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Original Communication

The apolipoprotein E mimetic peptide AEM-2 attenuates mitochondrial injury and apoptosis in human THP-1 macrophages

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ABSTRACT

Cardiovascular disease, specifically atherosclerosis, is exacerbated by hypercholesterolemia. Current therapies that target lipid lowering, however, are not effective in all patients. Apolipoprotein E (apoE) plays an important role in mediating the clearance of plasma cholesterol and also exerts numerous cytoprotective responses. Our laboratory has synthesized novel therapeutics that mimic the ability of apoE to decrease plasma cholesterol. The apoE mimetic peptide AEM-2 is a dual domain peptide composed of an amphipathic helical region that binds phospholipids and a positively charged region that mediates the hepatic clearance of lipoproteins. Administration of AEM-2 to apoE-null mice reduced plasma cholesterol concentration by 80% one hour post-administration. Since apoE is also known to exert anti-inflammatory effects that are independent of its ability to lower cholesterol, we tested effects of AEM-2 on lipopolysaccharide-induced responses in human THP-1 macrophages. Pre-treatment of THP-1 cells with AEM-2 significantly reduced the lipopolysaccharide (LPS)-induced secretion of interleukin-6 (IL-6) and tumor necrosis factor α (TNFα). Since LPS administration is associated with an increase in mitochondrial injury, we monitored effects of AEM-2 on mitochondrial function. AEM-2 significantly reduced mitochondrial superoxide formation, prevented the LPS-induced decrease in mitochondrial membrane potential and attenuated the release of cytochrome c. AEM-2 also inhibited the activities of initiator caspases 8 and 9 and effector caspase 3. The attenuation of apoptosis in AEM-2-treated cells was associated with an increase in cellular autophagy. These data suggest that AEM-2 attenuates cellular injury in LPS-treated THP-1 macrophages and facilitates the removal of cellular debris and damaged organelles *via* induction of autophagy.

KEYWORDS: apoE mimetic peptide, apoptosis, inflammation, mitochondria.

ABBREVIATIONS

βME	:	β-mercaptoethanol
$\Delta\Psi_m$	:	mitochondrial membrane potential
ABCA1	:	ATP binding cassette transporter A1
ABCG1	:	ATP binding cassette transporter G1
AEM	:	apolipoprotein E mimetic peptide
AEM-2	:	Ac-Aha-LRRLRRRLLR-DWLKAFYDKVAEKLKEAF-NH_2
Aha	:	α-aminohexanoic acid
CQ	:	chloroquine
FBS	:	fetal bovine serum
FCCP	:	carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
Fmoc	:	fluorenylmethyloxycarbonyl chloride
HSPG	:	heparan sulfate proteoglycan
IL-6	:	interleukin-6

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LPS : lipopolysaccharide
LC3 : microtubule-associated protein 1A/1B-light chain 3
PMA : phorbol 12-myristate 13-acetate
TFA : trifluoroacetic acid
TMRM : tetramethylrhodamine methyl ester
TNFα : tumor necrosis factor α
Trp : tryptophan
WD : Western diet

INTRODUCTION

Apolipoprotein E is an exchangeable apolipoprotein that is associated with high-density lipoprotein (HDL), very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) remnant particles. It is synthesized by hepatocytes and macrophages and plays an important role in reducing plasma cholesterol levels. Native apoE contains a lipid-associating domain (residues 203-266) and a globular domain (residues 1-191) containing an LDL receptor binding site (residues 141-150 with the sequence LRKLRKRLLR) [1]. The presence of the LDL receptor-binding domain facilitates the hepatic clearance of cholesteryl esters, resulting in a significant decrease in plasma cholesterol [2]. While LDL is cleared by the LDL receptor, other apoE-containing lipoproteins can be cleared by alternate receptors. These include the LDL-receptor-related proteins (LRP) and heparan sulfate proteoglycans (HSPGs) located in the space of Disse [3]. Thus, apoE plays a prominent role in clearing pro-atherogenic, apoB-containing lipoproteins such as chylomicrons, VLDL, and remnant lipoproteins, all of which possess cholesterol levels per particle that are much higher than that of LDL.

ApoE also exerts anti-inflammatory effects that are independent of its cholesterol-lowering property. These include a repression of macrophage activation, inhibition of thrombosis, anti-oxidant effects and activation of cell survival signaling cascades [4, 5]. It follows that apoE gene polymorphisms are associated with an enhancement of the inflammatory response [6, 7]. Previous studies suggest that upregulation of apoE in monocytes induces an anti-inflammatory phenotype in these cells and reduces their recruitment to sites of injury [8-11]. Monocytosis increases tissue injury in models of sepsis and atherosclerosis [12, 13]. HDL-associated apoE regulates this process by inhibiting hematopoietic stem cell proliferation, mobilization, and the production of monocytes [13]. The protective role of apoE under these conditions was associated with apoE binding to monocyte HSPGs and an interaction with the cholesterol transporters ATP binding cassette transporter A1/ATP binding cassette transporter G1 (ABCA1/ABCG1) to reduce cellular cholesterol content [13, 14]. HDL-associated apoE thus plays a critical role in ameliorating inflammatory cell injury.

HMG-CoA reductase inhibitors (statins) represent the gold standard for the treatment of lipid disorders by inhibiting the *de novo* synthesis of cholesterol. A significant number of patients with coronary artery disease, however, are resistant to statin therapy [15, 16]. Further, the use of other cholesterol-lowering therapies may be limited by unwanted side effects and adverse reactions [17]. apoE is an additional target for therapy; however, at present, there are no pharmacological agents that increase levels of the apolipoprotein. Due to the importance of apoE in not only reducing levels of atherogenic lipoproteins but also exerting cholesterol-independent atheroprotective effects, our laboratory initiated the development of synthetic peptides which are structurally and functionally similar to native apoE but possess unique sequences [18, 19]. In this report, we describe effects of an apoE mimetic peptide analog bearing these structural motifs on physiological responses *in vivo* and *in vitro*.

