Apolipoprotein E Interrupts Interleukin-1β Signaling in Vascular Smooth Muscle Cells

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Objectives—Apolipoprotein E (apoE) exerts antiatherogenic effects but precise mechanisms remain unclear. We here investigated the effect of apoE on intracellular signaling by interleukin-1β (IL-1β), a proinflammatory cytokine present in atherosclerotic lesions.

Methods and Results—IL-1β–induced expression and activation of inducible nitric oxide synthase and cyclooxygenase-2 were inhibited by apoE in vascular smooth muscle cells (VSMCs). These inhibitory effects were linked to the suppression of both NF-κB and activating protein-1 (AP-1) transactivation, suggesting that the interruption of IL-1β signaling occurs upstream of transcription factors. Studies in VSMCs overexpressing IL-1β signaling intermediates revealed that NF-κB transactivation was inhibited by apoE in MyD88- and IRAK1- but not in TRAF6-transfected cells. Furthermore, apoE prevented IRAK1 phosphorylation and IRAK1-TRAF6 but not MyD88-IRAK1 complex formation. Inhibitory effects of apoE on IL-1β signaling were abolished after silencing LDL receptor–related protein-1 (LRP1) expression with siRNA. In addition, inhibitors of adenylyl cyclase and protein kinase A (PKA) restored IL-1β signaling in apoE-treated VSMCs, whereas apoE stimulated PKA activity. ApoE inhibited VSMC activation in response to IL-18 but not to tumor necrosis factor-α or polyinosinic:polycytidylic acid.

Conclusion—ApoE targets IRAK-1 activation and thereby interrupts IL-1β and IL-18 signaling in VSMCs. This antiinflammatory effect represents a novel antiatherogenic activity of apoE. (Arterioscler Thromb Vasc Biol. 2007;27: 1610-1617.)

Key Words: apolipoprotein E ■ IL-1β ■ vascular smooth muscle cells ■ inflammation ■ atherosclerosis

Apolipoprotein E (apoE) is a major protein component of plasma lipoproteins and plays an important role in preventing atherosclerosis. The antiatherogenic effects of apoE are usually attributed to its ability to promote cholesterol efflux from peripheral cells for reverse cholesterol transport and to facilitate hepatic clearance of very low density lipoprotein (VLDL) and chylomicron remnants. However, apoE protects against atherosclerosis even in experimental settings, in which its effects on plasma cholesterol are negligible. For instance, transgenic expression of apoE in arterial wall led to inhibition of atheroma formation without affecting plasma lipoprotein profile.1,2 Conversely, acceleration of atherosclerosis was observed in apoE−/− mice after transplantation of bone marrow from apoE-deficient mouse.3 These results suggest that apoE directly inhibits the development of atherosclerosis in a manner independent of its role in cholesterol transport.

Atherosclerosis is commonly regarded as a chronic inflammatory disease. Inflammation mediators such as cytokines and chemokines were shown to significantly contribute to the formation of atherosclerotic lesions, whereas antiinflammatory factors play the opposite role. ApoE has been previously suggested to suppress the expression of adhesion molecules on endothelial surface and thereby to prevent monocyte recruitment into the arterial intima.4 However, little information is available concerning the involvement of apoE in initiation and perpetuation of inflammation within the vascular wall. In the present study, we examined the influence of apoE on cellular effects of interleukin-1β (IL-1β), a proinflammatory cytokine produced by macrophages and present in atherosclerotic lesions. Our results indicate that apoE interferes with intracellular signaling in response to IL-1β. Along this way, apoE prevents IL-1β–induced activation of vascular smooth muscle cells (VSMCs), constituting an important component of the inflammatory sequel in the vascular wall.

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Methods

Cell Culture
VSMCs derived from rat thoracic aortas were maintained in Dulbecco modified Eagle medium (DMEM) containing 10% fetal calf serum. Human aortic smooth muscle cells (hASMCs, third passage) were purchased from a commercial supplier and cultured in SmGM-2 medium. In most experiments cells were preincubated for 1 hour with apoE (0 to 10 μg/mL) and stimulated with 10 ng/mL IL-1β.

Transient Transfections and Reporter Assays
Starved VSMCs were transfected with p(κB)-Luc and p(AP-1)-Luc plasmids using LipofectAmine 2000 (Invitrogen). Cotransfection experiments with MyD88, IRAK-1, or TRAF-6 expression plasmids were carried out by nucleofection. Lysates were assayed for luminescence intensity using Luciferase Assay System (Promega). β-galactosidase activity was used to normalize luciferase activity.

siRNA Silencing
The siRNA vectors based on pGeneClip-hMGFP and expressing 4 individual predesigned siRNA plus GFP as a reporter gene were transfected into VSMCs with LipofectAmine 2000. The overall transfection efficiency was assessed by fluorescence microscopy.

General Procedures
Data are presented as means±SD from at least 3 separate experiments or as results representative for at least 3 repetitions, unless indicated otherwise. Comparisons between the groups were performed with 2-tailed Student t test. Probability values less than 0.05 were considered significant.

Detailed description of all methods can be found in the supplemental materials, available online at http://atvb.ahajournals.org.

Results

ApoE Inhibits IL-1β–Induced iNOS and COX-2 Expression in VSMCs
Addition of IL-1β to VSMCs led to accumulation of iNOS and COX-2 mRNA, which was markedly reduced in cells pretreated with apoE (Figure 1A). This was accompanied by decreased iNOS and COX-2 synthesis. As shown in Figure 1B, the amounts of both enzymes were elevated after stimulation with IL-1β. ApoE alone failed to affect iNOS and COX-2 expression, but reduced the amounts of both enzymes, when added to VSMCs before IL-1β stimulation. The suppressing effects of apoE on IL-1β–induced iNOS and COX-2 synthesis were reflected by reduced enzymatic activities: IL-1β-stimulated nitrite/nitrate and prostaglandin E2 (PGE2) levels were reduced in a concentration-dependent fashion in the presence of apoE (Figure 1C).

