Apolipoprotein E and Peptide Mimetics Modulate Inflammation by Binding the SET Protein and Activating Protein Phosphatase 2A

Dale J. Christensen, Nobutaka Ohkubo, Jessica Oddo, Michael J. Van Kanegan, Jessica Neil, Fengqiao Li, Carol A. Colton and Michael P. Vitek

*J Immunol* 2011; 186:2535-2542; doi: 10.4049/jimmunol.1002847
http://www.jimmunol.org/content/186/4/2535

References

This article cites 40 articles, 22 of which you can access for free at:
http://www.jimmunol.org/content/186/4/2535.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Apolipoprotein E and Peptide Mimetics Modulate Inflammation by Binding the SET Protein and Activating Protein Phosphatase 2A

Dale J. Christensen,* Nobutaka Ohkubo, ‡ Jessica Oddo,* Michael J. Van Kanegan,* Jessica Neil,* Fengqiao Li,* Carol A. Colton, ‡ and Michael P. Vitek*‡

The molecular mechanism by which apolipoprotein E (apoE) suppresses inflammatory cytokine and NO production is unknown. Using an affinity purification approach, we found that peptide mimetics of apoE, derived from its receptor binding domain residues 130–150, bound to the SET protein, which is a potent physiological inhibitor of protein phosphatase 2A (PP2A). Both holo-apoE protein and apoE-mimetic peptides bound to the C-terminal region of SET, which is then associated with an increase in PP2A-mediated phosphatase activity. As physiological substrates for PP2A, the LPS-induced phosphorylation status of signaling MAPK and Akt kinase is reduced following treatment with apoE-mimetic peptides. On the basis of our previous report, in which apoE-mimetic peptides reduced IκB kinase and NF-κB activation, we also demonstrate a mechanism for reduced production of inducible NO synthase protein and its NO product. These data provide evidence for a novel molecular mechanism by which apoE and apoE-mimetic peptides antagonize SET, thereby enhancing endogenous PP2A phosphatase activity, which reduces levels of phosphorylated kinases, signaling, and inflammatory response. The Journal of Immunology, 2011, 186: 2535–2542.
cular mechanism by which apoE and its peptide mimetics are able to suppress the immune response. Clues to the molecular mechanism can be derived from the reports of Kawamura et al. and Singh et al. (20, 21) Kawamura and coworkers (20) reported that the apoE holoprotein inhibits IL-1β signaling in vascular smooth muscle cells, resulting in reduced production of inflammatory mediators, including NO and PGE2. This effect was shown to be mediated through decreased phosphorylation and reduction of nuclear localized NF-κB leading to reduced production of inducible NO synthase (22). Our collaborators, Singh et al. (21), also reported that COG112, another apoE-mimetic peptide composed of the apoE [133–149] peptide fused to the antenmapedia protein transduction domain peptide, also reduced activation of the NF-κB pathway. In each case, it was noted that phosphorylation of the 1-kb kinase (IKK) was reduced by treatment with apoE or its mimetic peptide.

To understand the role of apoE and determine the mechanism by which these apoE and apoE-mimetic peptides inhibit inflammatory processes and reduce phosphorylation of NF-κB, we sought to determine if the suppression of TNF-α production following LPS stimulation was mediated by the known apoE receptors, LRP and LDLR, and to identify other cellular proteins that bound to the apoE[133–149] peptide. We found a significant dose-dependent suppression of TNF-α production by two apoE-mimetic peptides, apoE[133–149] and COG1410, following LPS stimulation of either primary mouse peritoneal macrophages or BV2 microglial cells. This is noteworthy because both LRP and LDLR are expressed in peritoneal macrophages, but they are not expressed in the BV2 cell line, suggesting that the anti-inflammatory activity occurs by a receptor-independent mechanism. Beyond the mechanism of uptake, we now report that apoE and apoE-mimetic peptides bind to the SET oncoprotein and that the apoE holoprotein interacts with SET through its C-terminal region from amino acids 177–277. The protein SET is also known as inhibitor-2 of protein phosphatase 2A (I2PP2A) and exists at elevated levels in the brains of those with Alzheimer’s disease relative to normal age-matched controls (23) and in leukemia cancer cells (24–26).