MATERIALS AND METHODS

ApoE mimetic peptide synthesis

A synthetic peptide mimicking properties of endogenous apoE was synthesized using the solid phase peptide synthesis method using 4-methylbenzhydrylamine hydrochloride resin (0.49 mM/g) [19]. We previously reported that 18A, a class A amphipathic peptide (DWFKAFY DKVAEKFKEAF) binds to phospholipids and mediates cholesterol efflux from macrophages [18]. This peptide was covalently linked to an arginine-rich peptide sequence (LRRLRRRLLR) that shares sequence similarities with the LDL receptor-binding domain present in native apoE.

Fmoc-aminohexanoic acid (Aha) was added to the amino terminus of the arginine-rich region. This modification increases the hydrophobicity of the resulting peptide and thus enhances binding to cell membranes. After removal of Fmoc-group, the peptide resin was acetylated, and peptide was cleaved from the resin using trifluoroacetic acid (TFA) (in presence of scavengers to protect tryptophan (Trp)) to yield the stable peptide Ac-Aha-LRRLRRRLLR-18A-NH_2 (AEM-2). Peptide was purified using preparative HPLC and purity was confirmed by analytical HPLC and mass spectrometry. In this report, AEM-2 will be used to denote this dual domain peptide.

Animals

Ten-week-old, female apoE-null (B6.129P2-*ApoE*^{tm1Unc}/J) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and allowed a 1 week recovery period prior to initiating experimental protocols. Mice were fed a Western diet (WD/Research Diets, Inc) for 1 week, and water was provided *ad libitum*. As expected, WD feeding increased the plasma cholesterol values from approximately 450 mg/dL to approximately 900 mg/dL. Mice were maintained at constant humidity (60 ± 5%), temperature (24 ± 1 °C), and light cycle (6 am to 6 pm). All protocols were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham and were consistent with the Guide for the Care and Use of Laboratory Animals: Eighth Edition (National Research Council. 2011). After one week, mice were randomized to receive a single dose of AEM-2 (100 µg/mL; n = 5) or an equivalent volume of saline (n = 4) *via* retro-orbital injection. Blood samples were collected after 1, 5 and 24 hrs for measurement of plasma cholesterol.

Cell culture

THP-1 monocytes were purchased from American Type Culture Collection (Manassas, Virginia) and grown in RPMI Medium 1640 containing 10% heat-inactivated fetal bovine serum (FBS), 0.05 mM β-mercaptoethanol (βME), and penicillin/streptomycin. THP-1 cells were differentiated to a macrophage phenotype by addition of 10 ng/ml phorbol 12-myristate 13-acetate (PMA) for 72 hrs. Cells were then treated with AEM-2 (20 and 50 µg/mL) for 24 hrs in RPMI 1640 media containing 10% lipoprotein-deficient serum. Culture medium was then replaced, followed by addition of LPS (1 µg/mL) for a 6-hr treatment period.

Cellular cholesterol measurements

The cholesterol content of THP-1 macrophages was measured in cell lysates 24 hrs after AEM-2 treatment. Cholesterol levels were assessed using an Amplex Red Cholesterol Assay kit (Invitrogen) and were normalized to cell protein content.

Cytokine expression

Conditioned medium was collected from AEM-2-treated THP-1 cells 6 hrs after exposure to LPS. Levels of TNFα and IL-6 were measured by ELISA (BD Biosciences). Data are expressed as a percent of the maximum response to LPS.

Mitochondrial oxidant formation

Mitochondrial oxidant formation was assessed using the fluorescent probe Mitosox (Invitrogen). THP-1 macrophages were treated with AEM-2 (20 and 50 µg/mL) or saline for 24 hrs. Cells were then exposed to LPS for 6 hrs. At the end of the treatment period, cells were washed, and Mitosox (5 µM) was added for 10 min. After washing, Mitosox-dependent fluorescence ($510_{ex}/580_{em}$) was monitored on a plate reader. Hydrogen peroxide (0.3%) (H_2O_2) was added to control groups for 15 min to elicit maximal reactive oxygen species formation. Data are expressed as a percent of the maximum response to H_2O_2.

Mitochondrial membrane potential ($\Delta\Psi_m$)

$\Delta\Psi_m$ was measured using the fluorescent probe tetramethylrhodamine methyl ester (TMRM/ThermoFisher Scientific). Cells were treated with AEM-2 (20 and 50 µg/mL) or saline for 24 hrs, followed by LPS treatment for 6 hrs. Cells were then washed, and TMRM (200 nM) was added for 30 min at 37 °C. After washing, TMRM-dependent fluorescence ($540_{ex}/590_{em}$) was monitored on a plate reader. In control experiments, cells were treated with the mitochondrial uncoupling agent carbonyl cyanide 4-(trifluoromethoxy)phenyl-hydrazone (FCCP) (10 µM) prior to measurement of fluorescence, in order to disrupt the proton electrochemical gradient and $\Delta\Psi_m$.

Cytochrome c release

AEM-2-treated THP-1 macrophages were exposed to LPS, as described previously. At the end of the treatment period, cells were lysed, and the cytosolic fraction was collected by centrifugation. The concentration of cytochrome c in this fraction was measured using a commercially available ELISA kit (Abcam Inc).

Caspase activity assays

THP-1 macrophages were grown on 100 mm dishes and treated with AEM-2 followed by LPS. Cells were scraped, collected and centrifuged (1,000 rpm, 5 min). Pellets were resuspended in 100 µl of lysis buffer. Caspase 3, 8, and 9 activities were then assessed using commercially available kits (BioVision Inc.).

Western blot analysis

Cell lysates (30 µg) of THP-1 cells treated with AEM-2 and LPS were subjected to SDS-PAGE (15% gel), transferred to nitrocellulose membranes and probed for microtubule-associated protein 1A/1B-light chain 3 (LC3) using a rabbit polyclonal antibody (Sigma Inc.). The housekeeping protein β-actin was detected using a rabbit polyclonal antibody (Sigma Inc.). After labeling with appropriate secondary antibodies, proteins were visualized using Millipore Immobilon chemiluminescent substrate.