The inhibitory effects exerted by apoE on iNOS and COX-2 were additionally assessed in hASMCs. Whereas IL-1β failed to induce iNOS expression and nitrite/nitrate generation in hASMCs, both COX-2 expression and PGE2 production were potently stimulated by IL-1β and these effects were completely abolished in the presence of apoE (Figure 1D; see also supplementary materials.). Endotoxin contamination did not account for the inhibitory effects of apoE on VSMCs activation (see supplementary materials).

ApoE Inhibits IL-1β–Induced NF-κB Activation and 1κB Phosphorylation in VSMCs
As a first step to investigate mechanisms underlying the inhibitory effect of apoE on iNOS and COX-2 expression, we

Figure 1. Effect of apoE on the IL-1β–induced expression and activation of iNOS and COX-2. VSMCs or hASMCs were incubated for 1 hour with or without apoE (10 μg/mL) or with increasing concentrations of apoE and exposed for 24 hour to IL-1β (10 ng/mL). A, mRNA levels were assessed by RT-PCR. Shown are representative agarose gels of amplified iNOS, COX-2, and GAPDH DNA fragments. B, Total protein was subjected to Western blot using anti-iNOS, anti–COX-2, and anti-actin antibodies. Results are representative for 1 experiment out of 5. C, Nitrite/nitrate and PGE2 levels in cell media were determined by fluorescence spectroscopy or EIA. D, COX-2 expression and PGE2 levels in human smooth muscle cells (hASMCs). Data represent mean±SD from 3 to 5 experiments. *P<0.05, **P<0.01, apoE-treated vs untreated cells.
examined the activity of the transcription factor NF-κB, which controls iNOS and COX-2 expression in VSMCs. To this aim, the IL-1β–induced binding of p65/RelA, a component of the NF-κB complex, to NF-κB binding DNA sequence was investigated in VSMCs in the presence or absence of apoE. As shown in Figure 2A, IL-1β activated NF-κB DNA binding and this effect was inhibited in VSMCs pretreated with apoE. To verify the inhibitory effect of apoE on NF-κB activation, transient transfection assays were performed using NF-κB–responsive reporter vector. Treatment of VSMCs with IL-1β increased NF-κB transcriptional activity, whereas apoE alone was ineffective. However, the stimulatory effect of IL-1β was reversed by apoE in VSMCs and hASMCs (Figure 2B).

NF-κB activation is preceded by phosphorylation and degradation of IkB, a component of the NF-κB complex. Therefore, we investigated total and phosphorylated IkB in VSMCs exposed to IL-1β in the absence or presence of apoE. Consistent with previous studies, IL-1β reduced total amount and increased phosphorylated IkB in VSMCs (Figure 2C). Both effects were reduced in cells pretreated with apoE, and IkB phosphorylation was inhibited in a concentration-dependent manner (Figure 2C). Inhibitory effects of apoE were also seen in hASMCs (Figure 2C). IkB phosphorylation is mediated by IkB kinases (IKK). Figure 2D demonstrates that apoE inhibited IL-1β–increased phosphorylation of IKK-α.

ApoE Inhibits IL-1β–Induced AP-1 Activation in VSMCs

In addition to NF-κB, the expression of iNOS and COX-2 in VSMCs is transcriptionally controlled by activating protein-1 (AP-1). The IL-1β–induced activation of AP-1 and upstream regulatory kinases p38MAPK and JNK were inhibited in the presence of apoE (see supplementary materials).

ApoE Prevents IL-1β–Induced IRAK-1/TRAF-6 Complex Formation and Inhibits IRAK-1 Phosphorylation in VSMCs

The reduced activity of both NF-κB and AP-1 in the presence of apoE suggests that the interruption of IL-1β signaling occurs upstream of transcription factors. Therefore, we assessed the effects of apoE on the function of myeloid differentiation factor 88 (MyD88), IL receptor-associated kinase-1 (IRAK-1), and TNF-α receptor-associated factor-6 (TRAF-6)–signaling intermediates consecutively activated in VSMCs on stimulation with IL-1β. To this aim, VSMCs were cotransfected with plasmids encoding wild-type MyD88, IRAK-1, or TRAF-6 together with the p(κB)5-Luc reporter plasmid. Transfection of cells with either of 3 IL-1β signaling intermediates enhanced NF-κB transcriptional activity (Figure 3A). ApoE reduced NF-κB activity in VSMCs transfected with MyD88 or IRAK-1 but not with TRAF-6.

Figure 2. Effect of apoE on the IL-1β–induced NF-κB activation. A, VSMCs exposed for 1 hour to apoE were stimulated with IL-1β (10 ng/mL). Lysates were analyzed for p65/RelA binding to specific DNA sequences. Shown are original presentation of solid phase-binding assay (left panel), quantitative effects of apoE on p65/RelA binding (middle panel), and dose-dependence of apoE-induced inhibition (right panel). Data represent mean±SD from 3 to 5 experiments. *P<0.05, apoE-treated vs untreated cells. B, VSMCs or hASMCs were transfected with p(κB)5-Luc reporter plasmid, stimulated for 24 hours with IL-1β (10 ng/mL) with or without apoE, and analyzed for luciferase. Data represent mean±SD from 3 to 5 experiments. **P<0.01, apoE-treated vs untreated cells. C, VSMCs or hASMCs were stimulated for indicated times with IL-1β (10 ng/mL) or preincubated for 1 hour without or with apoE (10 μg/mL or increasing concentrations) and stimulated for 10 minutes with IL-1β. Lysates were analyzed for total (t) and phosphorylated (p) IkB. Shown are blots representative for 3 to 5 experiments. Data represent mean±SD from 3 experiments. D, Lysates from VSMCs stimulated for 10 minutes with IL-1β (10 ng/mL) in the presence of apoE were analyzed for total (t) and phosphorylated (p) IKKα.
To further narrow the location of the signaling interference produced by apoE, we examined the formation of MyD88–IRAK-1 and IRAK-1–TRAF-6 complexes in response to IL-1β. We transfected an expression vector for Myc-IRAK-1 into VSMCs and assessed the anti-Myc immunoprecipitate for the presence of MyD88 or TRAF-6. As shown in Figure 3B, IRAK-1 coimmunoprecipitated MyD88 or TRAF-6 in VSMCs exposed to IL-1β/H9252. Preincubation of cells with apoE slightly increased MyD88–IRAK-1 complex formation but severely impaired the IRAK-1–TRAF-6 complex formation. Because activation of IRAK-1 precedes the formation of IRAK-1–TRAF-6 complex formation, we assessed the activation state of IRAK-1 in IL-1β-stimulated cells in the presence or absence of apoE exploiting the property of IRAK-1 to autophosphorylate. As shown in Figure 3C, immunoprecipitation of IRAK-1 from VSMCs exposed to IL-1β led to appearance of a typical migration pattern consisting of several bands corresponding to sequential steps of IRAK-1 phosphorylation at multiple sites. The IL-1β–induced IRAK-1 phosphorylation was inhibited by apoE.

