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Apolipoprotein E-mediated cell cycle arrest linked to p27 and the Cox2-dependent repression of miR221/222

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ABSTRACT

Objective: In addition to its effects on cholesterol levels, apoE3 has lipid-independent effects that contribute to cardiovascular protection; one of these effects is the ability to inhibit cell cycling in VSMCs. The goal of this study was to identify and characterize cell cycle-regulatory mechanisms responsible for the anti-mitogenic effect of apoE.

Methods and results: Primary VSMCs were stimulated with serum in the absence or presence of apoE3. apoE3 upregulated expression of the cdk inhibitor, $p27^{kip1}$, in primary VSMCs, and this effect required Cox2 and activation of PGI_2-IP signaling. The microRNA family, miR221/222 has recently been identified as a post-translational regulator of p27, and apoE3 inhibited miR221/222 expression in a Cox2- and PGI_2/IP-dependent manner. Moreover, reconstituted miR222 expression was sufficient to override the effects of apoE on p27 expression and S phase entry. The ability to repress expression of miR221/222 is shared by apoE3-containing HDL but is absent from apoA-1, LDL and apoE-depleted HDL. All three apoE isoforms regulate miR221/222, and the effect is independent of the C-terminal lipid-binding domain. miR221/222 levels are increased in the aortae of apoE3-null mice and reduced when apoE3 expression is reconstituted by adeno-associated virus infection. Thus, regulation of miR221/222 by apoE3 occurs in vivo as well as in vitro.

Conclusions: ApoE inhibits VSMC proliferation by regulating p27 through miR221/222. Control of cell cycle-regulatory microRNAs adds a new dimension to the spectrum of cardiovascular protective effects afforded by apoE and apoE-HDL.

1. Introduction

Apolipoprotein E (apoE), a component of high density and triglyceride-rich lipoproteins, regulates lipid homeostasis and plays an important role in preventing atherosclerotic disease [1,2]. ApoE3 is composed of a C-terminal, 10-kDa domain that is required for lipid-binding and an N-terminal, 22-kDa that binds to the LDL receptor [1,3]. Other reported properties that contribute to the anti-atherogenic behavior of HDL include anti-inflammation, anti-oxidation, anti-thrombosis and vasodilation. ApoE may also protect against cardiovascular disease by inhibiting vascular smooth muscle cell (VSMC) proliferation [4–8]. Transgenic expression of apoE inhibits, while deletion of apoE increases, VSMC proliferation after vascular injury in vivo [9].

ApoE is a polymorphic protein with three major isoforms, apoE2, apoE3 and apoE4. ApoE3 is the most common and is considered to be the parent form of the protein [1,3]. The polymorphism in apoE occurs at residues 112 and 158; the apoE4 isoform contains R at both positions while the apoE3 and apoE2 isoforms contain C/R and C/C, respectively, at these sites. The C/R interchange at position 112 that distinguishes apoE3 and apoE4 has little effect on LDLR binding activity whereas the C/R substitution at position 158 dramatically lowers the binding of apoE2 to the LDL receptor (LDLR). This is the primary defect in type III hyperlipoproteinemia. Besides being a risk factor for atherosclerosis, apoE4 polymorphism is a major genetic risk factor for Alzheimer's disease [1,10].

Abbreviations: Apo, apolipoprotein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VSMC, vascular smooth muscle cell; cdk, cyclin-dependent kinase; qPCR, quantitative PCR.

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The mechanism by which apoE controls VSMC proliferation is not well understood. Others have reported that its anti-mitogenic effect is associated with a partial reduction in the expression of cyclin D1 mRNA [4]. We have not seen a strong effect of apoE on cyclin D1 [6], but did find that apoE3 increases Cox2 expression and prostacyclin (PGI_2) production in primary VSMCs [6,11]. Furthermore, we showed that these effects led to a PGI_2- and cyclin E/cdk2-dependent inhibition of VSMC cycling [6]. Cyclin-cdk2 complexes are typically inhibited by the binding of cip/kip family cyclin-dependent kinase (cdk) inhibitors ($p21^{cip1}$, $p27^{kip1}$, and $p57^{kip2}$). Of these cdks, p27 has been closely linked to regulation of VSMC cycling, especially after vascular injury [12,13]. Deletion of p27 also accelerates atherosclerosis in apoE-null mice [14]. Regulators of p27 therefore have the potential to strongly influence neointima formation in atherosclerosis and during the response to injury. In this report, we show that apoE and apoE-containing HDL strongly inhibit the expression of miR221/222, a microRNA family that regulates p27 levels post-transcriptionally. Additionally, we show that the effect of apoE on miR221/222 leads to an upregulation of p27, and that the change p27 expression is sufficient to explain the anti-mitogenic effect of apoE in VSMCs.

