Regulated expression of apolipoprotein E by human retinal pigment epithelial cells

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Abstract In early age-related macular degeneration (AMD), lipid-containing deposits (drusen) accumulate in Bruch's membrane underlying the retinal pigment epithelium (RPE). Recent studies indicate that apolipoprotein E (apoE) may play a role in lipid trafficking in AMD. Compared with the apoE3 allele, the apoE4 and apoE2 alleles are associated with decreased and increased risk for AMD, respectively; drusen contain high levels of apoE, and apoE null mice develop lipid deposits in Bruch's membrane similar to those observed in AMD. Primary cultures of human RPE cells expressing the apoE3 allele were grown on Transwell® culture plates. Western blotting, ELISA assay, and mass spectrometry confirmed that apoE3 was secreted into the apical and basal chambers and that secretion was upregulated by thyroid hormone, 9-*cis*-retinoic acid, and 22(*R*)-hydroxycholesterol. In addition, basally secreted apoE associated with exogenously added HDL. These results indicate that apoE secretion can be regulated by specific hormones and that apoE associates with HDL. The findings are consistent with a role for apoE in lipid trafficking through Bruch's membrane and may be relevant to AMD.—Ishida, B. Y., K. R. Bailey, K. G. Duncan, R. J. Chalkley, A.L. Burlingame, J. P. Kane, and D. M. Schwartz. **Regulated expression of apolipoprotein E by human retinal pigment epithelial cells. *J. Lipid Res.* 2004.** 45: **263–271.**

Supplementary key words age-related macular degeneration • Bruch's membrane • retina • high density lipoprotein • thyroid hormone

Age-related macular degeneration (AMD) is the leading cause of severe visual loss in the developed world (1, 2). In the early stages of the disease, before visual loss occurs from choroidal neovascularization, there is progressive accumulation of lipids in Bruch's membrane (3–6). Bruch's membrane lies at the critical juncture between the outer retina and its blood supply, the choriocapillaris. Lipid deposition causes reduced hydraulic conductivity and macromolecular permeability in Bruch's membrane and is thought to impair retinal metabolism (7–9). Interestingly, lipid accumulation in Bruch's membrane similar to that in AMD has been observed in apolipoprotein E (apoE) null mice (10, 11). Because of the additional association between apoE alleles and other age-related degenerations, such as Alzheimer's disease and atherosclerosis, there has been recent investigation into a potential role for apoE in AMD.

Several studies of apoE polymorphism in AMD have been conducted (12–14). In contrast to Alzheimer's disease, the apoE4 allele has been associated with a reduced prevalence of AMD. The apoE2 allele is slightly increased in patients with AMD. Further supporting a role in AMD pathogenesis, apoE has been detected in drusen, the Bruch's membrane deposits that are the hallmark of AMD (13, 15). Immunohistochemical studies of postmortem eyes have demonstrated apoE in the basal aspect of the retinal pigment epithelium (RPE) (15). Cultured RPE cells synthesize high levels of apoE mRNA, comparable to the levels found in brain (15).

Although the role of apoE in AMD is not established, this apolipoprotein has several functions that may affect the course of this disease. ApoE has anti-angiogenic (16), anti-inflammatory (17), and anti-oxidative (18) effects. These are all considered atheroprotective attributes of apoE, but they may also be important in protecting against the progression of AMD. Although atheroprotective effects of apoE were initially thought to stem from effects on plasma lipid levels, local effects on vascular macrophages are probably equally important. Thus, selective enhanced expression of macrophage apoE in the arterial wall reduces atherosclerosis in spite of hyperlipidemia

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Abbreviations: AMD, age-related macular degeneration; HC, 22(*R*)-hydroxycholesterol; LXR, liver X receptor; POS, photoreceptor outer segment; RA, 9-*cis*-retinoic acid; RPE, retinal pigment epithelium; RXR, retinoid X receptor; T_3, thyroid hormone (3,3′,5-triiodothyronine); TR, thyroid hormone receptor.

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(19–21). Conversely, reconstitution of apoE null macrophages in C57BL/6 wild-type mice induces atherosclerosis (22). Atheroprotective effects of arterial apoE expression are thought to derive in part from facilitation of reverse cholesterol transport (23, 24). The mechanisms by which apoE facilitates reverse cholesterol transport are incompletely understood. ApoE expression increases cholesterol efflux to HDL_3 in J774 macrophages (25) and to lipid-free apolipoprotein A-I (26). Cell surface apoE is also hypothesized to induce cholesterol efflux from the plasma membrane (24).

