

Structure–function properties of the apoE-dependent COX-2 pathway in vascular smooth muscle cells

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Abstract

Apolipoprotein (apoE) E is a multifunctional protein that plays a critical role in atherogenesis, in part by regulating the intimal proliferation of vascular smooth muscle cells. Recently, a novel cyclooxygenase (COX)-2 pathway was shown to contribute to the anti-proliferative action of human apoE3 in vascular smooth muscle cells (VSMC). Here, we provide insight into the structure–function properties by which apoE mediates these effects. ApoE3 is most effective in promoting COX-2 expression as a lipid-free protein and is less active after lipidation. Alterations in the stability of the helix bundle N-terminal domain of apoE that contains the binding site for the low density lipoprotein (LDL) receptor and heparin do not affect the up-regulation of the COX-2 pathway. In addition, the apoE2, 3, and 4 isoforms are all capable of up-regulating the COX-2 pathway. Finally, the effect of apoE on COX-2 was found to be independent of expression on the VSMC surface of the LDL receptor and heparan sulfate proteoglycans (HSPG). In summary, our data indicates that apoE, particularly in the lipid-free state, can up-regulate COX-2 in murine vascular smooth muscle cells apparently independently of binding to the LDLR, LRP or HSPG.

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1. Introduction

Apolipoprotein E (apoE) is a component of high density lipoproteins (HDL) and triglyceride-rich proteins (VLDL, chylomicrons) and, as such, is an important regulator of lipid homeostasis. The apoE molecule is composed of 22 kDa (residues 1–191) and 10 kDa (residues 216–299) domains connected by a hinge region. Each of these domains has distinct functions [1]. The 22 kDa N-terminal domain is important for receptor binding while the 10 kDa C-terminal domain is involved predominantly in lipid binding [2,3].

The lipid transport functions of apoE depend on its interaction with the LDL receptor (LDLR) family and heparan sulfate proteoglycans (HSPG) [4,5]. These apoE receptors are involved in lipoprotein metabolism, cholesterol homeostasis, brain development, vascular integrity, and protection against the development of atherosclerosis [6–8].

Independent of its ability to regulate lipoprotein metabolism, apoE affects the vascular system by modulating inflammation [9], suppressing oxidative stress [10], and inhibiting the proliferation of vascular smooth muscle cells [11–15]. The role of apoE in the proliferation of vascular smooth muscle cells (VSMC) in particular has important implications for atherosclerosis, vascular remodeling and restenosis.

The cyclooxygenase (COX) enzymes convert arachidonic acid to prostaglandin H₂ that eventually leads to the

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biosynthesis of eicosanoids; these include prostaglandins (PGE₂, PGF_{2α}, PGD₂), prostacyclin (PGI₂) and thromboxane (TXA₂). COX exist as two isoforms: COX-1 is constitutively expressed whereas COX-2 is up-regulated by various pro-inflammatory mediators, such as lipopolysaccharide, growth factors, cytokines and apolipoproteins [16]. The major eicosanoids produced by the activation of COX-2 in endothelial cells are prostacyclin (PGI₂) and prostaglandin E₂ (PGE₂) [17]. The PGI₂ signaling cascade is dependent upon activation of its prostacyclin receptor (IP), which is generally associated with a G stimulatory protein coupled receptor [18]. The physiological consequences of eicosanoid metabolite production by the COX-2 enzyme are tissue- and cell-specific. In vascular systems, COX-2-dependent production of prostacyclin can affect platelet coagulation [19–21] and leukocyte adhesion [22,23]. COX-2 activation in endothelial cells and vascular smooth muscle cells plays a vital role in the development and stability of atherosclerotic plaque. COX-2 is up-regulated in atherosclerotic lesions either by inflammatory cytokines or platelet activation [24,25].