2. Methods

2.1. Cell culture

Early passage explant cultures of mouse vascular smooth muscle cells (VSMCs) were isolated from 8 to 10 week old male C57BL/6 mouse (Jackson Labs) or IP-null mice on the C57BL/6 background ([15]; kindly provided by Garret FitzGerald, University of Pennsylvania). Explant culture VSMCs were isolated from aortae (aortic arch plus the descending thoracic aorta) of these mice as described [6] and maintained in growth medium (1:1 Dulbecco's modified Eagle's Medium (DME)/Ham's F-12 supplemented with 2 mM L-glutamine and 10% FBS). The FBS was not depleted of bovine apoE before use. Cells were used between passages 2–5. For cell cycle experiments, 60–90% confluent monolayers of wild-type, $IP^{-/-}$, or $p27^{-/-}$ VSMCs were grown in 60-mm (for RT-qPCR, S phase assays, or transfections) or 100-mm (for RT-qPCR and western blotting) culture dishes. The cells were G0-synchronized by incubation in serum-free DME containing 1 mg/ml heat-inactivated, fatty acid-free BSA (DME-BSA) for 48 h before stimulation with fresh growth medium in the absence or presence of 200 nM cicaprost (kindly provided by Bayer Schering Pharma AG), 50 μg/ml lipoprotein, 60 ± 5 μg/ml apolipoprotein, 1 μM nimesulide (a Cox-2 inhibitor) or 1 mM SC560 (a Cox-1 inhibitor). Recombinant human apoA-I, apoE2, apoE3, apoE4, the 22 kD N-terminal fragment (amino acids 1–191) of apoE3, and the 10 kD C-terminal fragment (amino acids 222–299) of apoE3 were expressed in E. coli and purified as described [16,17]. ApoE3 and its N- and C-terminal domains were tested at equivalent molarities (2 μM), as were the three apoE isoforms. Samples were dialyzed against PBS immediately before use. When S phase entry was measured, BrdU or EdU was added at the time of serum stimulation and remained in the cultures throughout the experimental incubation. LDL, total HDL, and apoE-depleted HDL were purified similarly to published procedures [18,19].

2.2. Transfections

We transfected near confluent VMSCs in 60-mm dishes containing coverslips with 3 μg of either an expression plasmid for microRNA-222 (Origene) or pCDNA (control) using 25 μl Lipofectamine 2000. After 4 h, the transfected cells were allowed to recover overnight in regular growth medium. The cells were then starved for 48 h in DME/BSA and directly stimulated with fresh growth medium containing 10% FBS and apoE3.

2.3. Quantitative real-time reverse transcriptase-PCR (qPCR)

To measure steady-state levels of miR-221 and miR-222, total RNA was isolated from cells or isolated aortae with TRIZOL and reverse transcribed using 15–40 ng of RNA in a 10-μl reaction with TaqMan MicroRNA reverse transcription kit (Applied Biosystems). An aliquot (20%) of the reaction was used for qPCR using TaqMan universal master mix, Mature MicroRNA assay ID #524 (miR221), #2276 (miR222), and #1232 (snoRNA202) (Applied Biosystems). To measure Cox-2 mRNA levels, ~50 ng of RNA was reverse transcribed in a 20-μl reaction, and the cDNA was subjected to qPCR using TaqMan gene expression assays Mm00478374_m1 (Applied Biosystems) respectively. RT-qPCR results were calculated using the standard curve or ddCt methods using 18S and SnoRNA202 as the reference for mRNAs and microRNAs, respectively.

2.4. Immunoblotting and immunofluorescence microscopy

Cells for immunoblotting were collected and lysed as described [20]. Equal amounts of protein (15–25 μg) were resolved on reducing SDS mini-gels and immunoblotted using antibodies specific for p27 (BD Biosciences Pharmingen), GAPDH (sc-25778, Santa Cruz Biotechnology) or actin (sc-8432, Santa Cruz Biotechnology). The resolved proteins were detected using ECL (Amersham). S phase incorporation assays were performed similarly to that described [21] using BrdU or EdU. Images were captured using a Nikon Eclipse 80i microscope, 20×/0.45 PL Plan Fluor objective, Hamamatsu C4742-95 digital camera and camera controller. Images were analyzed using Image-Pro Plus software, and the number of BrdU-positive and Dapi-positive nuclei was manually counted.

2.5. In vivo experimentation

The aortic arch and thoracic aorta were isolated from euthanized 11-week male wild-type and apoE-null mice on the C57BL/6J background. The isolated aortae were cleaned and stored in RNAlater (Qiagen) until isolation of total RNA using RNeasy (Qiagen). For the in vivo adeno-associated virus reconstitution experiments, an AAV8-TBG-hapoE3 vector was prepared by the Vector Core of the Penn Gene Therapy Program. Male apoE3-deficient mice (9-week old) were injected with AAV8-TBG-hapoE3 via the tail vein at a dose of 1×10^{12} genome copies. Mice were sacrificed at 11-weeks, and total RNA was prepared as described above. See Kitajima et al. [22] for methodological details of AAV vector construction, tail vein injection, cholesterol levels, and apoE3 expression.

3. Results

3.1. ApoE3 regulates p27 through the Cox2-PGI_2 pathway in VSMCs

We previously reported that the anti-mitogenic effect of apoE3 requires the induction of Cox2 and production of PGI_2 [6]. More recently, we showed that PGI_2 inhibits VSMC cycling in a p27-dependent manner [23]. We therefore investigated the importance of p27 to the anti-mitogenic effects of apoE. Consistent with our previous studies [23], p27 levels were downregulated in response to serum-stimulation in wild-type VSMCs, and treatment with the PGI_2 mimetic, cicaprost, prevented this decrease in p27 (Fig. 1A; top panels). ApoE3 also antagonized the serum-induced downregulation of p27 in wild-type VSMCs (Fig. 1A; top panels). This apoE effect was blocked by the Cox2 inhibitor, nimesulide, but not by the Cox1 inhibitor, SC560 (Fig. 1A; top panels). The level of