Reverse cholesterol transport may be important in the pathogenesis of AMD because of its role in lipid efflux from RPE into Bruch's membrane. Like intimal macrophages, RPE cells progressively accumulate lipid deposits throughout life; however, unlike intimal macrophages, the source of RPE lipid is thought to be the retinal photoreceptor outer segment (POS) (27). A schematic representation of POS uptake by the RPE and lipid efflux across Bruch's membrane is shown in **Fig. 1**. Every day, each RPE cell phagocytoses and degrades more than 1,000 POS discs via lysosomal enzymes. These POS discs are enriched in phospholipid and contain the photoreactive pigment rhodopsin. Incompletely digested POS lipids accumulate as lipofuscin in RPE. By age 80 years, ~20% of RPE cell volume is occupied by lipofuscin (28).

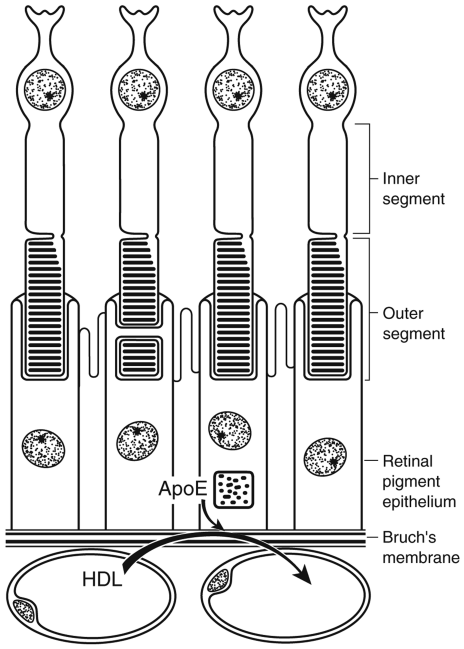

Fig. 1. Schematic representation of photoreceptor outer segment (POS) lipid uptake and trafficking by the retinal pigment epithelium (RPE). The diagram shows one possible pathway for POS-derived lipids to traverse Bruch's membrane. Shown are photoreceptor cells with inner and outer segments labeled, RPE, Bruch's membrane, and blood vessels. Microvilli of RPE cells aid in the phagocytosis of lipid-rich POS at the RPE apical surface. The POS lipids are digested by lysosomes. Lipids that are not recycled to the photoreceptors for new POS synthesis, in particular oxidized lipids, may be secreted across the basal surface, perhaps bound to apolipoprotein E (apoE). Secreted lipid and apoE may bind HDL from the circulation in Bruch's membrane, where they can enter the circulation after crossing Bruch's membrane.

Analysis of Bruch's membrane lipid reveals an age-related accumulation of phospholipid, triglyceride, cholesterol, and cholesterol ester (4). The origin of these lipids also is thought to derive principally from POS rather than from the circulation (4, 5). POS lipids are hypothesized to efflux from the RPE into Bruch's membrane. Although cholesteryl ester deposition in Bruch's membrane suggests a contribution from plasma lipids, biochemical analysis of these esters suggests esterification of intracellular cholesterol by RPE cell-derived acetyl cholesteryl acyltransferase (29). Although trafficking of lipids from the retina to RPE cells has been studied extensively, the mechanisms of lipid efflux from RPE to Bruch's membrane are not well understood. Furthermore, from a pathogenic standpoint, the regulation of lipid efflux into Bruch's membrane may be important in determining the rate of lipid-induced thickening that occurs in aging.

Nuclear hormone receptor ligands regulate reverse cholesterol transport in macrophages via their effects on ATP binding cassette receptor A1 (ABCA1) and apoE expression. Liver X receptor (LXR) and retinoid X receptor (RXR) ligands increase the levels of these transporters and increase reverse cholesterol transport in macrophages (30–32). Thyroid hormone (T_3) has also been demonstrated to increase the expression of apoE in HepG2 cells (33).

To evaluate the role of apoE in reverse cholesterol transport in the RPE, we have used primary cultures of human RPE cells to evaluate the secretion and regulation of apoE expression by nuclear hormone receptor ligands. In the current study, we report that primary cultures of human RPE cells secrete apoE from their apical and basal surfaces. We also report that apoE expression is upregulated by T_3 receptor (TR), LXR, and RXR agonists.