The activation of IRAK-1 is negatively regulated by other members of the IRAK family as well as by the suppressor of cytokine signaling-1 (SOCS-1). ApoE failed to induce the expression of IRAK-2, IRAK-M, and SOCS-1 in VSMCs (Figure 3D).

**Figure 3.** Effect of apoE on the IL-1β–induced IRAK-1 activation. A, VSMCs were cotransfected with wild-type MyD88 (left panel), IRAK-1 (middle panel), and TRAF-6 (right panel) along p(β)5-Luc reporter plasmid, incubated for 24 hours without or with apoE (10 μg/mL), and analyzed for luciferase. Data are expressed as fold increase over empty vector-transfected cells and represent mean±SD from 3 to 6 experiments. *P<0.05, **P<0.01, apoE-treated vs untreated cells. B, VSMCs transfected with wild-type IRAK-1 were preincubated for 1 hour with apoE and stimulated for 1 or 10 minutes with IL-1β (10 ng/mL). IRAK-1 was pulled down using anti-Myc antibody and immunoprecipitates analyzed for MyD88 (1 minute stimulation) and TRAF-6 (10 minutes stimulation). C, [32P]orthophosphate-labeled VSMCs were stimulated with IL-1β (10 minutes) in the presence of apoE. Phosphorylated IRAK-1 was immunoprecipitated and visualized by autoradiography or anti–IRAK-1 Western blot. Results are typical for 1 experiment of 5. Lower panel, Radioactivity associated with IRAK-1 bands was analyzed by scintillation spectrometry. Results represent mean±range from 2 experiments. D, VSMCs were stimulated with apoE (10 μg/mL). Lysates were analyzed for IRAK-2, IRAK-M, and SOCS-1.

**LDL Receptor-Related Protein 1 and Protein Kinase A Are Involved in the Inhibitory Effects of ApoE on IL-1β–Induced Signaling in VSMCs**

Next, signaling pathways utilized by apoE to interrupt IL-1β signaling were characterized. Initially, we compared apoE with apolipoprotein A-I (apoA-I), which interacts with some but not all apoE receptors. As shown in Figure 4A, the activation of NF-κB DNA binding and NF-κB transcriptional activity as well as the phosphorylation of IκB in response to IL-1β remained unchanged in the presence of apoA-I. To further analyze the receptor involvement in the inhibitory effects of apoE, we made either use of receptor associated protein (RAP), which interferes with apoE binding to the low density lipoprotein (LDL) receptor family members, or heparinase, which abolishes apoE binding to heparan sulfate-containing proteoglycans. As shown in Figure 4B, inhibitory effects of apoE on the activation of NF-κB DNA binding, NF-κB transcriptional activity, and IκB phosphorylation in response to IL-1β were reversed in the presence of apoA-I. To address more specifically the identity of the receptor mediating inhibitory effects of apoE, we made either use of receptor associated protein (RAP), which interferes with apoE binding to the low density lipoprotein (LDL) receptor family members, or heparinase, which abolishes apoE binding to heparan sulfate-containing proteoglycans. As shown in Figure 4B, inhibitory effects of apoE on the activation of NF-κB DNA binding, NF-κB transcriptional activity, and IκB phosphorylation in response to IL-1β were reversed in the presence of RAP, but not heparinase. To address more specifically the identity of the receptor mediating inhibitory effects of apoE, we downregulated the expression of LDL receptor-related protein 1 (LRP1)—a member of LDL receptor family responsible for the suppressing effects of apoE on VSMCs proliferation—with a combination of 4 vectors encoding distinct siRNAs specific for LRP1 and a green fluorescent protein (GFP) as a reporter gene. As shown in Figure 4C, VSMCs were effec-
tively transfected with siRNA-encoding vectors and the expression of LRP1 was substantially reduced as assessed by polymerase chain reaction (PCR). Figure 4D demonstrates that the inhibitory effects of apoE on NF-κB transcriptional activity and IκB phosphorylation in response to IL-1β were absent in VSMCs transfected with LRP1-siRNA.

ApoE binding to LRP1 is associated with activation of protein kinases protein kinase A (PKA) and Akt. In addition, Akt and calcium/calmodulin-dependent protein kinase (CaMK) phosphorylate and inhibit IRAK-1. As shown in Figure 5A, the inhibitory effects of apoE on NF-κB transcriptional activity and IκB phosphorylation in response to IL-1β were absent in VSMCs transfected with LRP1-siRNA.