Binding of the apoE-mimetic peptide to SET results in activation of protein phosphatase 2A (PP2A), with a corresponding dephosphorylation of PP2A targets. Signaling modules consisting of various kinases and PP2A have been reported to increase the fidelity of a signal transduction pathway by tightly regulating activation and deactivation processes. Evaluation of these modules has identified numerous kinases that interact with PP2A, including the MAPK proteins p38 kinase (27), JNK (28), and ERK (29, 30). Furthermore, PP2A negatively regulates Akt and its target, IKK, which normally participates in the activation of the NF-κB transcription factor (31, 32). NF-κB has been shown to positively regulate production of inducible NO synthase (22) from the NOS2 gene, resulting in increased NO production. Accordingly, we found reduced levels of phosphorylated p38 MAPK and the Akt kinase that activates the NF-κB pathway. We also report that SET is essential for normal inflammatory responses to LPS because small interfering RNA (siRNA)-mediated reduction of the SET protein level significantly inhibits the inflammatory response following LPS stimulation. Taken together, these results suggest a novel mechanism by which apoE and apoE-mimetic peptides modulate biological pathways that are critical for inflammatory signaling by antagonism of SET, resulting in activation of PP2A.

Materials and Methods

Materials

All peptides were synthesized by PolyPeptide Laboratories (San Diego, CA), using standard Fmoc-based chemistry and purified to >95% purity. The sequences synthesized were apoE[133–149]: acetyl-LRVRLASHLRK-LRKKLL-amide and COG1410: acetyl-AS(aib)LRLK(aib)KRLL-amide, where (aib) signifies amino-isoo-butyric acid. All buffers and reagents were from Sigma unless otherwise specified.

Detection of LRP and LDLR by Western blotting

Primary mouse peritoneal macrophages were obtained by i.p. injection of four 25- to 30-g male C57BL/6 mice with 1 ml of 5 mM sodium periodate and repeating this injection 72 h later. Peritoneal macrophages were harvested by lavage with cold PBS containing 10 U/ml heparin sulfate, 24 h after the second sodium periodate injection. Cells were collected by centrifugation and washed with 1 ml chilled PBS and lysed in Nonidet P-40 (NP-40) buffer (50 mM Tris, 0.2% NP-40, 150 mM NaCl). BV2 microglial cells were grown to log phase in a T75 flask, stimulated with LPS (100 ng/ml), and cells were lysed in NP-40 buffer. Protein concentration of the lysate was determined using the BCA assay (Pierce) and adjusted to 5 mg total protein per milliliter of solution. Laemmli protein sample buffer (4×, 75 μl) was added to the cell lysate (25 μl), and the solutions were heated to 90°C for 5 min. Protein solutions were loaded onto duplicate gels and were separated by SDS-PAGE, blotted onto nitrocellulose membranes (Bio-Rad). The nitrocellulose membranes were blocked using 5% nonfat milk in TBST (containing 0.1% Tween 20) for 3 h, then washed with TBST. One membrane was incubated overnight at 4°C in 1× PBS containing anti-LRP Ab (CalBiochem) and a rabbit anti–β-GAPDH Ab (Santa Cruz). Membranes were washed with TBST for 1 h, with three changes of the wash solution, and incubated with the appropriate IRDye 680 or IRDye 800 secondary Abs. Protein bands were visualized and quantitated using an Odyssey Infrared scanner (LiCor). Following the first read of the LDLR blot, a second membrane was blocked, washed thoroughly and mounted in Vectashield mounting media (Vector Labs) and probed for LDL receptor with a rabbit polyclonal anti-LDLR Ab (Cayman Chemical). The other membrane was incubated in a mouse monoclonal anti-LRP Ab (CalBiochem) and a rabbit anti–β-GAPDH Ab (Santa Cruz). Membranes were washed with TBST for 1 h, with three changes of the wash solution, and incubated with the appropriate IRDye 680 or IRDye 800 secondary Abs. Protein bands were visualized and quantitated using an Odyssey Infrared scanner (LiCor). Following the first read of the LDLR blot, a second membrane was incubated for 24 h at 37°C in 5% CO2 to stimulate BV2 cells were grown overnight and seeded at 5 × 104 cells per well in a 48-well plate. Cell treatments were performed as described for the peritoneal macrophages, except LPS (200 ng/ml) was used without IFN-γ to stimulate TNF-α production. After 24 h, media were removed and TNF-α quantified using ELISA (Invitrogen) Cell numbers were determined with the MTT assay (Sigma). Values for TNF-α were normalized to MTT values prior to plotting.

BV2 uptake of biotin-labeled apoE[133–149] peptide and SET immunofluorescence staining