MATERIALS AND METHODS

Cell culture

Primary cultures of normal human RPE cells from a 35 year old male donor (apoE phenotype E3/E3) were prepared as described (34, 35). Cells from passages 5–10 were used for the experiments described below. RPE cells were grown to confluence on laminin-coated six-well Costar Transwell® tissue culture plates (Fisher Scientific, Los Angeles, CA) with DMEM H21 containing 5% FBS, 2 mM glutamine, 5 mg/ml gentamicin, 100 IU/ml penicillin, 100 mg/ml streptomycin, 2.5 mg/ml Fungizone, 1 ng/ml basic fibroblast growth factor, and 1 ng/ml epidermal growth factor in the top and bottom chambers. Cells were grown for at least 1 week at confluence before treatment. Cells to be treated with drugs were incubated in DMEM H21 containing serum substitute for 6–8 h before drug addition. Drug treatments were in DMEM H21 containing 6 g/l nonessential amino acids and 0.39 g/l methylcellulose (serum free medium) with or without 10^{-7} M T_3, 10^{-7} M 9-*cis*-retinoic acid (RA), or 2.5×10^{-6} M 22(*R*)-hydroxycholesterol (HC) in both chambers for the times indicated. No differences in cell morphology or apoE expression were observed in cultures from different passages.

RT-PCR

Confluent cell cultures were harvested and total RNA was purified using RNeasy (Qiagen, Valencia, CA) according to the manufacturer's instructions. The RT-PCR primer sequences used are given followed by the predicted RT-PCR product size: apoE forward, 5′-TAA GCT TGG CAC GGC TGT CCA AGG A; apoE reverse, 5′-ACA GAA TTC GCC CCG GCC TGG TAC AC; 241 bp product (detects both apoE3 and apoE4). PCR was conducted for 40 cycles at 55°C in buffer containing 2.0 mM $MgCl_2$. The RT-PCR product of the predicted size for apoE was excised from the gel, and its identity was confirmed by DNA sequencing. RT-PCR of RPE cell mRNA revealed mRNA for only the apoE3 allele.

Western blotting

Media were concentrated 20-fold by centrifugal ultrafiltration using VIVA SPIN 20 columns with a 5,000 kDa molecular weight cutoff (MWCO) (Vivascience, Hannover, Germany) and dialyzed against 0.15 M NaCl, 1 mM sodium EDTA, and 0.025% sodium azide (SalEN). Total protein content was determined by a modified Lowry assay (DC kit; Bio-Rad, Richmond, CA). Concentrated media (50 μg of protein) were made to start buffer (0.025 M NaCl, 0.010 M Tris, pH 8.5, and 5 mM $MnCl_2$) and adsorbed onto a 0.1 ml column containing Heparin-Sepharose CL-4B (Pharmacia, Uppsala, Sweden). After a 2 ml wash in start buffer, the apoE-containing bound fraction was eluted with 0.5 M NaCl in start buffer. The eluate was concentrated to 20 μl and buffer-exchanged to SalEN by centrifugal ultrafiltration (Biomax; 5,000 kDa MWCO, Millipore, Bedford, MA). The apoE was resolved by Tris-Tricine-buffered SDS-PAGE (5–25% linear acrylamide gradient), and proteins were electrophoretically transferred (55 V, 18 h) to nitrocellulose membrane filters (Schleicher and Shuell, Keene, NH). Membranes were blocked with 10% BSA at room temperature and probed with 1% goat anti-human apoE antiserum (18 h, 3°C) prepared in 0.15% NaCl, 1 mM EDTA, pH 7.4, and 0.1% Triton X-100 (SalET). The primary-bound anti-apoE antibodies were detected colorimetrically with horseradish peroxidase-conjugated rabbit anti-goat Ig (H+L) and $NiCl_2$-enhanced diaminobenzine staining. Stained bands were compared densitometrically from the digitized scanned image (NIH Image, version 1.62). Anti-apoE antibodies were obtained by hyperimmunization of goats with purified apoE or obtained from Assay Designs (A299; Ann Arbor, MI).

Lipoprotein fractions were prepared from conditioned medium that was adjusted with solid KBr to a density of 1.21 g/ml. Samples were ultracentrifuged in a Beckman 42.2 Ti rotor at 40,000 rpm for 18 h at 10°C. The lipoprotein and lipoprotein-free fractions, the top and bottom 50 μl, respectively, were dialyzed against SalEN before analysis by Western blotting.

Continuous KBr gradient centrifugation

Purified apoE was also analyzed on continuous KBr gradients. The apoE was isolated from RPE cell culture medium as described above. The purified apoE was concentrated to 0.5 ml, adjusted to a density of 1.35 g/ml with solid KBr, and layered under a preformed KBr gradient (d = 1.18–1.30). The gradients were ultracentrifuged at 45,000 rpm in a Beckman 50.2 Ti rotor (10°C, 48 h). After centrifugation, 0.3 ml fractions were collected from the bottom. Density was determined as a function of conductivity using calibrated KBr solutions. Density gradients measured in mock samples did not differ in range or slope. The apoE was detected by ELISA as described below.