Statistical analysis

Data are reported as mean ± SEM. Statistical analysis was performed using SigmaPlot 12 software (Systat Software). Differences between treatment groups were assessed by one-way analysis of variance with *post hoc* testing (Student-Newman-Keuls test). A P value of less than 0.05 was considered statistically significant.

RESULTS

We hypothesized that the dual domain structure of AEM-2 would facilitate cholesterol clearance *in vivo*. Thus, the capacity of AEM-2 to lower plasma cholesterol was assessed in apoE-null mice fed a WD. Cholesterol levels were measured in mice at baseline and 1, 5 and 24 hrs after retro-orbital injection of AEM-2 (n = 5) or an equivalent volume of saline (n = 4). At baseline, plasma cholesterol was 895 ± 66 mg/dL and 905 ± 24 mg/dL in mice randomized for saline and AEM-2 treatment, respectively. While there was a modest reduction in plasma cholesterol in saline-treated mice over the 24-hr treatment period, this did not reach statistical significance. AEM-2 treatment, however, reduced cholesterol levels by 80% and 82% at 1 and 5 hrs after administration, respectively. At the end of the 24-hr treatment period, plasma cholesterol levels were similar in saline and AEM-2-treated mice (Fig. 1).

Since apoE is known to exert cytoprotective effects that are independent of its ability to lower plasma cholesterol, we tested effects of AEM-2 on inflammatory responses in human THP-1 macrophages. THP-1 cells were differentiated to a macrophage phenotype by exposure to PMA for 72 hrs. Cells were then treated with AEM-2 (20 and 50 µg/mL) for 24 hrs. In initial studies, we monitored effects of AEM-2 on cell cholesterol content. Administration of AEM-2 modestly reduced the cholesterol content of THP-1 macrophages, but these values were not significantly different from the cholesterol content of control cells receiving saline treatment (Fig. 2). Since previous studies suggested that apoE attenuates the activation of monocytes/macrophages, we tested effects of AEM-2 on inflammatory responses in THP-1 cells [4]. THP-1 macrophages were incubated with AEM-2 (20 and 50 µg/mL) for 24 hrs, followed by replacement with medium containing LPS (1 µg/mL) for 6 hrs. Pre-treatment with 20 µg/mL AEM-2 did not significantly reduce the LPS-induced release of IL-6 or TNFα; however, 50 µg/mL AEM-2 reduced cytokine release by 37% and 41%, respectively (Fig. 3).

Since the activation of macrophages by LPS is associated with an increase in mitochondrial oxidant production, we monitored effects of AEM-2 on the redox status of THP-1 macrophages [20, 21]. Cells were treated with saline or AEM-2, followed by addition of LPS. Untreated cells served as control for basal oxidant formation in THP-1 macrophages. At the end of the incubation period, Mitosox (5 µM) was added for 10 min. In the absence of AEM-2 treatment, Mitosox-dependent fluorescence was significantly increased in LPS-treated THP-1 macrophages compared to saline-treated control cells (Fig. 4A). Pre-treatment

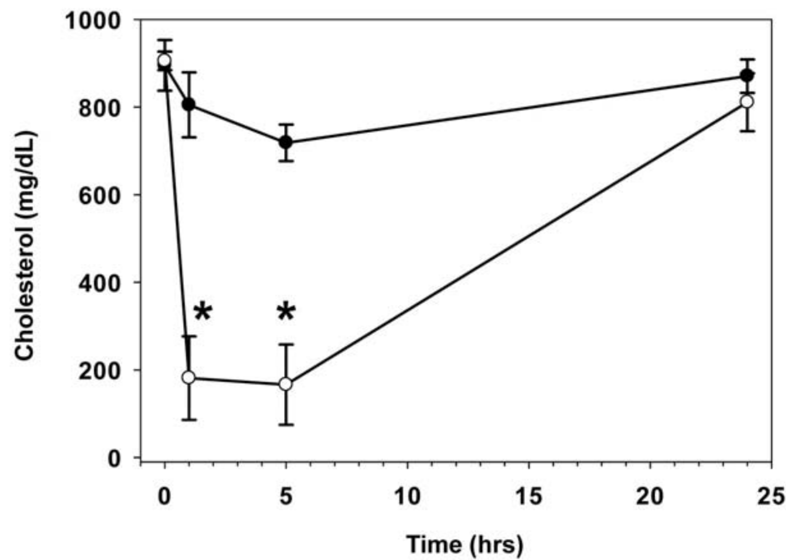

Fig. 1. AEM-2 reduces plasma cholesterol in apoE-null mice. A blood sample was collected from mice at baseline. Mice were then randomized to receive 100 µg/mL AEM-2 (○, n = 5) or an equivalent volume of saline (●, n = 4) by retro-orbital injection. Blood samples were collected after 1, 5 and 24 hrs for measurement of plasma cholesterol. Data represent the mean ± SEM. * denotes a significant difference ($P < 0.05$) compared to control.

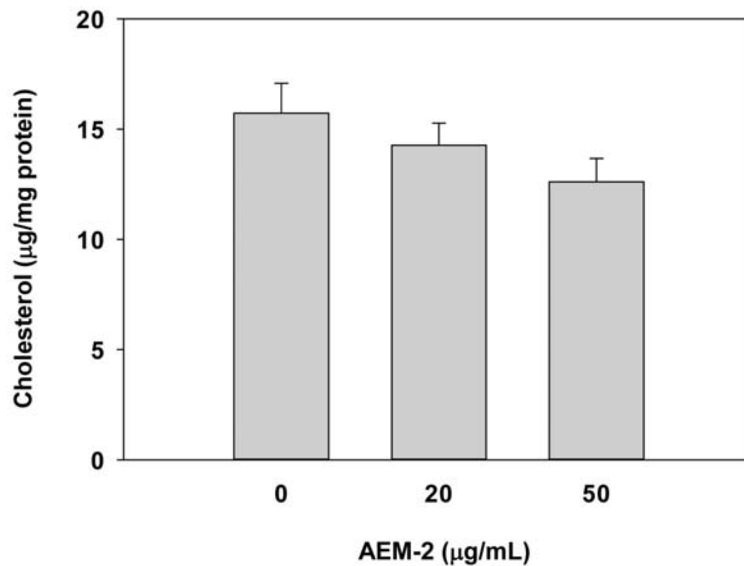

Fig. 2. Measurement of cellular cholesterol content in THP-1 macrophages. THP-1 cells were treated with saline or AEM-2 for 24 hrs. Cells were then collected and lysed prior to measurement of cholesterol content. Data were normalized to cellular protein and represent the mean ± SEM (n = 12 per treatment).