BV2 cells were plated at a density of 8 × 104 cells per well onto poly-d-lysine-coated 12-mm glass coverslips and grown overnight in complete DMEM/10% FBS/1% pen-strep/1% HEPES/1% t-glutamine media (Life Technologies). Cells were incubated at 2 × 104 cells/cm2 in a humidified 5% CO2 tissue culture plate and incubated in 5% CO2 at 37°C overnight to adhere to the plate. Prior to plotting, a loading control protein band of COG1410 was read, a rabbit anti-cyclophilin B Ab (Abcam) was added, and the solutions were heated to 90°C for 5 min. Protein solutions were loaded onto duplicate gels and were separated by SDS-PAGE, blotted onto nitrocellulose membranes (Bio-Rad). The nitrocellulose membranes were blocked using 5% nonfat milk in TBST (containing 0.1% Tween 20) for 3 h, then washed with TBST. One membrane was incubated overnight at 4°C in 1× PBS containing anti-LRP Ab (CalBiochem) and a rabbit anti–β-GAPDH Ab (Santa Cruz). Membranes were washed with TBST for 1 h, with three changes of the wash solution, and incubated with the appropriate IRDye 680 or IRDye 800 secondary Abs. Protein bands were visualized and quantitated using an Odyssey Infrared scanner (LiCor). Following the first read of the LDLR blot, a second membrane was blocked, washed thoroughly and mounted in Vectashield mounting media (Vector Labs) and probed for LDL receptor with a rabbit polyclonal anti-SET primary Ab (Santa Cruz) diluted in blocking buffer for 2 h at room temperature. Following a thorough wash, cells were then labeled with an Alexa Fluor 488-labeled goat anti-rabbit IgG secondary Ab (Invitrogen) and Hoechst-33342 nuclear stain (Pierce) for 1 h at 37°C. Cells were then washed with PBS and rinsed with 2 M acetic acid (15 s) to remove extracellular bound ligand, and then cells were washed thoroughly with PBS (3 × 500 μl) and fixed with 4% paraformaldehyde for 20 min. Cells were blocked and permeabilized by treatment with 10% normal goat serum/1% BSA/0.1% Triton X-100 for 1 h, washed, and incubated with Alexa Fluor 555-labeled streptavidin (Invitrogen) for 30 min at 37°C. Next, cells were washed and incubated with a rabbit polyclonal anti-SRF primary Ab (Santa Cruz) diluted in blocking buffer for 2 h at room temperature. Following a thorough wash, cells were then labeled with an Alexa Fluor 488-labeled goat anti-rabbit IgG secondary Ab (Invitrogen) and Hoechst-33342 nuclear stain (Pierce) for 1 h at room temperature. Stained cells were then washed thoroughly and mounted in Vectashield mounting media (Vector Labs) and imaged with a Nikon Eclipse Ti-S fluorescence microscope. Images were processed using Nikon NIS-Elements Basic Research software.
Affinity purification of apoE[133–149] binding proteins

BV2 microglial cells were grown to log phase in a T75 flask, stimulated with LPS (100 ng/ml), and biotin-labeled apoE[133–149] (10 μM) or biotin (10 μM) was added for 2 h; a lysate was prepared in NP-40 buffer (50 mM Tris, 0.2% NP-40, 150 mM NaCl) by a single freeze–thaw cycle. Protein concentration of the lysate was determined using the BCA assay (Pierce) and adjusted to 5 mg total protein per milliliter of solution. Streptavidin–agarose beads (1 ml) were washed with 10 ml NP-40 buffer, and 0.5 ml beads were added to 1 ml extract. After incubation the beads were collected by filtration through a disposable minicolumn (Bio-Rad) and the flow-through extract collected for analysis. Following washing with 100 ml chilled NP-40 buffer, the beads were removed from the column and collected by centrifugation, and 75 μl 4X Laemmli buffer was added. Beads were vortexed and heated to 90°C for 10 min to ensure that all proteins were released from the beads. Proteins were separated by SDS-PAGE, visualized by Coomassie staining, and identified by mass spectrometry.

Affinity purification and Western blotting of mouse and human brain lysate

Frozen mouse and human brain samples were ground to a fine powder, using a mortar and pestle cooled with liquid nitrogen. Extracts were prepared by suspending 100 mg brain powder in NP-40 buffer and performing a freeze–thaw cycle. Lysates were diluted to 1 mg/ml and 1 ml aliquots precleared with streptavidin–agarose beads, as above. Biotin–apoE[133–149] (10 μg) was added to 1 mg protein extract from BV2 cells, mouse brain, and human brain. As a negative control, biotin (0.1 μg) and non–biotin-labeled apoE[133–149] peptide (10 μg) were added to another 1 mg sample of total protein extract. After incubation for 2 h (4°C), 50 μl fresh, washed beads were added to the lysate/peptide mixtures, with incubation being continued for an additional 2 h. Following four washes with 1 ml chilled NP-40 buffer, 75 μl 4X Laemmli buffer was added and the beads were heated to 90°C for 10 min. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes (Bio-Rad), and bound SET protein was detected by Western blotting. Nitrocellulose membranes were blocked using 5% nonfat milk in TBST, and incubated overnight at 4°C in a rabbit anti-SET Ab (Santa Cruz). Membranes were washed with TBST, and incubated overnight at 4°C in binding buffer (50 mM Tris pH 7.5, 0.5 mM EDTA, 0.1% Tween 20, and 5% nonfat milk). Membranes were washed with TBST, and incubated overnight at 4°C in a rabbit anti-SET Ab (Santa Cruz), then incubated with phospho-p38 or total p38 Abs (Cell Signaling). Secondary Abs on an Odyssey scanner, as described above. Equal amounts of cellular protein were added to 4X Laemmli buffer and subjected to SDS-PAGE. After transferring to nitrocellulose membranes, the blots were blocked for 30 min with Odyssey Blocking Buffer (LiCor) and incubated with phospho-Akt (BioSource) or total Akt Abs (Cell Signaling) in Super Block (ThermoSci). Proteins were visualized using LiCor secondary Abs on an Odyssey scanner, as described above. Similar blots were incubated with phospho-p38 or total p38 Abs (Cell Signaling).