Mass spectrometry

ApoE was purified from RPE cell culture medium for mass spectrometric analysis by anti-apoE immunoaffinity chromatography followed by resolution of the bound material on silver-stained SDS-polyacrylamide gels. The gels were stained as described (36) modified to omit the glutaraldehyde fixative (37). Bands were excised, cysteines were reduced and carbamidomethylated, and then the protein was subjected to in-gel digestion using sequencing-grade trypsin. (Promega, Madison, WI). Tryptic peptides were then analyzed by liquid chromatography-tandem mass spectrometry (LC-MS-MS). Peptides were separated on a 0.075 mm (inner diameter) × 150 mm C18 Pepmap column (Dionex, Sunnyvale, CA) using an Ultimate liquid chromatography pump (Dionex). As peptides eluted off the column, they were introduced directly into a QSTAR Pulsar Mass Spectrometer (MDS Sciex; Applied Biosystems, Foster City, CA) in which peaks were automatically selected for fragmentation analysis in a data-dependent manner. Control samples included human apoE that had been biochemically purified from VLDL delipidated with ethanol-diethyl ether (3:1) essentially as described (38).

HDL binding

RPE cells were grown on Transwell® plates with DMEM H21 containing 5% FBS in the apical chamber, as described above. The basal chambers contained serum-free medium with 0, 50, or 200 μg/ml mouse HDL (d = 1.063–1.210 g/ml) prepared by ultracentrifugation of mouse plasma (PanBio, Columbia, MD). After 36 h, HDL was purified from the basal medium by ultracentrifugation, proteins were resolved by SDS-PAGE, and human apoE was detected by immunoblotting as described above.

ELISA assays

Samples treated with 0.1% Tween 20 containing 1% BSA were incubated (37°C, 4 h) on 96-well plates previously coated with apoE affinity-purified goat anti-apoE antibody. The apoE was detected using a secondary antibody-peroxidase conjugate and 3,3′,5,5′,-tetramethylbenzidine substrate (Kirkegaard and Perry Labs, Gaithersburg, MD). Optical density was measured at 450 nm. The assay was calibrated with purified apoE. The dynamic range was 1–40 ng/ml, with a coefficient of variation <5%.

RESULTS

To distinguish apical from basal apoE secretion, cells were cultured on laminin-coated Transwell® plates. In an initial set of experiments, cells were cultured for 36 h with defined serum-free medium in both apical and basal chambers. Total protein and apoE-specific protein concentrations were measured from media pooled and concentrated from three replicate wells. To assess apoE-specific secretion, apoE was purified from apical and basal media by Heparin-Sepharose affinity chromatography. The apoE was detected by immunoblotting. The results of two experiments are shown in **Fig. 2**. As shown, immunoblotting revealed a 35 kDa protein in the apical (Fig. 2, lanes 1 and 3) and basal (Fig. 2, lanes 2 and 4) medium corresponding to the molecular weight of apoE. RPE cell-secreted apoE comigrated with human serum apoE purified from VLDL (Fig. 2, lane 5).

Because we observed other bands by anti-apoE immunoblotting that could be apoE aggregates, apoE proteolytic fragments, or nonspecific binding, we sought additional proof that our immunoblotting was specific for

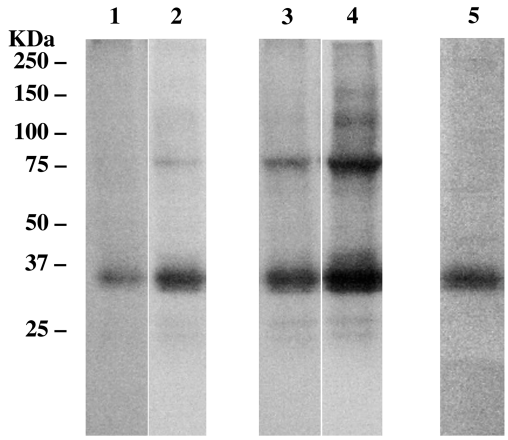

Fig. 2. Apical and basal secretion of apoE. RPE cell culture media from apical (lanes 1 and 3) and basal (lanes 2 and 4) chambers were harvested and chromatographed on Heparin-Sepharose. The apoE-containing fraction (bound) was analyzed by SDS-PAGE and Western blotted with antibodies to apoE. Lane 5, apoE purified from human VLDL.