with 50 µg/mL AEM-2 significantly reduced mitochondrial oxidant formation. The stimulatory effect of LPS on mitochondrial oxidant formation was associated with an increase in mitochondrial injury and a reduction in $\Delta\Psi_m$ (Fig. 4B). In contrast, treatment with 50 µg/mL AEM-2 was associated with an increase in $\Delta\Psi_m$.

A reduction in $\Delta\Psi_m$ induces matrix remodeling, resulting in the release of cytochrome c from the inner mitochondrial membrane to the cytosol [22].

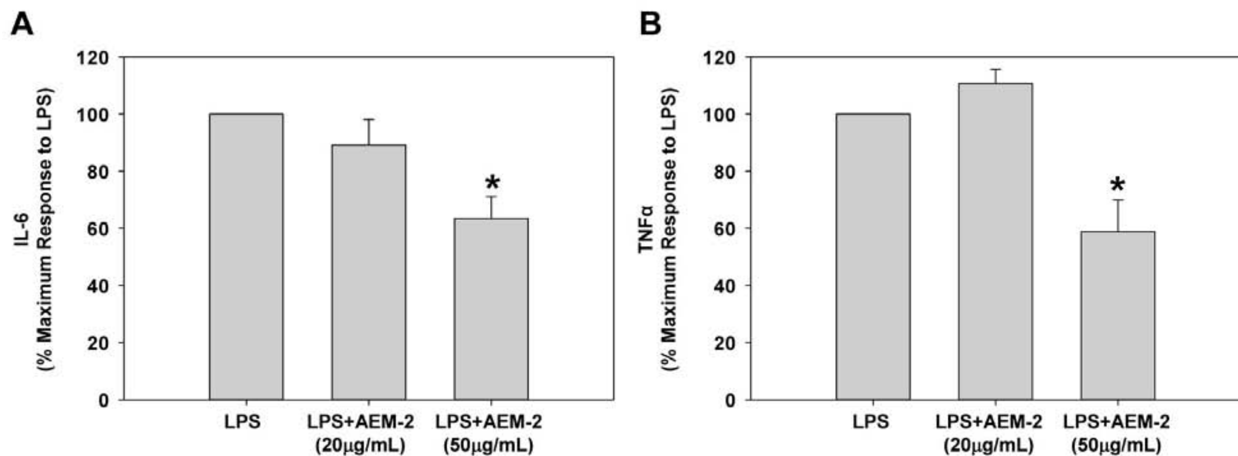

Fig. 3. AEM-2 pretreatment attenuates IL-6 and TNF-α secretion in THP-1 macrophages. THP-1 cells were treated with saline or AEM-2 for 24 hrs. Cells were thoroughly washed, followed by addition of LPS (1 μg/ml) for 6 hr. The secretion of IL-6 (Panel A) and TNFα (Panel B) in conditioned media was then determined by ELISA. Data represent the mean ± SEM (N = 12 per treatment). * denotes a significant difference ($P < 0.05$) compared to LPS treatment.

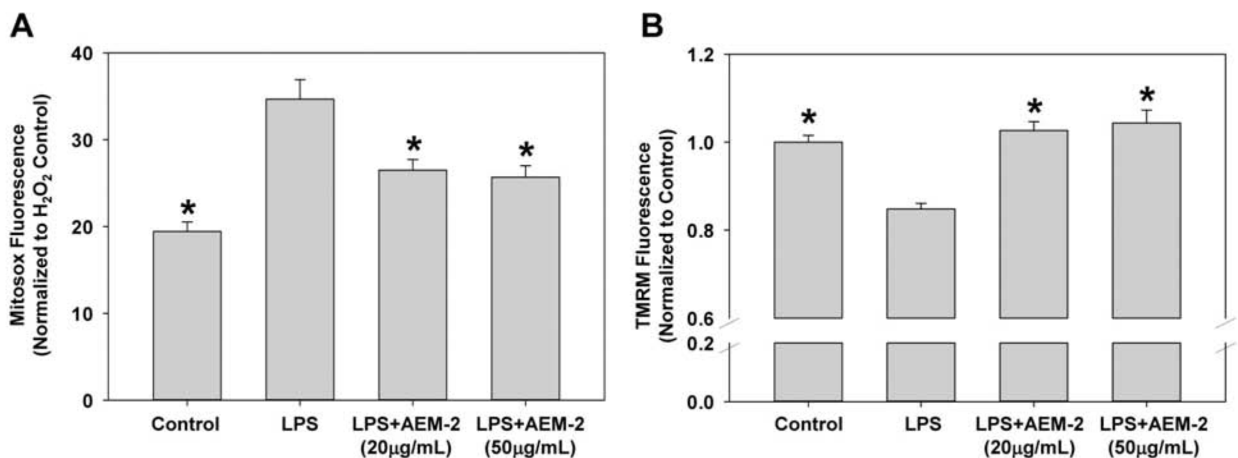

Fig. 4. AEM-2 attenuates mitochondrial injury in THP-1 macrophages. THP-1 cells were treated with saline or AEM-2 for 24 hrs. LPS (1 μg/ml) was added for 6 hr, followed by incubation with 5 μM Mitosox for 10 min. Superoxide-dependent fluorescence was then monitored at $510_{ex}/580_{em}$ (Panel A). Under these experimental conditions, $\Delta\Psi_m$ was measured using the indicator TMRM. Fluorescence was monitored at $540_{ex}/590_{em}$ (Panel B). Data represent the mean ± SEM (N = 6-12 per treatment). * denotes a significant difference ($P < 0.05$) compared to LPS.