Figure 4. Involvement of LRP1 in the inhibitory effects of apoE on VSMCs activation. A, Native or p(κB)5-Luc reporter plasmid-transfected VSMCs exposed for 1 hour to apoE (10 μg/mL) or apoA-I (10 μg/mL) were stimulated for 10 minutes with IL-1β. Lysates were analyzed for NF-κB binding (upper panel), NF-κB activity (middle panel), or IκB phosphorylation (lower panel). *P<0.05, **P<0.01, apolipoprotein-treated vs untreated cells. B, Native or p(κB)5-Luc reporter plasmid-transfected VSMCs exposed for 1 hour with apoE (10 μg/mL) in the absence or presence of RAP (100 μg/mL) or heparinase III (1.0 U/mL) were stimulated with IL-1β (10 ng/mL). Lysates were analyzed for NF-κB binding (upper panel), NF-κB activity (middle panel), or IκB phosphorylation (lower panel). *P<0.05, inhibitor-treated vs untreated cells. C, VSMCs were transfected with pGeneClip-hMGFP vectors encoding green fluorescent protein (GFP) and siRNA specific for LRP1 (LRP1-siRNA) or scrambled RNA (scrRNA). Left panel, Demonstration of transfection efficiency by fluorescence microscopy. Right panel, PCR analysis of LRP1 expression. D, VSMCs were cotransfected with p(κB)5-Luc reporter plasmid and LRP1-siRNA- or scrRNA-encoding plasmids, preincubated with apoE (10 μg/mL, 1 hour), and stimulated with IL-1β (10 ng/mL). Lysates were analyzed for NF-κB activity (upper panel) or IκB phosphorylation (lower panel).

Effect of ApoE on VSMC Activation in Response to IL-18, TNF-α, and Polyinosinic:Polycytidylic Acid

In addition to IL-1β, apoE inhibited VSMC activation induced by IL-18, which predominantly exploits MyD88-IRAK-1–TRAF-6 signaling pathway, but not by TNF-α and polyinosinic:polycytidylic acid (poly(I:C)) (Toll-like receptor 3 [TLR-3] agonist), which use IRAK-1 or TRAF-6 as auxiliary signaling molecules (see supplementary materials).

Discussion

ApoE protects against atherosclerosis, and antiatherogenic effects of apoE are partly explained by the capacity of this apolipoprotein to interact with and to modulate VSMC function. For instance, apoE inhibits VSMC proliferation and migration and promotes VSMC-dependent extracellular matrix expansion. However, as yet no effects of apoE on the inflammatory activation of VSMCs have been reported. In the present study we show that the expression and activation of COX-2 and iNOS—2 enzymatic markers of inflammation—in response to IL-1β was downregulated in the presence of apoE. The inhibitory effects of apoE were seen both in rat and human VSMCs and occurred within a concentration range expected in the vasculature. Taken together, these
findings demonstrate that apoE exerts suppressing effects on the inflammatory activation of VSMCs.

Previous studies highlighted the central role of NF-κB and AP-1 in the regulation of COX-2 and iNOS expression. These transcription factors are activated by inflammatory molecules commonly released in the vascular environment and detected within atherosclerotic lesions. Our results show that interaction of apoE with VSMCs leads to impaired NF-κB and AP-1 promoter binding and transcriptional activation. To our knowledge, this is the first demonstration of the antagonistic effect of apoE on NF-κB and AP-1 activation evoked by proinflammatory stimuli in vitro. Several molecular mechanisms can be invoked to explain the inhibitory influence of apoE on NF-κB and AP-1 activation. Decreased promoter binding or nuclear translocation of p65/RelA or c-jun or reduced bioavailability of coactivators such as CBP/p300 were all postulated to account for the negative regulatory effects exerted on NF-κB and AP-1 activation by other antiatherogenic factors. Whereas our results do not preclude that apoE perturbs 1 or more of the above mechanisms, the inhibition of IL-1β–induced IκB and c-jun phosphorylation as well as IKK-α and JNK activation suggests that apoE interferes with upstream signaling pathways controlling both NF-κB and AP-1. The activation of IL-1 receptor (IL1-R1) results in the recruitment of IRAK-1 to receptor complex via an adapter molecule MyD88, followed by the formation of IRAK-1–TRAF-6 complex, which in turn is involved in NF-κB and AP-1 activation. The present study demonstrates that apoE inhibits transcription factor activation in MyD88 and IRAK-1 but not in TRAF-6–overexpressing cells and that this apolipoprotein prevents IRAK-1–TRAF-6 but not IRAK-1–MyD88 complex formation. Taken together, these results suggest that apoE disrupts IL-1β signaling upstream to NF-κB and AP-1 and identifies IRAK-1 as a molecular target of antiinflammatory effects of apoE in VSMCs.

The mechanism underlying the inhibitory effects of apoE on IRAK-1 function remains to be elucidated. During activation IRAK-1 becomes phosphorylated by IRAK-4, which terminates its interaction with MyD88 and initiates the aggregation with TRAF-6. The formation of the IRAK-1–TRAF-6 complex is interrupted by IRAK-M, IRAK-2, or SOCS-1, and factors increasing their expression suppress NF-κB and AP-1 activation. However, in the present study expression of IRAK-M, SOCS-1, or IRAK-2 remained unaltered in VSMCs exposed to apoE. As apoE prevented IRAK-1 phosphorylation, it may be assumed that the inhibition of IRAK-1 function occurs before its dissociation from the IL-1-R1–MyD88 complex. Posttranslational modifications such as phosphorylation by Akt or CaMK were previously proposed to prevent IRAK-1 autophosphorylation. However, in the present study the apoE-induced suppression of IκB phosphorylation and NF-κB activation were both retained in the presence of Akt and CaMK inhibitors, thus
excluding the involvement of both kinases in the inhibitory effects of apoE on IL-1β signaling. Hence, apoE seems to target IRAK-1 function by other mechanisms that may include functional modifications of either IRAK-1 or IRAK-4.