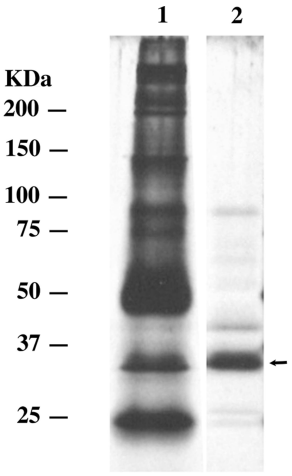

Fig. 3. Immunoaffinity-purified apoE. ApoE was purified from RPE cell culture media by immunoaffinity chromatography for identification by mass spectrometry. Shown is a silver-stained SDS-polyacrylamide gel with molecular mass calibration to the left. Lane 1, immunoaffinity-purified apoE fraction from conditioned media; lane 2, apoE (arrow) purified from human VLDL.

apoE. ApoE was purified for identification by mass spectrometry by passing RPE cell culture media (serum free) over a column of affinity-purified anti-apoE antibody-coupled Sepharose prepared from the same anti-apoE antibodies used for immunoblotting. After extensive washing, the bound material was eluted and resolved by SDS-PAGE and visualized by silver staining. Anti-apoE immunoaffinity chromatography yielded a 35 kDa band (**Fig. 3**, lane 1) that electrophoresed identically with biochemically pure apoE purified from human plasma VLDL (Fig. 3, lane 2). Although other bands were also observed, mock purification column runs did not yield the 35 kDa protein (data not shown). Two of the proteins that eluted with apoE, of 50 and 25 kDa, were later identified by mass spectroscopy as immunoglobulin heavy and light chains, respectively, that dissociated from the column support matrix. The 35 kDa protein was excised from the gel and digested with trypsin. The resulting tryptic peptides were separated and analyzed by LC-MS-MS. Peptide fragmentation spectra were queried against a National Center for Biotechnology Information nonredundant database using the search engine MASCOT (http://www.matrixscience.com). Twenty-one spectra matched to sequences present in human apoE corresponding to 72% of the full-length protein (**Fig. 4A**). Identification of peptides containing residues Cys_{112} (Fig. 4B) and Gly_{127} (data not shown) is consistent with the RT-PCR assignment of the E3/E3 phenotype for the human cells used (data not shown). Although we were unable to assign sequence data at amino acid position 158, we did identify a tryptic peptide corresponding to $apoE_{159–167}$ (Fig. 4A). The presence of Cys_{158} in apoE2 would preclude cleavage by trypsin. Barring nonspecific proteolysis at this position, generation of this peptide is consistent with the presence of Arg_{158} of apoE3.

Because apoE and HDL are known to be involved in reverse cholesterol transport, we determined if secreted apoE was associated with lipid and if it could interact with an HDL acceptor. Concentrated RPE cell culture media (serum free) were ultracentrifuged at a density of 1.21 g/ml. The density fractions were collected and analyzed for apoE content by immunoblotting. As shown in **Fig. 5**, apoE was localized entirely in the lipoprotein-poor fraction ($d > 1.21$ g/ml) (Fig. 5, lane 2), suggesting that apoE is initially secreted in a lipid-free or a lipid-poor state. In contrast, human plasma apoE was localized in the lipid-rich fraction ($d < 1.21$ g/ml) (Fig. 5, lane 3). Minimally lipidated apoE has been demonstrated to form small phospholipid discs with densities of ~1.20–1.26 g/ml (39, 40). To better characterize the lipidation state of newly secreted apoE, apoE was analyzed on continuous density gradients. As shown in **Fig. 6**, 70% of the apoE secreted by RPE cells under the conditions of our cell culture had a density of 1.263–1.282 g/ml. However, 30% of the apoE had a density of 1.193–1.256 g/ml, indicating that a significant proportion of the apoE may have been complexed with phospholipids as phospholipid discs.

To assess whether apoE secreted into the medium would interact with an HDL acceptor, we included mouse plasma HDL in the basal medium. As shown in **Fig. 7**, antibodies that detect human apoE, but not mouse apoE, detected human apoE in the lipoprotein fraction when mouse plasma HDL was included in the basal medium (Fig. 7, lanes 1 and 2). When mouse HDL was stained with anti-human apoE antibodies, no human apoE was detected in the lipoprotein fraction (Fig. 7, lane 3).