This is an important initiating step in apoptotic cell death [23]. Our results show that LPS administration increased the translocation of cytochrome c to the cytosol in THP-1 macrophages (Fig. 5A). Consistent with the inhibitory effect of AEM-2 on mitochondrial oxidant formation and a reduction in $\Delta\Psi_m$, the peptide significantly reduced the release of cytochrome c (Fig. 5A). We next tested whether AEM-2 inhibited the activation of caspase 9. We found that the induction of caspase 9 by LPS was significantly reduced by pretreatment with AEM-2 (Fig. 5B). LPS has also been shown to activate the extrinsic cell death pathway *via* stimulation of caspase 8 activity [24]. Our results confirm that LPS induces caspase 8 in THP-1 macrophages, and this activity was attenuated by

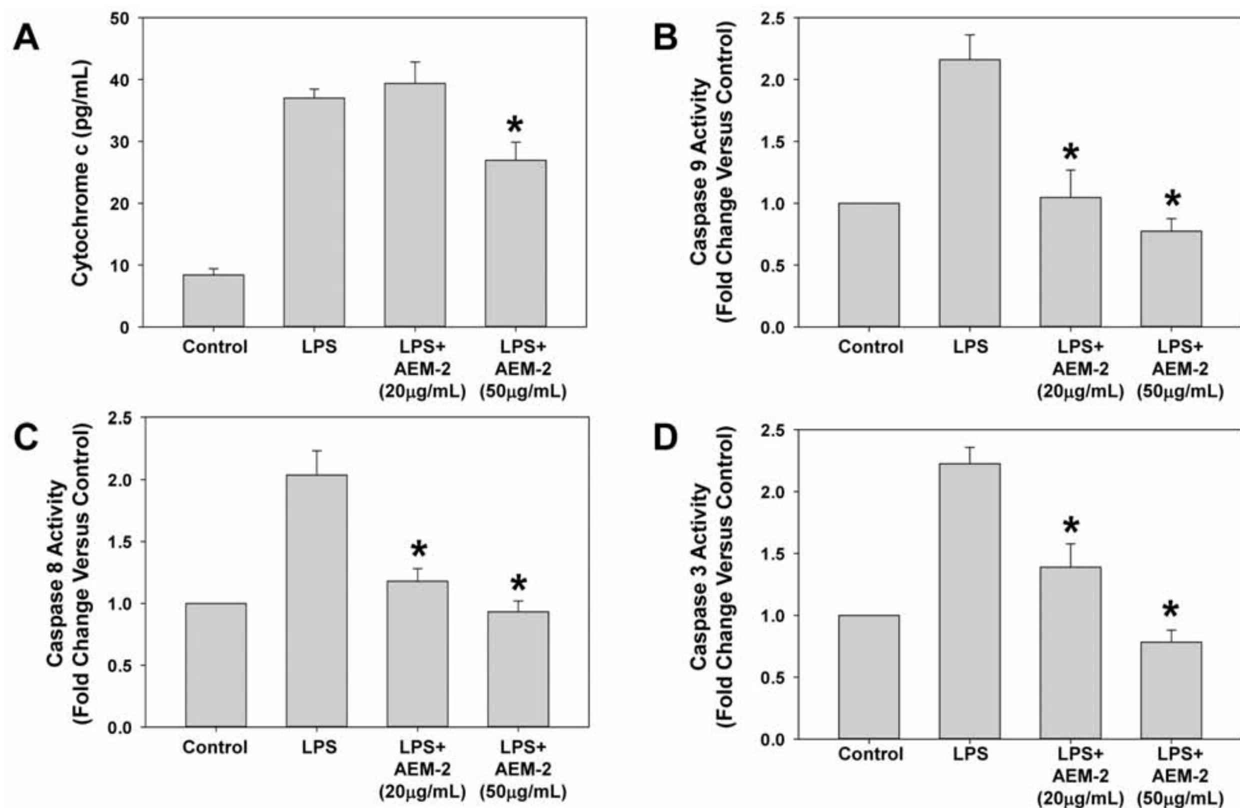

Fig. 5. AEM-2 inhibits apoptosis in THP-1 macrophages. THP-1 cells were treated with saline or AEM-2 for 24 hrs, followed by addition of LPS (1 μg/ml) for 6 hr. The translocation of cytochrome c was measured in the isolated cytosolic fraction by ELISA (Panel A). The activities of caspases 8, 9 and 3 were measured using commercially available kits (Panels B-D). Data are presented as fold changes relative to control and represent the mean ± SEM (N = 6-9 per treatment). * denotes a significant difference ($P < 0.05$) compared to LPS.

AEM-2 (Fig. 5C). Both caspase 8 and caspase 9 can cleave caspase 3 that acts as the final mediator of apoptosis. Similar to the effect of AEM-2 on caspase 8 and 9, the peptide inhibited the activation of caspase 3 (Fig. 5D). Taken together these data suggest that AEM-2 decreases LPS-induced apoptosis.

Apoptosis and autophagy are homeostatic mechanisms that play an important role in determining cell survival and death [25]. Since there is crosstalk between these pathways, we monitored the conversion of LC3-I to LC3-II, a mediator of autophagosome formation. Entry into the autophagy pathway is initiated by the cleavage of cytosolic LC3-I. The subsequent lipidation of LC3-I yields LC3-II that plays a role in autophagosome membrane expansion. The ratio of LC3-II to LC3-I is thus an index for autophagy induction. The accurate measurement of this ratio can be complicated by the degradation of LC3-II along with the internal contents of the autophagosome [26]. Thus, it is difficult to directly measure the conversion of LC3-I to LC3-II. To overcome this obstacle, we measured the LC3-II/LC3-I ratio in the presence of chloroquine (CQ), an inhibitor of autophagosomal degradation [25]. Under these conditions, 50 μg/ml AEM-2 induced autophagic flux in LPS-treated cells, while treatment with LPS alone was without effect (Fig. 6). These data suggest that AEM-2 increases flux through the autophagy pathway by stimulating both the induction and the turnover of autophagosomes.