The preservation of apoE-induced NF-κB suppression in cells overexpressing MyD88 or IRAK-1 suggests that its inhibitory effects do not arise as a consequence of IL-1β displacement from IL-1 receptor. As apoE absorbs cholesterol from cell membranes, it might induce a perturbation of microenvironment, in which IL-1 receptor complex is localized. However, apoA-I—another apolipoprotein depleting membrane cholesterol—failed to affect IL-1β signaling, suggesting that the inhibitory effects are apoE-specific. It is more likely that the interaction of apoE with cells generates intracellular signals that interfere with IRAK-1 activation. Previous studies demonstrated that members of the LDL receptor family and heparin-sulfate proteoglycans serve as apoE binding partners in VSMCs.8,15–17 The observation that inhibitory effects do not arise as a consequence of IL-1, while interpreting results obtained with apoE-deficient mice. Moreover, IL-1β is a proinflammatory cytokine that promotes cholesterol accumulation by macrophages, expression of endothelial adhesion molecules, and VSMC migration at sites of vascular injury. Genetic ablation of IL-1β or IL-1R1 reduces lesion formation.28,29 Conversely, IL-1RA, a naturally occurring inhibitor of IL-1β signaling, decreases the severity of atherosclerosis.30 Hence, the inhibition of IL-1β signaling may contribute to the antiatherogenic potential of apoE. However, IRAK-1—the principal target of apoE ant-inflammatory signaling in VSMCs—is shared by various signal transduction pathways used by proatherogenic factors. For instance, IL-18, a cytokine aggravating atherosclerosis in mice, promotes IRAK-1 phosphorylation and IRAK-1–TRAF-6 complex formation.13 Moreover, MyD88–IRAK-1–TRAF-6 pathway is also exploited, albeit to various extent, by TNFα and by Toll-like receptors (TLRs) that both initiate formation of atherosclerotic lesions.12,13,31,32 The present study demonstrates that IL-18 signaling is negatively regulated by apoE. By contrast, proinflammatory effects of TNFα and poly(I:C)—the TLR-3 ligand—were not influenced by apoE. The considerable diversity of TNFα and TLR signaling may account for this observation. Whereas MyD88–IRAK-1–TRAF-6 represents a primary signal transducing module in cells exposed to IL-1β and IL-18, various mutually redundant pathways are switched on upon stimulation with TNF-α or TLR ligands. For instance, TAB-1/2, TAK-1, and TICAM-1 may be preferentially used by TLR-3 instead of MyD88, IRAK-4, and IRAK-1 to secure TRAF-6 recruitment to the TLR receptor.13 Likewise, activation of JAK–STAT pathway rather than NF-κB is involved in TNF-α–induced VSMC activation.33

In conclusion, the present study demonstrates that by targeting IRAK-1, apoE restricts cytokine-induced activation of VSMCs. This antiinflammatory effect may significantly contribute to the antiatherogenic potential attributed to this apolipoprotein.

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Disclosures
None.

References


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SUPPLEMENTARY MATERIALS (R2)

METHODS

Materials – Human apoE isolated from pooled human plasma was obtained from Biodesign Int., Saco, MN, and contained more than 90% of E3 isoform. IL-1β was purchased from PeproTech EC, London, UK. Receptor associated protein (RAP) was a generous gift of Dr. Marcus Merkel, University Hospital Hamburg-Eppendorf, Hamburg, Germany. Heparinase III was provided by Sigma, Deisenhofen, Germany. Pharmacological kinase inhibitors or activators SQ22536, LY294002, H89, KN93 and 8Br-cAMP were from MERCK Biosciences, Darmstadt, Germany. Antibodies against phosphorylated and native isoforms of mitogen-activated protein kinases p38 and jun N-terminal kinase (JNK) as well as against IκB and c-jun were obtained from Cell Signaling Technology, Beverly, MA. Polyclonal antibodies against interleukin receptor associated kinase-1 (IRAK-1) and tumor necrosis factor-α receptor-associated factor-6 (TRAF-6) were from SantaCruz Biotechnology, Karlsruhe, Germany. Antibodies against inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), myeloid differentiation factor 88 (MyD88), IRAK-M, IRAK-2 and suppressor of cytokine signaling-1 (SOCS-1) were from BD Transduction Laboratories, San Jose, CA, Lab Vision, Fremont, CA, ΨproSci, Poway, CA, Abcam, Cambridge, UK, StressGen, Victoria, Canada and Imgenex, San Diego, CA, respectively. Rat MCP-1 ELISA was provided by R&D Systems, Minneapolis, MN. QCL-1000 Chromogenic LAL endpoint assay was purchased by Cambrex, Vervier, Belgium. Other chemicals were from Sigma, Deisenhofen, Germany, and were of highest purity available.

Cell culture - Cell culture experiments were carried out using vascular smooth muscle cells (VSMCs) derived from rat thoracic aortas from 6-month-old male normotensive Wistar-Kyoto rats. Cells were incubated in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (Biochrom, Berlin, Germany), 100 U/mL penicillin G, 100
µg/mL streptomycin and 2 mmol/L L-glutamine. Cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The medium was changed initially after 24 h and then every 2–3 days. When cells had formed a confluent monolayer after about 8–10 days, they were harvested by addition of 0.05% trypsin, and the culture was continued for up to eight passages. To verify that cultured cells were VSMCs, we carried out immunocytochemical localization of smooth muscle-specific α-actin using a fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies ASM-1 (Progen, Heidelberg, Germany). Cells were made quiescent by incubation in serum-free medium containing 0.1% bovine serum albumin, 100 U/mL penicillin and 100 µg/mL streptomycin for 48 h.

In some experiments, human aortic smooth muscle cells (hASMCs) were used. hASMCs (third passage) were purchased from BioWhittaker and cultured in SmGM-2 medium, also from BioWhittaker, under a humidified atmosphere of 5% CO₂/95% air at 37°C. Cells used for experiments were between passages 4 and 6.