Comimunoprecipitation of apoE and SET

GST–SET fusion constructs were a kind gift from Dr. Ye (Emory University, Atlanta, GA). GST fusion proteins were expressed in E. coli bacterial strain BL21 and purified using B-Per GST Fusion Protein Purification Kit (Pierce) according to the manufacturer’s protocol. Purified GST–SET was eluted from the column, using 50 mM reduced glutathione, dialyzed in SET buffer (50 mM Tris pH 7.5, 0.5 mM EDTA, 1 μM benzamidine, 0.5 μM PMSF, 10% glycerol, and 0.25% NP-40). Glutathione-agarose or apoE Ab E667 (Calbiochem) with protein G beads (Santa Cruz) was added and incubated for 45 min. Beads were washed 4× with TBS + 0.1% NP-40 and extracted in SDS sample buffer for 10 min at 90°C. Samples were run on 12.5% SDS-PAGE gel, transferred to nitrocellulose, and immunoblotted with rabbit anti-GST (Cell Signal) and rabbit anti-apoE (Calbiochem). IRDye-labeled secondary Abs, goat anti-rabbit and anti-mouse Abs (LiCor) were used for imaging on a LiCor Odyssey imaging system, as described above.

Competitions with COG1410 and apoE[133–149] peptides

Following the procedure above, competitions were performed using the BV2 lysate. Non–biotin-labeled apoE[133–149] or COG1410 was added to individual aliquots of lysate in increasing concentrations and incubated for 1 h prior to affinity purification and Western blotting of the SET, as described above.

PP2A activity in mouse brain

C57BL/6 mice were intravenously injected (5 ml/kg: n = 5 per group) with either COG1410 (5 mg/kg in lactated Ringer’s solution) or lactated Ringer’s solution. After 30 min the mice were anesthetized, sacrificed by cervical dislocation, their brains removed, and flash frozen. The brain was ground to a fine powder, as above, and lysed using Qiagen’s mammalian proteome lysis kit. Protein was quantified using BCA (Pierce), and 500 μg total protein was combined with 4 μl anti-PP2A Ab (1D6; Upstate) and 50 μl protein A–agarose beads in a total volume of 500 μl. The mixture was shaken for 2 h at 4°C, and then beads were collected by centrifugation. Following four washes, 50 μl phosphatase assay buffer (Upstate) was added to the beads, vortexed, and 50 μl bead slurry was added to one well of a 96-well plate. A 10 mM stock of 6,8-difluoro-4-methylumbelliferyl phosphate (Innoven) was diluted to 100 μM in assay buffer, and 50 μl was added to each well of the plate; then the fluorescence intensity of a fluorescent product produced by cleavage of phosphate from 6,8-difluoro-4-methylumbelliferyl phosphate, a synthetic phosphatase substrate, was measured using a plate reader. Fluorescence reads were performed every 3 min, and shaking every 30 s, over a 30-min period.

Analysis of Akt and p38 phosphorylation following LPS stimulation

BV2 cells were plated at 5 × 10⁶ cells per well and were allowed to adhere overnight in a six-well plate in DMEM/10% FBS (Life Technologies). Cells were pretreated for 15 min with 5 μM apoE[133–149] peptide before adding 100 ng/ml LPS for 30 min. Protein extracts from cell cultures were lysed in Mammalian Protein Extraction Reagent (Pierce) with protease inhibitors. Equal amounts of cellular protein were added to 4X Laemmli buffer and subjected to SDS-PAGE. After transferring to nitrocellulose membranes, the blots were blocked for 30 min with Odyssey Blocking Buffer (LiCor) and incubated with phospho-Akt (BioSource) or total Akt Abs (Cell Signaling) in Super Block (ThermoSci). Proteins were visualized using LiCor secondary Abs on an Odyssey scanner, as described above. Similar blots were incubated with phospho-p38 or total p38 Abs (Cell Signaling).