We have previously demonstrated the presence of functional TRs in cultured human RPE cells (35). We have also detected mRNA for RXRα and LXRα in these cells by RT-PCR (data not shown). In an initial experiment, we tested the effect of TR, RXR and LXR agonists on basal apoE secretion from RPE cells by immunoblotting. Triplicate wells were treated for 36 h with the drugs indicated, and the basal chamber media were pooled for immunoblot-

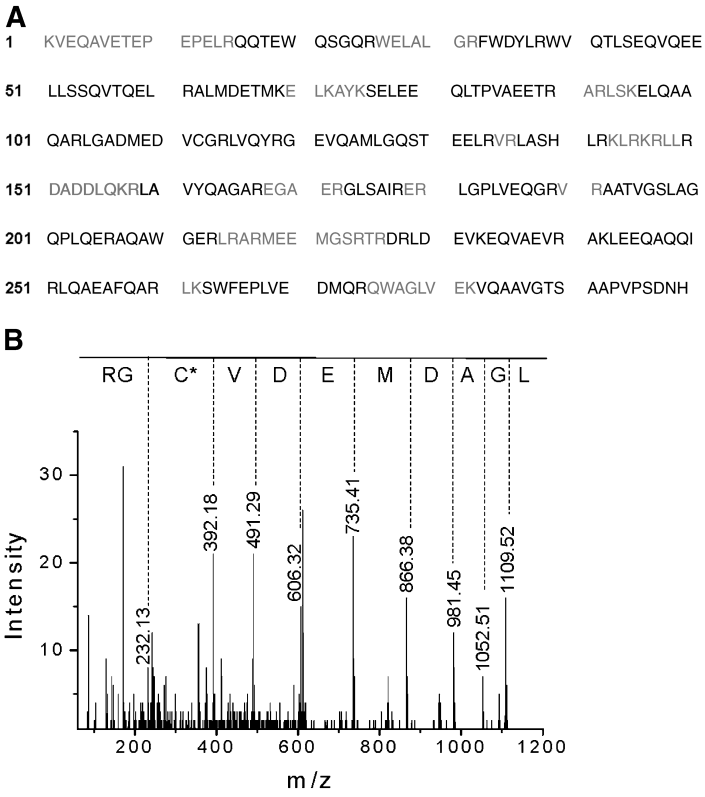

Fig. 4. Identification of apoE by mass spectrometry. Identification of the 35 kDa protein as the apoE3 allele. A: Liquid chromatography-tandem mass spectrometry analysis of tryptic peptides from the 35 kDa band identified it as apoE, with 72% sequence coverage of the protein (the observed sequence is shown in boldface). B: Tandem mass spectrum of peptide Leu_{104}–Arg_{114} (LGADMEDVC*GR) containing Cys_{112} is consistent with that for the E3 protein isoform. C*, carbamidomethylated cysteine.

ting. The results are shown in **Fig. 8**. Treatment with T_3 (Fig. 8, lane 2), HC (Fig. 8, lane 3), or RA (Fig. 8, lane 4) increased apoE protein in the basal medium compared with untreated cells (Fig. 8, lane 1). In a second set of experiments, apoE secretion was measured quantitatively by ELISA assays of the basal media after treatment for 36 h with the T_3, HC, RA, and combinations of the three, as indicated in **Fig. 9**. Treatment with the combination of T_3 and HC had the greatest effect on levels of basal apoE secretion, increasing them by 54% (Fig. 9). Treatment with all of the other drugs and drug combinations, except for the combination of T_3 and RA, significantly ($P < 0.05$, two tailed *t*-test) increased basal apoE secretion, but to a lesser degree. The experiments were performed once on tripli-

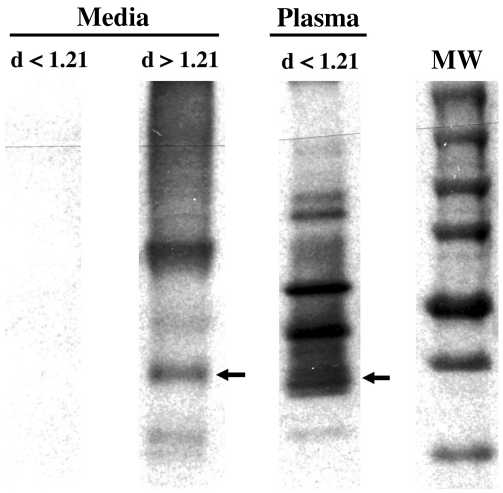

Fig. 5. Characterization of secreted apoE by density gradient ultracentrifugation. Conditioned basal medium was subjected to ultracentrifugation at a density of 1.21 g/ml. Lanes 1 and 2, the top (d < 1.21 g/ml) and bottom (d > 1.21 g/ml) fractions were analyzed for apoE content by Western blotting. Lane 3, a plasma lipoprotein fraction (d < 1.21 g/ml) stained for apoE is shown for comparison. Molecular mass (MW) protein gel calibrators (250, 150, 100, 75, 50, 37, and 25 kDa) are shown. Arrows indicate apoE band.