DNA expression vectors – Transcription factor-specific reporter gene vectors p(κB)₅-Luc and p(AP-1)₇-Luc were obtained from Stragene (La Jolla, CA). These vectors contain five or seven concatamerized consensus binding sites for NF-κB and activated protein-1 (AP-1), respectively, linked to a SV40 minimal promoter in front of the firefly luciferase (LUC) reporter gene. An eucaryotic expression vector for galactosidase (pCMV-SPORT-βgal) was obtained from Invitrogen, Karlsruhe, Germany. Expression vector for wild-type form of MyD88 (pCR3.V64-Met-Flag-MyD88-(1-306) was kindly provided by Dr. Jürg Tschopp (Univ. of Lausanne, Lausanne, Switzerland). Epitope-tagged wild-type forms of IRAK-1 (pCMV5-Myc-IRAK1-(1-710)) and TRAF-6 (pCMV5-Flag-TRAF-6-(1-524)) were generously donated by Dr. Ken Yanagisawa (Jichi Medical School, Tochigi, Japan). The generation of constructs used in this study has been described elsewhere (1,2).

Transient transfections and reporter assays – VSMCs were seeded in 24-well plates at a density of 0.5 x 10⁶ cells/well. 24 h prior to transfection cells were incubated in serum-free
DMEM. 1.0 µl LipofectAmine 2000 transfection reagent (Invitrogen, Karlsruhe, Germany) and 1 µg of pNF-κB-Luc and pAP1-Luc plasmids were added for 18 h to cells in serum-free DMEM (0.6 mL final volume) according to manufacturer’s recommendations. Thereafter, cells were incubated with IL-1β in the absence or presence of apoE as indicated in figure legends. To determine luciferase activity, cells were lysed using passive reporter lysis buffer (Promega, Madison, WI), transferred to polystyrene tubes, and assayed for luminescence intensity using Luciferase Assay System (Promega) and Sirius Luminometer (Berthold Detection Systems, Bad Wildbad, Germany). β-galactosidase activity was determined by standard protocol and used to normalize firefly luciferase activity in relation to transfection efficiency.

Co-transfection experiments were carried out by nucleofection as recommended by Amaxa (Köln, Germany). Briefly, VSMCs (1.5 x 10⁶ cells) were resuspended in Nucleofector Solution™ (Amaxa, 0.1 mL) in the presence of 1 µg MyD88, IRAK-1 or TRAF-6 expression plasmid and 1 µg of pNF-κB-Luc reporter plasmid. The amount of DNA transfected was kept constant by addition of appropriate amount of empty vector plasmid. A pool of cells was transfected using Amaxa nucleofection protocol U-25 optimized for VSMCs. Thereafter, cells were seeded in a 24-well plate, allowed to recover for 18 h and used for reporter assays. In some experiments, VSMCs were transfected only with Myc-IRAK-1, seeded in a 6-well plate and used for co-immunoprecipitation as described below.

Transcription factor binding – the DNA binding activities of transcription factors NF-κB and AP-1 were determined using EZ-Detect assay system (Perbio, Rockford, IL) according to the supplier recommendations. Briefly, quiescent VSMCs were stimulated with IL-1β in the presence or absence of apoE. Cells were lysed in PBS containing 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, and 0.1% (w/v) SDS, and equal amounts of lysates were exposed against solid phase-immobilized DNA fragments containing consensus binding sequences for
p65/RelA or c-jun. After extensive washing bound transcription factors were detected with specific anti-p65/RelA or anti-c-jun antibodies followed by HRP-conjugated secondary antibodies. The binding specificity was confirmed using appropriate competitor duplexes.

**Determination of mRNA levels** - Total RNA from VSMCs was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized from 1 µg of total RNA with the iScript cDNA Synthesis Kit (Bio-Rad, München, Germany). The resulting cDNA was subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) with gene-specific primers for rat iNOS and COX-2 genes and mouse/rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) obtained from R&D Systems (Minneapolis, MN). For the diagnostic semi-quantitative PCR against LRP-1, primers were designed from Acc# BC088327 (sense primer 1: CTAACAACAGCACCTGCACC, antisense primer 1: GAGCCACCATTGTACAGTG, antisense primer 2: ATATGCCAGGTGTTGCTG). In some experiments, the intactness of the cDNA was controlled by diagnostic PCR for human Ribosomal Protein 27 (hRP27; sense-primer: CCA GGA TAA GGA AGG AAT TCC TCC TG, antisense primer: CCA GCA CCA CAT TCA TCA GAA GG). For PCR reaction, 2 µg of cDNA were incubated at standard conditions with 2.5 U of HotStarTaq DNA Polymerase (Qiagen) and 7.5 µmol/L of each primer pair. After an initial denaturation at 95°C for 2 min, the reaction was carried out in 25 cycles (95°C, 1 min; 55°C, 1 min; 72°C, 1 min) followed by a final extension at 72 °C for 10 min. PCR products were visualized by agarose gel electrophoresis and quantified using the AlphaEase FC software system (Alpha Innotech, San Leandro, CA). iNOS and COX-2 mRNA were standardized against the level of the respective GAPDH controls.

**siRNA silencing** - The siRNA-expressing vectors were purchased from Super Array (SureSilencing shRNA plasmids, Frederick, USA). Vectors were based on the parent vector pGeneClip-hMGFP (Promega) and expressed four individual pre-designed siRNA from the U1 promoter plus GFP as a reporter gene. A fifth negative control vector contained a
scrambled artificial sequence with no resemblance to any human, mouse, or rat sequence instead of siRNA. Vectors were transformed into DH5α cells and purified using a commercial maxiprep kit (Invitrogen) including a precipitation step to remove bacterial endotoxins from the preparation. For transient transfection experiments VSMC were plated into 6-well plates the day before transfection and allowed to grow to 80% confluency. Prior to transfection, cells were kept in serum-reduced medium (5% FCS, v/v) for 2-3 h and subsequently allowed to recover in full medium for 1 h. Transfections were performed with LipofectAmin 2000 (Invitrogen). Briefly, 4 µg of endotoxin-free plasmid DNA was incubated with 10 µL of LipofectAmin2000 in DMEM without antibiotics or serum and given to the cells for 12 h. The transfecting compound was aspirated and the cells allowed recovering in full medium for 2 h prior to starving. The overall efficiency of transfection was assessed by estimation of GFP expression with fluorescence microscopy.