DISCUSSION

Results of the current study show that the apoE mimetic peptide AEM-2 mimics properties of native apoE. Administration of the peptide to

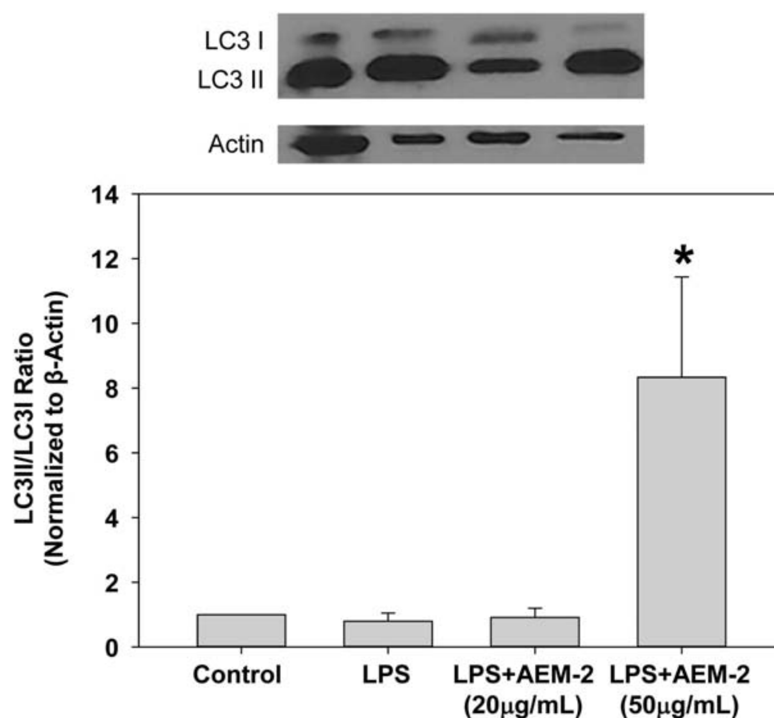

Fig. 6. AEM-2 induces autophagy in THP-1 macrophages. The effect of AEM-2 on cellular autophagy was assessed by measuring the ratio of LC3-II to LC3-I in CQ-treated cells. Immunoblotting studies demonstrate that, in the presence of an inhibitor of autophagosome-lysosome fusion, 50 μg/mL AEM-2 significantly increases the formation of LC3-II. Bands were normalized to β-actin and expressed relative to control. Data represent the mean ± SEM of N = 3 immunoblots. * denotes a significant difference ($P < 0.05$) compared to LPS.

apoE-null mice fed a WD significantly reduced plasma cholesterol within 1 hr post treatment (Fig. 1). The ability of AEM-2 to reduce plasma cholesterol is due to its dual domain structure. First, the lipid-binding domain is composed of a class A amphipathic helix that is known to bind with phospholipids in lipoprotein particles [18]. Second, the receptor-binding domain of the peptide contains an arginine-rich sequence that is similar in structure to the putative LDL receptor binding sequence of native apoE [19]. Arginine-rich peptides possess a strong positive charge and have been shown to bind to negatively-charged HSPGs located in the space of Disse in the liver [3]. At this locus, peptide-lipoprotein complexes are taken up and cleared from the circulation [1, 19]. In this manner, AEM-2 may associate with chylomicron remnants, VLDL, and LDL and target them to hepatocytes for clearance. Support for this route of elimination is provided by the observation that pre-treatment of apoE-null mice with heparinase, followed by apoE mimetic administration, does not reduce plasma cholesterol [27]. The response to a single injection of AEM-2 was transient since the plasma cholesterol level was similar to that of saline-treated apoE-null mice 24 hrs post treatment. It is likely that the peptide was completely cleared from the circulation by this time point.

Polymorphisms in the apoE gene are associated with an increase in inflammation and mortality in mice and humans [6, 7]. Further, the deletion of apoE in mice is associated with an increase in circulating levels of atherogenic lipoproteins and pro-inflammatory cytokines compared to wild type mice [28-30]. Conversely, adenoviral delivery of apoE has been shown to reduce inflammation in apoE-null mice [31]. We now appreciate that apoE exerts anti-inflammatory effects that are independent of its ability to reduce plasma cholesterol [4, 30, 31]. Monocytes and macrophages are important sites for apoE action. apoE-containing HDL particles reduce circulating levels of

pro-inflammatory monocytes, thus attenuating monocyte adhesion to the vessel wall [4]. Several studies also show that increasing monocyte apoE expression reduces the inflammatory potential of these cells and limits their recruitment to sites of injury [8, 9, 11]. Consistent with the anti-inflammatory properties of native apoE, we found that 50 μg/mL AEM-2 significantly reduced the ability of LPS to induce the secretion of IL-6 and TNFα in THP-1 macrophages (Fig. 3A-B). These were residual responses to AEM-2 treatment since the peptide was absent from cell culture medium containing LPS. Previous studies suggest that apoE reduces the expression of pro-inflammatory markers in monocytes by a mechanism involving a reduction in cellular lipid content [4]. In the current study, AEM-2 induced a modest reduction in cellular cholesterol, but this was not significantly different from cells treated with saline (Fig. 2). Thus, the ability of AEM-2 to reduce cytokine secretion likely proceeds by an alternate mechanism.