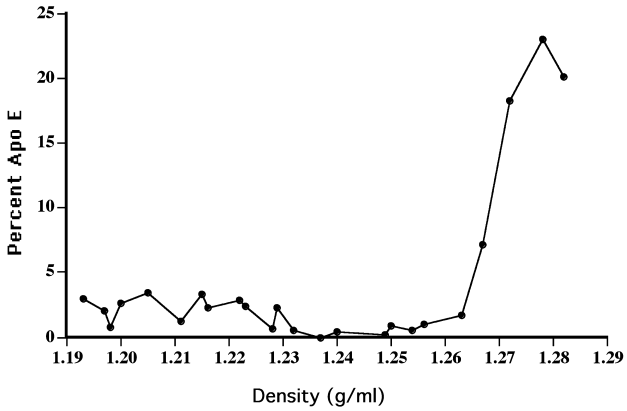

Fig. 6. Characterization of secreted apoE by continuous gradient ultracentrifugation. Conditioned medium was subjected to ultracentrifugation on a continuous KBr gradient (d = 1.193–1.282). The apoE was detected by ELISA. Results are shown as the percentage of total apoE in each density fraction.

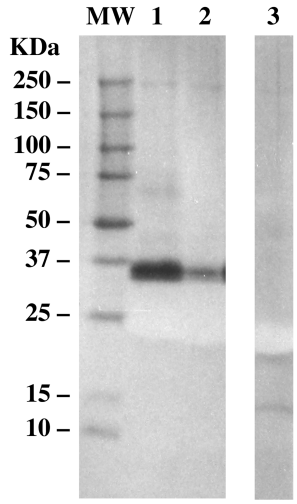

Fig. 7. Association of RPE-secreted apoE with HDL. HDL purified from mouse plasma was cultured with RPE for 36 h. HDL was repurified by density gradient ultracentrifugation and analyzed by SDS-PAGE. Depicted are apoE Western blots of media samples incubated in the presence of 50 and 200 μg/ml HDL (lanes 1 and 2, respectively). ApoE staining of mouse HDL is shown in lane 3.

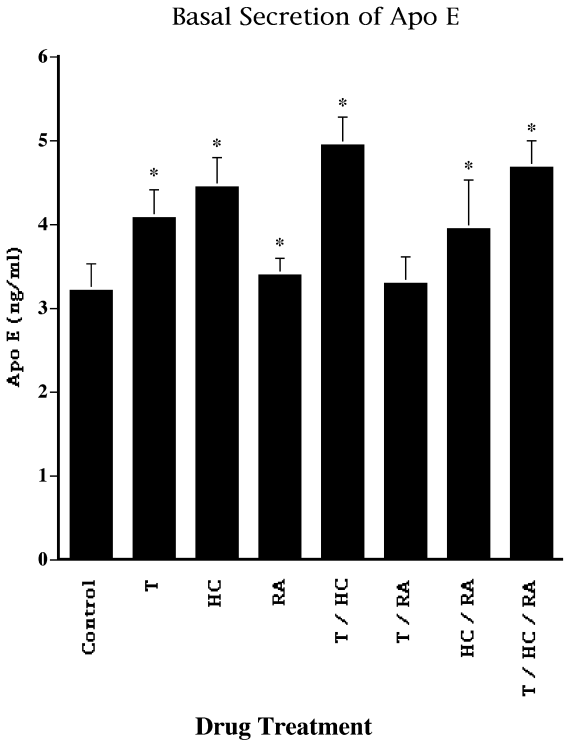

Fig. 9. Regulation of apoE secretion as assessed by ELISA. RPE cells were cultured in the presence of thyroid hormone (T), 22(*R*)-hydroxycholesterol (HC), and 9-*cis*-retinoic acid (RA), singly and in combinations, for 36 h. ApoE in basal media was measured by ELISA. The data shown (means ± SD) are from media samples (n = 6) each measured by ELISA in triplicate. Statistical significance (two-tailed *t*-test, $P < 0.05$) versus control media is indicated (*).

cate wells of cell culture media (data not shown) and once on sextuplicate wells of cell culture media (Fig. 9) with similar results. ELISA assays were performed in triplicate. Apical secretion of apoE was similarly increased in both experiments (data not shown).

DISCUSSION

In addition to its well-characterized expression by the liver, apoE has been shown to be expressed by several peripheral tissues, in which it has been postulated to function in local, cell-to-cell transport of cholesterol (41, 42). These include kidney, adrenal glands, spleen, brain, lymph node, lung, thymus, and retina (15, 41–43). Because apoE polymorphisms have been associated with both increased and decreased propensity for AMD, there has been recent interest in this apolipoprotein by retinal biologists. RT-PCR and immunohistochemical analyses have localized apolipoproteins, including apoE, to the retina, RPE, and Bruch's membrane deposits, including drusen (15, 44).