In vitro studies have implicated several molecular pathways by which apoE may directly influence the proliferation of VSMC. Ishigami et al. showed that apoE inhibits platelet derived growth factor (PDGF)-induced VSMC proliferation by mitogen-activated protein kinase (MAPK) mediated suppression of the G₁ cell cycle gene, *cyclin D* [14,15]. The initial signal transduction mechanism describing apoE's anti-proliferative activity in VSMC involves binding of the C-terminal domain of apoE to perlecan HSPG through PDGF stimulation to induce iNOS which suppresses MAP kinase activity [13,14]. This suppression subsequently arrests cells in the G₀ phase of the cell cycle (21). We have identified another signaling pathway by which apoE binds to an unknown receptor to up-regulate COX-2 and prostacyclin production [11]. Prostacyclin activates the G_s protein-coupled (IP) receptor which eventually leads to inhibition of the CREBP and pocket protein-E2F of cyclin A, thereby, preventing cell progression to the S-phase [12]. In this study, we sought to further characterize important structure–functional properties of apoE regarding its ability to mediate up-regulation of the COX-2 pathway.

2. Materials and methods

2.1. VSMC culture

Primary murine aortic smooth muscle cells were isolated from wild-type C57BL/6 (Jackson Laboratory, Bar Harbor, Maine) or LDLR/apobec double knockout mice as described [26] and used between passage three and five. The cells were maintained in 40:40 ratio of Dulbecco's modified Eagle's/Ham F12 medium containing 10% heat-inactivated fetal bovine serum (HI-FBS) and 25 mM HEPES, penicillin, streptomycin, and glutamine. The purity of the VSMC

population was determined by the expression of α-smooth muscle actin as detected by immunofluorescence. For experiments, VSMC were rendered quiescent when the cells were a near-confluent monolayer in serum-free defined medium [12] for 2 days followed by re-stimulation in 10% HI-FBS DMEM/Ham F12 media in the absence or presence of 2 μM recombinant lipid-free apoE proteins: human apoE3, apoE2, and apoE3, mutants apoE3 (K146E), N-terminal domain of apoE-22 kDa (apoE22), C-terminal domain of apoE3-10 kDa (apoE10), and apoE3 22 kDa quadruple mutant (L141K, K143L, K146L, L148K) (4apoE3-22). The expression and purification of recombinant apoE proteins were described previously [27]. The 1,2-dimyristoyl phosphatidylcholine (DMPC)-apoE complexes were prepared as described previously [27].

2.2. Quantitative real time polymerase chain reaction (QRT-PCR)

VSMC were serum-starved and then stimulated with the 10% HI-FBS media described above in the absence or presence of 2 μM apoE. After 6 h (near optimum expression of COX-2), total RNA was extracted by Trizol, treated with DNase and 1.5 μg total RNA reversed transcribed into cDNA using Superscript II reverse transcriptase enzyme (Invitrogen). mRNA levels were quantitatively determined on an ABI Prism 7000 sequence detection system (Applied Biosystems) using SYBR-green detector or universal TaqMan. Murine primers COX-2, COX-1 and GAPDH containing unlabeled PCR primers and/or FAM dye-labeled Taqman MGB probe were purchased from assays-on-demand gene expression products (Applied Biosystems). The relative mRNA of COX-2 was normalized to the housekeeping genes, cyclooxygenase-1 (COX-1) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and then fold-induction was determined above the control levels of 10% FBS conditioned media. There was no expression of COX-2 above control levels in unstimulated VSMC.

2.3. Immunoblotting

Subconfluent VSMC were serum-starved for 48 h and then restimulated as described above in 10% FBS media supplemented with 2 μM apoE for 24 h. Total protein was isolated by incubation for 15 min at room temperature with MPER lysis reagent (Pierce biotech) supplemented with phosphatase inhibitors and sodium orthovanadate, and the protein concentration determined by BCA assay. An equal amount of total protein (10–20 μg) was resolved on 4–12% NUPAGE, transferred onto nitrocellulose membrane (BioRad), and blocked with 5% non-fat dried milk in Tween20 phosphate-buffered saline (PBS). The nitrocellulose membrane was incubated with 1:1000 dilution of primary antibody and then with 1:5000 dilution of horseradish peroxidase (HRP) conjugated secondary antibody. The protein bands were visualized with ECL Western blot kit and quantified by densitometric anal-

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