In addition to stimulating cytokine secretion in macrophages, LPS damages cells by inducing mitochondrial oxidant formation [32]. To test whether the inhibitory effect of AEM-2 on cytokine secretion was associated with a reduction in oxidant formation, we monitored effects of the peptide on LPS-induced mitochondrial superoxide formation. In these studies, Mitosox, a mitochondrially-targeted fluorescent probe, was used to detect oxidant formation. As shown in Fig. 4A, treatment of THP-1 cells with LPS significantly increased Mitosox-dependent fluorescence compared to cells treated with saline. Mitochondrial injury and a decrease in $\Delta\Psi_m$ are characteristic responses to oxidant formation [33]. Consistent with these observations, LPS treatment reduced $\Delta\Psi_m$ as measured by TMRM fluorescence (Fig. 4B). In contrast to these findings, pretreatment of cells with 50 μg/mL AEM-2 significantly reduced LPS-induced superoxide formation and was associated with a significant increase in $\Delta\Psi_m$ (Figs. 4A-B).

The development of mitochondrial injury is a trigger for the activation of apoptosis, which can be mediated by either the intrinsic or extrinsic pathway [24]. The intrinsic cell death pathway is activated by a decrease in $\Delta\Psi_m$ and the release of cytochrome c from mitochondria. Cytochrome c induces the cleavage of the initiator caspase 9, which in turn cleaves effector caspase 3. In contrast, the extrinsic pathway is associated with caspase 8 expression and is induced by cell surface receptors (eg., TNFα receptor type 1) [34]. Previous data suggest that LPS can activate both the intrinsic and extrinsic pathways [35, 36]. Our data show that the LPS-induced decrease in $\Delta\Psi_m$ was associated with the translocation of cytochrome c to the cytosol (Fig. 5A). Further, the activities of caspases 8, 9 and 3 were significantly increased compared to THP-1 cells treated with saline (Fig. 5B-D). Pretreatment of THP-1 macrophages with 50 μg/mL AEM-2 significantly reduced cytochrome c translocation (Fig. 5A). Similarly, AEM-2 at both 20 and 50 μg/mL reduced the activities of all three caspases (Fig. 5B-D).

Apoptosis has been described as programmed cell death, while autophagy can mediate both cell death and cytoprotection. Ultimately, the cellular response to stress is determined by crosstalk between the two pathways [37]. Autophagy was first described in 1963 as a cellular recycling mechanism [38]. Cellular proteins and organelles, destined for degradation, become engulfed by a double-membraned vesicle called the autophagosome. The autophagosome fuses with the lysosome, resulting in the formation of the autophagolysosome which degrades its contents. Measurement of protein levels for ubiquitously expressed LC3 is a commonly used method for the assessment of autophagy [39]. Upon activation of the autophagy pathway, LC3-I is cleaved and lipidated, followed by insertion into the autophagosomal membrane. In this manner, LC3-I is converted to LC3-II, a protein whose molecular weight is reduced by 2 kDa. The ratio of LC3-II to LC3-I can then be used to assess the progression of autophagy. Interestingly, LC3-II can be degraded in the autophagosome, and, under conditions where autophagy flux is accelerated, LC3-II levels may be significantly underestimated. This complication is overcome by treatment of cells with chloroquine (CQ). By inhibiting the fusion of the lysosome and the autophagosome, CQ prevents the degradation of LC3-II and allows an accurate determination of autophagic flux [26, 39]. In the current studies, treatment of THP-1 macrophages with 50 μg/ml AEM-2 decreased apoptosis and

increased autophagy flux. AEM-2 may thus play an important role in regulating the homeostatic balance and crosstalk between these pathways.

CONCLUSION

Results of these studies show that the apoE mimetic peptide AEM-2, similar to native apoE, significantly reduces plasma cholesterol in apoE-null mice. While HMG-CoA reductase inhibitors are commonly used to reduce plasma cholesterol levels in humans, statin resistance is a common phenomenon which may preclude their usage in some subjects [15, 16]. The apoE mimetic peptide AEM-2 represents a potential alternative therapy. Since apoE is known to possess anti-inflammatory properties that are independent of its cholesterol lowering capacity, we tested whether the peptide exerts cytoprotective effects at the cellular level. Indeed, results of our studies show that AEM-2 induces prominent anti-inflammatory and anti-apoptotic effects in THP-1 macrophages. While peptide administration at a concentration of 20 µg/mL elicited variable responses in different assays, AEM-2 at 50 µg/mL consistently inhibited pro-inflammatory and apoptotic responses induced by LPS. While some data suggest that reducing cellular cholesterol prevents the activation of macrophages and promotes cell survival, our results show that AEM-2 only modestly reduces cholesterol content [13, 14]. AEM-2 thus appears to exert its effect *via* an alternate mechanism. This is currently under investigation in our laboratory.

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CONFLICT OF INTEREST STATEMENT