siRNA knockdown of SET

A confluent flask of BV2 cells was diluted 1:50 in normal growth media, and 500 μl per well was plated into 24-well plates. The cells were allowed to adhere overnight before transfection. Once the cells adhered, 80 μl Dharmafect-1 was added to 1960 μl Optimem, and 350 μl of each siRNA (2 μM in water) was added to 1960 μl Optimem. These solutions were incubated at room temperature for 5 min before addition of the Dharmafect mixture (0.25 μl) to each siRNA mixture. After 20 min, 5 ml media (DMEM, penicillin/streptomycin, nonessential amino acids, sodium pyruvate, and 1% FBS) was added to each siRNA mixture. After 20 min, 5 ml media (DMEM, penicillin/streptomycin, nonessential amino acids, sodium pyruvate, and 1% FBS) was added to each siRNA/Dharmafect mixture. Treatment of cells with the siRNA preparations was accomplished by aspirating media from cells, washing once with 250 μl PBS, and adding 500 μl siRNA/Dharmafect preparation to the cells. After incubation for 24 h, media were removed, cells were washed with 250 μl PBS, and fresh media (500 μl) containing treatments (no treatment control, 500 ng/ml LPS) was added. Following incubation (16 h) cells were harvested with washing once with 500 μl PBS and lifting with Versene. Cells were collected by centrifugation (5 min at 1000 × g) and washed with PBS to remove Versene. A Greiss assay (Promega) was performed on media immediately following the cell harvest, according to the manufacturer’s protocol. The effect of siRNA treatment on SET and PP2A was determined by lysing the cell pellets in Qiagen’s mammalian protein extraction buffer. Proteins were quantified and diluted such that 20 μg total protein was loaded per lane following addition of 4X Laemmli buffer and heating for 10 min at 90°C. Following transfer to nitrocellulose, Western blotting was performed as above with anti-SET (Santa Cruz), anti-β-actin (Santa Cruz), and anti-PP2A (Upstate) Abs.

Statistical analysis

All statistical analyses were performed using either one-way ANOVA or Student t test, with statistical significance defined as p < 0.05 and values expressed as mean ± SEM. For comparison of three or more values, ANOVA was used with the Neuman–Keuls post hoc test. When two groups were compared, the Student t test was used.

Results

ApoE-mimetic peptides suppress TNF-α production by an LDLR/LRP-independent mechanism

To determine if suppression of inflammatory cytokine production by apoE-mimetic peptides following LPS stimulation was due to cell surface apoE receptors, the expression of LRP and LDLR was evaluated by Western blotting on mouse peritoneal macrophages and on the BV2 cell line. Expression of both LRP and LDLR was
observed in the peritoneal macrophage, but expression of both receptors was notably absent in the BV2 cell line (Fig. 1A). With the established difference between LRP and LDLR expression in LPS-reactive cells of these two sources, we evaluated the ability of apoE[133–149] and the related COG1410 peptide to suppress TNF-α production following LPS stimulation. COG1410 is shorter than apoE[133–149] and was engineered to have a stabilized α-helical structure through introduction of helix-stabilizing aminoisobutyric acid residues, which provided for enhanced in vitro and in vivo anti-inflammatory activity relative to the natural apoE[133–149] peptide in other studies (33). Peritoneal macrophages showed no notable production of TNF-α when treated with no compound or with either of the apoE-mimetic peptides alone. Significant levels of TNF-α were produced following stimulation with LPS and IFN-γ, and the levels of TNF-α production were reduced in a dose-dependent manner by treatment with either apoE[133–149] or COG1410 (Fig. 1B). Treatment of BV2 cells in a similar manner also demonstrated significant suppression of TNF-α production following LPS stimulation (Fig. 1C). These data from BV2 cells that lack LDL/LRP receptors indicate that the anti-inflammatory activity of the apoE-mimetic peptides occurred through an LDLR/LRP-independent mechanism.

ApoE-mimetic peptides and apoE are transported into the cytoplasm and bind to SET/12PP2A

Because apoE[133–149] exerted anti-inflammatory effects independent of LRP/LDLR expression, we evaluated the ability of the apoE[133–149] peptide to become internalized into cells. BV2 cells were treated with an apoE[133–149] peptide that had biotin covalently attached to the N-terminal amine group of this peptide for use as an affinity tag, and with a nonbiotinylated apoE[133–149] peptide. After 2 h, the cells were washed with acid to strip cell surface/receptor-bound peptide, and the cells were fixed and subsequently permeabilized. After staining with Alexa Fluor 555-labeled streptavidin, red fluorescence from Cy3 was found in the cells treated with the biotin-labeled apoE[133–149] peptide, but was not observed in the cells treated with apoE[133–149] peptide lacking the biotin tag (Fig. 2A). The distribution of the red fluorescent signal indicated that apoE[133–149] peptide was transported into the cytoplasm of these cells.

