes 2 and 4) show the presence of greater amount of α -HDL and much less pre- β HDL, while the peptide treated samples (lanes 1 and 3) had predominantly pre- β HDL, just as in the samples that were obtained in presence of the cells.

Figure 4A

Figure 4. The effect of Ac-hE18A-NH₂ on the formation of preβ-HDL is seen even after removal of the peptide from the incubation medium

(A) Flow diagram of the experimental protocol. HepG2 cells (at 90% confluency) were incubated at 37°C with or without Ac-hE18A-NH₂ (20μg/ml). After an overnight incubation, the conditioned medium (medium 1) was removed and fresh medium **without the peptide** was added to both control as well as to cells that had previously been treated with the peptide. The medium (medium 2) was removed after an overnight incubation and subjected to agarose gel electrophoresis. Proteins were immunoblotted for apoA-I. **(B)** Panel A: Lanes 1 (peptide treated) and lane 2 (control) show the formation of preβ-HDL on Day 1 (medium 1). Panel B shows the HDL subspecies formed on Day 2 (medium 2) in the medium of cells that had been

previously exposed to Ac-hE18A-NH₂ for 18h followed by a 24h incubation without peptide (lane 3), and in the medium of cells that were not treated with the peptide at all (lane 4).

Figure 5. Ac-hE18A-NH₂ releases surface-bound apoE

A. Immunoprecipitation of [³⁵S]-apoE from conditioned medium and cell lysate of THP-1 monocyte-derived macrophages treated with or without Ac-hE18A-NH₂. Proteins were resolved by SDS-PAGE. Gels were stained, dried and were subjected to autoradiography. The autoradiogram shows apoE from medium and cell lysate in control (lanes 1 and 4) and peptide-treated cells (lanes 2 and 5). The intensities of the bands (IOD) were quantitated using computer assisted densitometry. The increase in apoE in the conditioned medium of Ac-hE18A-NH₂ treated cells was accompanied by a decrease in apoE in the cell lysate suggesting that the peptide induced secretion of apoE. **B.** THP-1 monocyte derived macrophages were pulsed with [³⁵S]-Met/Cys for 60min. After removal of surface bound apoE, the cells were treated with Ac-hE18A-NH₂ (20µg/ml) or without peptide. ApoE was immunoprecipitated using polyclonal antibody to human apoE. Proteins were resolved on SDS-PAGE. Gels were stained, dried and subjected to autoradiography. The autoradiogram shows medium from (1) control cells without heparin treatment (lane 1), (2) cells treated with Ac-hE18A-NH₂ and no heparin (lane 2), (3) Control cells treated with heparin (lane 3) and (4) cells treated with heparin and Ac-hE18A-NH₂ (lane 4). In peptide-treated (no heparin) cells, secreted apoE was four times greater than that of control cells. Heparin-treatment increased the secreted apoE in control cells suggesting that the peptide releases surface-bound apoE. **C.** RNA was extracted from cells (with or without Ac-hE18A-NH₂). ApoE mRNA levels (normalized to β actin) were determined

using real time PCR. There was no significant difference in apoE mRNA levels under the two experimental conditions.

Figure 6. Ac-hE18A-NH₂ stimulates cholesterol efflux in a concentration dependent manner
THP-1 derived macrophages were loaded with ¹⁴C-cholesterol. Efflux was monitored with different concentrations of Ac-hE18A-NH₂. Percent efflux increased with increasing concentration of peptide.

Figure 7B

Figure 7C

Figure 7. Ac-hE18A-NH₂ inhibits LPS-induced inflammatory responses

(A) Ac-hE18A-NH₂ inhibits the expression of VCAM-1 in HUVECs in a concentration dependent manner. HUVECs were treated with LPS (1 µg/ml) in the presence of Ac-hE18A-NH₂ (0-50 µg/ml) for 6h. Immunoblotting studies showed that VCAM-1 protein levels were reduced in the peptide treated samples in a concentration dependent manner. N=6 for each treatment. *p<0.05, **p<0.005 compared to the control (no peptide). (B) Co-incubation and pre-incubation of THP-1 macrophages with Ac-hE18A-NH₂ (50 µg/ml) and LPS (1 µg/ml) significantly inhibited IL-6 and MCP-1 secretion (C) Post-incubation of THP-1 macrophages with Ac-hE18A-NH₂ (50 µg/ml) after stimulation with LPS (1 µg/ml) and removal of LPS significantly inhibited IL-6 and MCP-1 secretion. Black bars: control, Grey bars: peptide treated.