For co-transfection experiments, the siRNA effector plasmids were mixed with a pNF-κB-Luc reporter vector at a ratio of 3:1 in favour of the effector plasmids. Cells were harvested in 1x passive lysis buffer (Promega) and luciferase activity was measured as described above.

**Western Blotting** - VSMCs were lysed in a buffer containing 50 mmol/L Tris-HCl, 0.15 mol/L NaCl, 1% (v/v) Nonidet P-40, 5% (v/v) sodium deoxycholate, 0.1% (v/v) SDS, 300 mmol/L NaF, 1 mmol/L EGTA, 1 mmol/L orthovanadate, and protease inhibitors (Complete, Roche, Mannheim, Germany). Cell lysates (50 µg/lane) were subjected to SDS-polyacrylamide gel electrophoresis. Thereafter proteins were transferred to nitrocellulose membranes, which were blocked overnight in Tris-buffered saline containing 5% non-fat dry milk prior to incubations with antibodies. For each blot with anti-phosphospecific antibodies loading controls were performed, using an antibody against non-phosphorylated isoform of the respective antigen.

**Co-immunoprecipitations** – VSMCs transiently expressing Myc-IRAK-1 were pre-treated with apoE and stimulated with IL-1β (10 ng/mL) for 10 min, washed and scraped into 0.4 mL of assay buffer (20 mmol/L Tris-HCl, 250 mmol/L NaCl, 3 mmol/L EDTA, 3 mmol/L EGTA,
pH 7.6), containing 0.5 % (v/v) Nonidet P-10 and protease inhibitors. After lysis on ice (10 min) and three freeze-thaw cycles, insoluble material was cleared by centrifugation. Soluble fraction was incubated for 1 h on ice with polyclonal anti-Myc antibody (5.0 µg). Thereafter, 0.01 mL protein agarose G was added and samples were incubated overnight at 4°C. Agarose beads were washed 4x with assay buffer and 1x with high-salt assay buffer (350 mmol/L NaCl). Captured proteins were resuspended in Laemmli buffer, boiled, and separated by SDS-PAGE. Proteins were analyzed by Western blotting, using polyclonal antibodies against MyD88 or TRAF-6, as described above.

IRAK-1 Immunoprecipitation - VSMCs were incubated in phosphate-free Krebs-Ringer buffer containing 0.1 mCi/mL [³³P]orthophosphate for 4 h at 37°C and stimulated with IL-1β in the absence or presence of apoE. Cells were then scraped into 0.5 mL of lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L EGTA, 25 mmol/L NaF, 10 mmol/L Na₄P₂O₇) containing 1% (v/v) Triton-X100 and protease inhibitors. Lysates were precleared for 30 min at 4°C with protein A/G-agarose. The immunoprecipitation with 1-2 µg of anti-IRAK-1 antibody and 500 µg of protein was performed overnight at 4°C. Phosphorylated IRAK-1 was visualized by autoradiography after proteins were captured by incubation for 2 h with protein A/G-agarose, separated by SDS-PAGE, and blotted. Radioactivity associated with IRAK-1 bands was quantitatively analyzed by scintillation spectrometry.

Determination of protein kinase A (PKA) activity – Quiescent VSMCs were incubated in serum-free DMEM for 30 min with apoE in the presence or absence of indicated inhibitors and collected into Omnia Cell Extraction Buffer (Biosource Int. Camarillo, CA). PKA activity was determined in cell lysates using a commercially available fluorescence assay kit (Omnia™, Biosource Int.) according to the manufacturer instructions.

Determination of nitrate and prostaglandin E₂ (PGE₂) levels - VSMCs were incubated with agonists in serum-free DMEM for indicated times and the media were collected after 24h.

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Nitrate levels in incubation solutions were determined using a nitrate/nitrite fluorimetric assay kit (Cayman Chemical, Ann Arbor, MI) according to manufacturer instructions. PGE$_2$ levels were determined using an enzyme-linked immunoassay (EIA) kit according to the manufacturer instructions (Cayman Chemical).

**Determination of endotoxin concentration** – Endotoxine content in apoE was determined using limulus amebocyte lysate (LAL) endpoint assay according to manufacturer’s instruction.

**General Procedures** - Data are presented as means ± S.D. form three separate experiments or as results representative for at least three repetitions, unless indicated otherwise. Comparisons between the groups were performed with two-tailed Student t-test using MedCalc 6.0 statistical software. $p$ values less than 0.05 were considered significant.

**RESULTS**

*The effect of apoE on IL-1β-induced activation of rat and human smooth muscle cells* - In the present study, IL-1β induced eNOS expression and activation only in rat but not in human vascular smooth muscle cells. Whereas consistent with previous observations (3), this finding documents that experimental results obtained in rat and human cell models may diverge. Although major inhibitory effects of apoE on IL-1β-induced VSMCs activation assessed as COX-2 expression and activation, NF-κB transcriptional activity and IκB phosphorylation could be recapitulated in hASMCs, caution is necessary while applying the results of the present study to mechanisms of atherosclerosis development in humans.

*Inhibitory effects of apoE on VSMCs activation are not due to endotoxin contamination* - We were concerned that the observed inhibitory effects of apoE on IL-1β-induced iNOS and COX-2 expression could be attributed to the contamination with endotoxin, which may bind to apoE (4). However, the plasma-isolated apoE used in the present study contained <15 pg endotoxin/µg of apoprotein as determined by the LAL method. In addition, the inhibitory
effects of apoE on IL-1β-induced iNOS and COX-2 expression as well as nitrite/nitrate and PGE2 generation were retained in the presence of polymyxin B (10 µg/mL), which is known to neutralize endotoxin activity.