To determine if the intracellular apoE[133–149] was binding to an internal protein, we used the biotin-labeled apoE[133–149] peptide to affinity purify cellular proteins that might bind to the apoE portion of the peptide. The biotin-tagged apoE-peptide was added to LPS-stimulated BV2 microglial cells. After incubation, the cells were lysed, and proteins bound to the biotinylated peptide were recovered as a complex by binding to agarose-conjugated streptavidin. After extensive washing of the agarose beads, bound proteins were eluted from the beads by heating in Laemmli buffer, separated by SDS-PAGE, and visualized with Coomassie staining (Fig. 2B). Two proteins of ∼39 and 25 kDa were readily observed in lanes containing the biotin-tagged apoE-mimetic peptide (lanes 4 and 5), but were absent in the control lane (lane 6) containing free biotin mixed with apoE[133–149] peptide lacking the covalently coupled biotin tag. Mass spectroscopy identified both bands as the protein SET, also known as 12PP2A, which exists in full-length and protease-cleaved forms (34, 35). To extend this observation to tissues, similar affinity purifications were performed with extracts from BV2 cells, mouse brain, and human brain. The binding of SET to the biotin-labeled apoE[133–149] peptide was observed by affinity purification followed by Western blotting in all three extracts (Fig. 2C). To confirm the localization of the SET protein in BV2 cells and to verify that SET and apoE[133–149] are found in the same intracellular compartment, we stained the BV2 cells treated with biotin-labeled apoE[133–149] with an anti-SET Ab. We detected the SET Ab with an Alexa Fluor 488-labeled secondary Ab and observed green fluorescence in the cytoplasm of BV2 cells. We previously observed that this same intracellular compartment contained the biotin-labeled apoE[133–149] peptide, as detected by Alexa Fluor 555-streptavidin staining (Fig. 2A).

To determine if apoE[133–149] binding to SET was consistent with its function as a mimetic of the apoE holoprotein, we investigated whether the apoE holoprotein bound to SET by performing interaction studies with purified apoE and GST-SET fusion proteins. Bacterially expressed GST-SET proteins were purified and represented the 1–77 N-terminal portion of SET, the 1–177 region that lacks the C-terminal fragment, and the full-length 1–277 SET protein (36). The purified GST-SET fusion proteins (and GST alone, as a control) were incubated with purified human apoE prior to affinity purification using either glutathione-agarose, or anti-apoE Ab/protein G–agarose complexes. Western blotting was used to detect SET–apoE interactions. An interaction was observed between the full-length GST-
SET fusion protein and human apoE (Fig. 3) in both GST-mediated affinity and anti–apoE-mediated affinity purifications. No interaction was observed between the GST control protein and apoE or for the GST-SET fusion proteins that lacked the C-terminal region from 177 to 277.

To confirm the specificity of the interaction, we used peptide-mediated affinity purification and Western blotting techniques to demonstrate that nonbiotinylated versions of the apoE[133–149] peptide and COG1410 could inhibit the interaction of the biotin–apoE[133–149] peptide with SET. Equal amounts of BV2 lysates were incubated with varying concentrations of nonbiotinylated peptides prior to addition of the biotin-labeled apoE[133–149] peptide, followed by incubation with streptavidin–agarose. Beads were washed repeatedly, heated in Laemmli sample buffer, and released proteins were run on an SDS-PAGE gel, as visualized with Coomassie staining. Lane 1, m.w. markers; lanes 2 and 3, total BV2 lysate; lanes 4 and 5, proteins affinity purified with biotin–apoE[133–149]; lane 6, proteins affinity purified with biotin alone. The arrowheads point to the 39-kDa SET protein and a 25-kDa fragment of SET.

ApoE-mimetic peptide treatment activates PP2A in vivo

Previous reports indicate that SET is a potent physiological inhibitor of PP2A. Because apoE peptides and apoE bind to SET, this suggests a novel mechanism whereby apoE/peptide increases PP2A activity within a cell by binding and forming a complex with SET, thereby reducing the amount of SET that is available to inhibit PP2A. To test this hypothesis, mice were injected with COG1410 or a vehicle control, and brains were removed 1 h later and assayed for PP2A activity. COG1410 could inhibit the interaction of the biotin–apoE[133–149] peptide with SET. Equal amounts of BV2 lysates were incubated with varying concentrations of nonbiotinylated peptides prior to addition of the biotin-labeled apoE[133–149] peptide, followed by affinity purification on streptavidin–agarose beads, which were washed and processed as above. Affinity purified proteins were subjected to Western blotting with an anti-SET Ab. For each blot, lane 1, m.w. markers; lane 2, 25 μg of total lysate; lane 3, pull-down with biotin only; lane 4, pull-down with biotin–apoE[133–149]. The major immunoreactive protein band is SET.