G. M. Anantharamaiah holds shares in LipimetiX LLC.

REFERENCES

1. Datta, G., Chaddha, M., Garber, D. W., Chung, B. H., Tytler, E. M., Dashti, N., Bradley, W. A., Gianturco, S. H. and Anantharamaiah, G. M. 2000, Biochemistry, 39, 213-220.
2. Segall, M. L., Dhanasekaran, P., Baldwin, F., Anantharamaiah, G. M., Weisgraber, K. H., Phillips, M. C. and Lund-Katz, S. 2002, J. Lipid Res., 43, 1688-1700.
3. Gonzales, J. C., Gordts, P. L., Foley, E. M. and Esko, J. D. 2013, J. Clin. Invest., 123, 2742-2751.
4. Gaudreault, N., Kumar, N., Posada, J. M., Stephens, K. B., Reyes de Mochel, S. N. R., Eberle, D., Olivas, V. R., Kim, R. Y., Harms, M. J., Johnson, S., Messina, L. M., Rapp, J. H. and Raffai, R. I. 2012, Arterio. Thromb. Vasc. Biol., 32, 264-272.
5. Davignon, J. 2005, Arterio. Thromb. Vasc. Biol., 25, 267-269.
6. Wang, H., Christensen, D. J., Vitek, M. P., Sullivan, P. M. and Laskowitz, D. T. 2009, Anaesth. Intensive Care, 37, 38-45.
7. Moretti, E. W., Morris, R. W., Podgoreanu, M., Schwinn, D. A., Newman, M. F., Bennett, E., Moulin, V. G., Mba, U. U. and Laskowitz, D. T. 2005, Crit. Care Med., 33, 2521-2526.
8. Bellosta, S., Mahley, R. W., Sanan, D. A., Murata, J., Newland, D. L., Taylor, J. M. and Pitas, R. E. 1995, J. Clin. Invest., 96, 2170-2179.
9. Baitsch, D., Hans, H., Engel, T., Telgmann, R., Muller-Tidow, C., Varga, G., Bot, M., Herz, J., Robenek, H., von Eckardstein, A. and Nofer, J-R. 2011, Arterio. Thromb. Vasc. Biol., 31, 1160-1168.
10. Fazio, S., Babaev, V. R., Murray, A. B., Hasty, A. H., Carter, K. J., Gleaves, L. A., Atkinson, J. B. and Linton, M. F. 1997, Proc. Natl. Acad. Sci. USA, 94, 4647-4652.
11. Potteaux, S., Gautier, E. L., Hutchison, S. B., van Rooijen, N., Rader, D. J., Thomas, M. J., Sorci-Thomas, M. G. and Randolph, G. J. 2011, J. Clin. Invest., 121, 2025-2036.
12. Hoekstra, M., Frodermann, V., van den Aardweg, T., van der Sluis, R. J. and Kuiper, J. 2013, PLoS One, 8, e80441. doi:10.1371/journal.pone.0080441.
13. Murphy, A. J., Akhtari, M., Tolani, S., Pagler, T., Bijl, N., Kuo, C. L., Wang, M., Sanson, M., Abramowicz, S., Welch, C., Bochem, A. E., Kuivenhoven, J. A., Yvan-Charvet, L. and Tall, A. R. 2011, J. Clin. Invest., 121, 4138-4149.

14. Murphy, A. J., Westerterp, M., Yvan-Charvet, L. and Tall, A. R. 2012, Biochim. Biophys. Acta, 1821, 513-521.
15. Josan, K., Majumdar, S. R. and McAlister, F. A. 2008, CMAJ, 178, 576-584.
16. Chen, Y., Ruan, X. Z., Li, Q., Huang, A., Moorhead, J. F., Powis, S. H. and Varghese, Z. 2007, Am. J. Physiol., 293, F680-F687.
17. Mohammadpour, A. H. and Akhlaghi, F. 2013, Clin. Pharmacokinet., 52, 615-626.
18. Garber, D. W., Datta, G., Chaddha, M., Palgunachari, M. N., Hama, S. Y., Navab, M., Fogelman, A. M., Segrest, J. P. and Anantharamaiah, G. M. 2001, J. Lipid Res., 42, 545-552.
19. Datta, G., Garber, D. W., Chung, B. H., Chaddha, M., Dashti, N., Bradley, W. A., Gianturco, S. H. and Anantharamaiah, G. M. 2001, J. Lipid Res., 42, 959-966
20. Suliman, H. B., Carraway, M. S., Welty-Wolf, K. E., Whorton, A. R. and Piantadosi, C. A. 2003, J. Biol. Chem., 278, 41510-41518.
21. Bulua, A. C., Simon, A., Maddipati, R., Pelletier, M., Park, H., Kim, K. Y., Sack, M. N., Kastner, D. L. and Siegel, R. M. 2011, J. Exp. Med., 208, 519-533.
22. Gottlieb, E., Armour, S. M., Harris, M. H. and Thompson, C. B. 2003, Cell Death and Differentiation, 10, 709-717.
23. Petrosillo, G., Ruggiero, F. M., Pistolese, M. and Paradies, G. 2001, FEBS Letters, 509, 435-438.
24. Liu, B., Sun, R., Luo, H., Liu, X., Jiang, M., Yuan, C., Yang, L. and Hu, J. 2017, Immunobiology, 222, 198-205.
25. El-Khattouti, A., Selimovic, D., Haikel, Y. and Hassan, M. 2013, J. Cell Death, 18, 37-55.
26. Mizushima, N. and Yoshimori, T. 2007, Autophagy, 3, 542-545.
27. Garber, D. W., Handattu, S., Aslan, I., Datta, G., Chaddha, M. and Anantharamaiah, G. M. 2003, Atherosclerosis, 168, 229-237.
28. de Bont, N., Netea, M. G., Demacker, P. N. M., Kullberg, B. J., van der Meer, J. W. and Stalenhoef, A. F. 2000, Eur. J. Clin. Invest., 30, 818-822.
29. Roselaar, S. E. and Daugherty, A. 1998, J. Lipid Res., 39, 1740-1743.
30. van Oosten, M., Rensen, P. C. N., van Amersfoort, E. S., van Eck, M., van Dami, A. M., Breve, J. J. P., Vogel, T., Panet, A., van Berkel, T. J. C. and Kuiper, J. 2001, J. Biol. Chem., 276, 8820-8824.
31. Ali, K., Middleton, M., Pure, E. and Rader, D. J. 2005, Circ. Res., 97, 922-927.
32. Suliman, H. B., Welty-Wolf, K. E., Carraway, M., Tatro, L. and Piantadosi, C. A. 2004, Cardiovasc. Res., 64, 279-288.
33. Vayssier-Taussat, M., Kreps, S. E., Adrie, C., Dall'Ava, J., Christiani, D. and Polla, B. S. 2002, Environ. Health Perspect., 110, 301-305.
34. Supinski, G. S., Ji, X., Wang, W. and Callahan, L. A. 2007, Journal of Applied Physiology, 102, 1649-1657.
35. Carlson, D. L., Willis, M. S., White, D. J., Horton, J. W. and Giroir, B. P. 2005, Crit. Care Med., 33, 1021-1028.
36. Supinski, G. S., Murphy, M. P. and Callahan, L. A. 2009, Am. J. Physiol., 297, R1095-R1102.
37. Gordy, C. and He, Y. W. 2012, Protein Cell, 3, 17-27.
38. De Duve, C. 1963, Sci. Am., 208, 64-72.
39. Yoshii, S. R. and Mizushimz, N. 2017, International Journal of Molecular Sciences, 18, 1-13.