ApoE inhibits IL-1β-induced AP-1 activation in VSMCs – In addition to NF-κB, the expression of iNOS and COX-2 in VSMCs is controlled by a group of transcription factors termed activating protein-1 (AP-1). Therefore, in the next step the effect of apoE on the IL-1β-induced AP-1 activation was examined. To this purpose, the IL-1β-induced binding of c-jun, a component of AP-1 complex, to the solid phase-immobilized DNA containing a specific AP-1 binding sequence was investigated. As shown in Fig. IA, IL-1β promoted AP-1 DNA binding and this effect was suppressed in a concentration-dependent fashion by apoE. To further ascertain the inhibitory effects of apoE on IL-1β-induced AP-1 activation, transient transfection assays using AP-1 responsive reporter plasmid p(AP-1)7-Luc were performed. As shown in Fig. IB, the transcriptional activity of AP-1 was increased in VSMCs exposed to IL-1β. The stimulatory effect of IL-1β was suppressed in the presence of increasing amounts of apoE.

The transcriptional activity of AP-1 is controlled by two mitogen-activated protein kinases p38MAPK and JNK that phosphorylate c-jun. To further elucidate the mechanisms by which apoE negatively regulates AP-1 in VSMCs, we evaluated its effects on the activation of p38MAPK and JNK and the phosphorylation of c-jun using phosphospecific antibodies. As shown in Fig. IC, incubation of cells with IL-1β resulted in an increase in p38MAPK, JNK and c-jun phosphorylation, indicating that both kinases were in an active state. The pre-treatment of VSMCs with increasing concentrations of apoE reduced the IL-1β-induced p38MAPK and JNK activation as well as c-jun phosphorylation.

Effect of apoE on VSMCs activation in response to interleukin 18 (IL-18), tumor necrosis factor-α (TNFα), and polyinosinic:polycytidylic acid (poly(I:C)).
In the final step, we sought to examine the effect of apoE on VSMCs activation by pro-inflammatory factors sharing signaling intermediates with IL-1β. To this aim, we employed IL-18, which predominantly exploits MyD88→IRAK-1→TRAF-6 signaling pathway (5), as well as TNFα and poly(I:C) (Toll-like receptor 3 (TLR-3) agonist), which utilize IRAK-1 and/or TRAF-6 as auxiliary signaling molecules (6,7). Though IL-18 failed to induce iNOS expression and nitrite/nitrate generation in VSMCs, it effectively stimulated COX-2 expression and PGE₂ production, and both stimulatory effects were abolished in cells pretreated with apoE (Fig. IIA). In addition, apoE inhibited IL-18-induced production of monocyte chemoattractant protein-1 (MCP-1). Furthermore, NF-κB transcriptional activity and IκB phosphorylation in response to IL-18 were suppressed in VSMCs in the presence of apoE (Fig. IIA). In contrast to IL-18, neither TNFα nor poly(I:C) induced COX-2 expression or PGE₂ release in VSMCs (not shown). Moreover, TNFα failed and poly(I:C) only weakly induced iNOS expression and nitrite/nitrate generation (Fig. IIB). By contrast, both TNFα and poly(I:C) potently stimulated MCP-1 production and, in addition, promoted NF-κB transcriptional activity (Fig. IIB). None of the stimulatory effects induced by TNFα and poly(I:C) in VSMCs was significantly influenced by pre-treatment of cells with apoE.

REFERENCES TO SUPPLEMENTARY MATERIALS


nuclear factor-kappaB (NF-kappaB) and c-Jun N-terminal kinase (JNK). Biochem. J. 2003;370:159-166


**Figure I. Effect of apoE on the IL1β-induced AP-1 activation** – A. VSMCs were pre-incubated for 1h without or with apoE and stimulated with IL-1β (10 ng/mL). Lysates were exposed against solid phase-immobilized DNA fragments containing c-jun binding sequence and bound transcription factors were visualized. Shown are original presentation of the solid phase-binding assay (left panel), quantitative effects of apoE on c-jun binding (middle panel) and dose-dependence of apoE-induced inhibition (right panel). Data represent mean ± SD from 3 to 4 experiments. ** p < 0.01, apoE-treated vs. untreated cells. B. VSMCs were transfected with p(AP-1)7-Luc reporter plasmid, stimulated for 24h with IL-1β (10 ng/mL) in the absence or presence of apoE and analyzed for luciferase. Data represent mean ± SD from 3 independent experiments. ** p < 0.01, apoE-treated vs. untreated cells. C. VSMCs were pre-incubated for 1h without or with apoE and stimulated for 10 min with IL-1β (10 ng/mL). Lysates were probed with antibodies against total (t) and phosphorylated (p) p38MAPK, JNK and c-jun. Shown are blots from 3 to 5 experiments.
Figure II. Effect of apoE on the IL-18-, TNFα– and poly(I:C)-induced expression and activation of iNOS and COX-2 - Native or p(κB)5-Luc reporter plasmid-transfected VSMCs were preincubated for 1 h with apoE (10 µg/mL) and stimulated for 10 min (IκB phosphorylation) or 24h with IL-18 (25 ng/mL), TNFα (25 ng/mL), poly(I:C) (50 µg/mL) or IL-1β (10 ng/mL). Total protein was subjected to Western blot using anti-iNOS, anti-COX-2, anti-phospho-IκB or IκB antibodies. Nitrite/nitrate, PGE2 and MCP-1 levels in cell media were determined by fluorescence spectroscopy, EIA or ELISA, respectively. Luciferase activity was determined for assessment of NF-κB promoter activity. A. Effect of apoE on IL-18-induced VSMCs activation. B. Effect of apoE on poly(I:C) and TNFα-induced VSMCs activation. Results are representative for or represents means ± SD from at least three separate determinations. * p < 0.05; ** p < 0.01, apoE-treated vs. untreated cells.