ApoE-mimetic peptide treatment activates PP2A in vivo

Previous reports indicate that SET is a potent physiological inhibitor of PP2A. Because apoE peptides and apoE bind to SET, this suggests a novel mechanism whereby apoE/peptide increases PP2A activity within a cell by binding and forming a complex with SET, thereby reducing the amount of SET that is available to inhibit PP2A. To test this hypothesis, mice were injected with COG1410 or a vehicle control, and brains were removed 1 h later and assayed for PP2A activity. COG1410 could inhibit the interaction of the biotin–apoE[133–149] peptide with SET. Equal amounts of BV2 lysates were incubated with varying concentrations of nonbiotinylated peptides prior to addition of the biotin-labeled apoE[133–149] peptide, followed by affinity purification on streptavidin–agarose beads, which were washed and processed as above. Affinity purified proteins were subjected to Western blotting with an anti-SET Ab. For each blot, lane 1, m.w. markers; lane 2, 25 μg of total lysate; lane 3, pull-down with biotin only; lane 4, pull-down with biotin–apoE[133–149]. The major immunoreactive protein band is SET.
phosphate cleavage was found to be ∼55% higher (p, 0.05) in brain extracts prepared from animals treated with COG1410 relative to vehicle-treated animals (Fig. 5).

ApoE-mimetic peptides modulate PP2A-regulated signaling pathways

On the basis of data showing that apoE-mimetic peptides bind and antagonize SET, resulting in activation of PP2A, we hypothesized that activation of PP2A by SET antagonists (i.e., apoE-mimetic peptides) would modulate PP2A-mediated signaling pathways. Therefore, we evaluated the effect of apoE-mimetic peptides on p38 MAPK and Akt phosphorylation following LPS stimulation of BV2 microglial cells. Following LPS stimulation, reduced phospho-p38 and phospho-Akt levels were observed in samples treated with apoE[133–149] relative to samples treated with only LPS (Fig. 6). These findings are also consistent with previously reported reductions of activation of the NF-κB pathway by reducing IKK phosphorylation with COG112, an apoE-mimetic peptide composed of the apoE[133–149] peptide fused to the antennapedia protein transduction domain peptide (21). These data provide mechanistic support for suppressed production of inflammatory cytokines, whose transcription is regulated by the MAPK and NF-κB pathways.

Finally, to validate that knockdown of SET produced a similar response to antagonism with apoE-mimetic peptides in modulating the inflammatory signaling process, production of the SET protein was silenced using siRNA techniques. Transfection of BV2 microglial cells with a noncoding, control siRNA did not reduce levels of LPS-induced NO production or the ability of COG1410 to significantly suppress NO production (Fig. 7A). However, transfection with SET-specific siRNAs resulted in significantly reduced production of NO relative to noncoding siRNA control samples upon LPS stimulation and was not further changed by the addition of COG1410. Although the amount of SET in the SET siRNA-transfected cells was greatly reduced, levels of the catalytic PP2Ac subunit of PP2A remained unchanged (Fig. 7B). Reduced NO production is consistent with increased PP2A functional activity without increased expression of the catalytic PP2Ac subunit in the

**FIGURE 4.** ApoE peptides compete with biotin–apoE[133–149] for binding to SET. A, BV2 microglial cell lysates were incubated with no competitor (–), apoE[133–149] (▲), or COG1410 (▼) at the indicated concentrations, followed by addition of biotin–apoE[133–149] and streptavidin–agarose beads. Beads were washed and heated, and released proteins were Western blotted and probed with anti-SET Abs. SET protein bands were quantified on a LiCor Odyssey fluorescence scanner and are plotted as arbitrary fluorescence units. Both the apoE[133–149] and the COG1410 peptides significantly competed with biotin–apoE[133–149] binding to SET in cell lysates. B, Western blots used for quantitation in A.

**FIGURE 5.** Subcutaneous administration of an apoE-mimetic peptide increases PP2A-mediated phosphatase activity in brain. C57BL/6 mice were injected with COG1410 (▼) or a vehicle control (●), and the activity of PP2A in brain extracts in a no extract control reaction (■) was measured using an immunoprecipitation/fluorometric assay, as described in Materials and Methods. The rate of phosphate cleavage in COG1410-treated brain extracts was significantly increased over the vehicle control or no extract control. p < 0.05 by ANOVA.

**FIGURE 6.** ApoE-mimetic peptides downregulate LPS-mediated phosphorylation of p38-MAP kinase and Akt kinase. A, BV2 microglial cells were treated with vehicle, LPS alone, apoE[133–149], and LPS plus apoE[133–149]. Total cell lysates were Western blotted, probed with anti-p38 MAP kinase or anti–phospho-p38 MAP kinase Abs, and proteins were quantified by fluorescence intensity (LiCor Odyssey scanner). The ratio of phospho-p38 MAP kinase divided by the total-p38 MAP kinase is plotted. Phospho-p38 MAP kinase levels are significantly higher in the LPS treatment than in the LPS plus apoE[133–149] treatment. B, The same lysates prepared for A were used in Western blots probed with anti-Akt or anti–phospho-Akt Abs, and proteins were quantified by fluorescence intensity (LiCor Odyssey scanner). The ratio of phospho-Akt divided by the total Akt is plotted. Phospho-Akt kinase levels are significantly higher in the LPS treatment than in the LPS plus apoE[133–149] treatment **p < 0.01.
LPS-mediated NO release. Given the simultaneous presence of phosphorylated signaling kinases and their associated activities, the regulation of inflammatory pathways can largely be viewed as a balance between the activating kinases and the deactivating phosphatases. In the case of the MAPK and NF-κB pathways, PP2A has been shown to mediate the deactivating dephosphorylation events. Therefore, inhibition of PP2A activity would be expected to result in an increased inflammatory response, whereas activation of PP2A would be expected to inhibit the inflammatory response.

Like the PP2A phosphatase complex, the SET protein, MAPK, and Akt kinases, we have demonstrated that the apoE-mimetic peptides are localized in the cytoplasm of BV2 cells. Similarly, exogenously applied biotin-labeled apoE holoprotein can be found in the cytoplasm and nuclei of Chinese hamster ovary cells (37). Furthermore, apoE protein has also been detected in the cytoplasm of neurons, astrocytes, and glial cells in human brain samples (38). In the case of the apoE holoprotein, reports suggested that apoE binds to LDLR and LRP and is taken up into cells by receptor-mediated endocytosis (2, 3). The apoE holoprotein is known to bind to LRP and LDLR, and the regions of the apoE protein ligand responsible for this finding are contained in the region from amino acids 130–150 (10). Similarly, an apoE[130–149] mimetic peptide that is 3 aa longer than the apoE[133–149] peptide used in these studies, binds to the LRP receptor with high affinity (19) through localized interactions between the complement repeat-17 region of LRP and the apoE[130–149] ligand, which were recently defined at the molecular structure level (39). Taken together, these data suggest that apoE and the apoE-mimetic peptides bind to LDLR or LRP or become internalized through receptor-mediated endocytosis (40). Although beyond the scope of the current work, the observation that apoE[133–149] gains entry into BV2 cells, which do not express either LDLR or LRP, suggests that other related receptors (40) may also allow for the apoE-mimetic peptides to gain entry into cells through endocytosis.

In this paper, we report that apoE and apoE-mimetic peptides bind to the SET protein, a potent physiological inhibitor of PP2A. Following activation of inflammatory signaling pathways via TLR-4 pathway stimulation with LPS, as the MAPK and NF-κB pathways become activated, binding of apoE and apoE-mimetic peptides to SET would be associated with an increased level of PP2A-mediated phosphatase activity, as we have reported. This increase in PP2A activity would account for the reduced levels of phosphorylated p38-MAP kinase and Akt kinase, as well as reduced NO release. We also observed that knockdown of SET using siRNA resulted in a decrease in NO production that was similar to the effect of antagonizing SET with the apoE-mimetic peptide.

These combined data suggest a novel molecular mechanism by which apoE and apoE-mimetic peptides positively modulate PP2A activity through antagonism of SET binding, with a concomitant reduction in inflammatory signaling due to decreased levels of activating phosphorylated signaling kinases. This mechanism is also consistent with previous reports that 1) both the apoE[130–149] peptide and the apoE holoprotein bind to LRP (3, 19); 2) apoE[133–149] can be detected inside cells (this paper), and the apoE holoprotein has also been found inside cells (37); 3) both apoE-mimetic peptides and apoE holoprotein inhibit production of NO, TNF-α, and other cytokines (15, 16, 18); and 4) both apoE-mimetic peptides and apoE holoprotein reduce NF-κB activation through inhibition of IKK phosphorylation (20, 21). These data, when coupled with the current demonstration that both the apoE-mimetic peptides and the apoE holoprotein bind to SET, suggest that the peptides are true mimetics of the apoE protein in receptor binding and SET binding activities. Furthermore, the molecular mechanism newly described in this paper, whereby apoE and apoE-mimetic peptides antagonize SET, increase cellular PP2A phosphatase activity, and decrease activation of inflammatory sig-
nal transduction pathways, may ultimately be a key for elucidation of the apoE genotype effects in human disease.

Disclosures
M.P.V., D.J.C., J.O., J.N., and F.L. are employees of Cognosci. M.P.V. owns shares of and serves on the Board of Directors of Cognosci. C.A.C., D.J.C., and M.P.V. have been reviewed and approved by the Duke Conflict of Interest Committee.

References