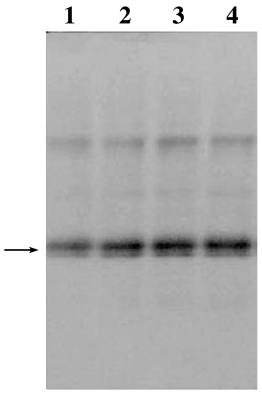

Fig. 8. Regulation of basal apoE secretion assessed by Western blotting. RPE cells were cultured in the presence or absence of thyroid hormone (lane 2), 9-*cis*-retinoic acid (lane 3), or 22(*R*)-hydroxycholesterol (lane 4) for 36 h. Lane 1, untreated control. ApoE in basal media was assessed qualitatively by Western blotting of basal cell culture media. The apoE band is indicated by the arrow.

Because of its important role in reverse cholesterol transport, and because of the importance of lipid and cholesterol transport to proper functioning of the retina and RPE, we have investigated the regulation of apoE secretion in cultured human RPE cells. Western blotting and ELISA assays demonstrated that RPE cells secreted apoE from both apical and basal surfaces. The identity of the secreted protein was confirmed by mass spectrometry. RPE cell-secreted apoE associated with the lipoprotein fraction when HDL was added to the cell culture medium, presumably by interacting with the added HDL. The observation that RPE-secreted apoE can interact with HDL suggests a possible role for apoE in trafficking basally secreted lipids from the RPE to an extracellular HDL acceptor (shown schematically in Fig. 1).

ApoE secretion by cultured human RPE cells was regulated by ligands for the nuclear hormone receptors TR, RXR, and LXR. T_3, RA, and HC all increased the basal se-

cretion of apoE. HC had the greatest effect on increasing basal secretion; the combination of HC with either T_3 or RA further increased the levels of apoE secretion. The regulation of apoE secretion we observed in RPE cells is analogous to its regulation in other cell types.

Basal secretion of apoE by RPE cells is consistent with previous findings that apoE is observed in Bruch's membrane deposits (15, 44). Although lipid deposition occurs throughout Bruch's membrane, a "wall" of lipid particles accumulates with age just beneath the basal lamina of the RPE (45). The presence of lipid and apoE at the inner aspect of Bruch's membrane suggests that both may be secreted by the RPE. Because apoE's role in reverse cholesterol transport is well established (20–25), it is possible that apoE may facilitate the efflux of lipids from the RPE into subjacent Bruch's membrane. Lin, Duan, and Mazzone (24) have shown that endogenously synthesized apoE facilitates cholesterol and phospholipid efflux from macrophages, perhaps by increasing lipid desorption from the plasma membrane. In macrophages, apoE-mediated lipid efflux is increased by cell surface proteoglycans (46), which likewise have been localized to the cell surface of RPE (47, 48).

Approximately 30% of the apoE secreted from RPE cells in the absence of exogenous HDL purified to a density of 1.193–1.256 g/ml, indicating that a significant amount of newly secreted apoE may be in the form of small phospholipid discs (39, 40). More detailed analysis will be necessary to fully characterize this apoE fraction. In the presence of exogenous HDL, all of the apoE purified to the $d < 1.21$ lipoprotein fraction. In macrophages, apoE promotes the binding of HDL_3 to the cell surface; furthermore, it has been speculated that secreted apoE and the associated lipid may bind to HDL containing apoA-I particles (49). If apoE serves to chaperone associated lipid residues to HDL, regulation of apoE secretion may be critical in determining the levels of accumulated lipid in Bruch's membrane. Future studies will address whether RPE cell-secreted apoE interacts with secreted POS lipid digestion products.

RPE cells also secreted apoE into the apical compartment, where it would be available to interact with photoreceptors. Interestingly, apoE has been shown to be expressed during optic nerve degeneration (42) and regeneration (50). In addition, lipid-free apoE has also been demonstrated to be capable of stimulating neurite outgrowth in Neuro-2a cells in culture (40). In light of these findings, one might speculate that apically secreted apoE may play a role not only in recycling POS lipids to the photoreceptors for use in synthesizing new POS but that apically secreted lipid-free apoE may play a role in maintaining the health and function of the neural retina. Future studies are needed to address these possibilities.

The regulation of apoE secretion in macrophages, myocytes, and adipocytes is controlled by nuclear hormone receptors. LXR-RXR heterodimers, stimulated by their appropriate ligands, upregulate apoE expression (32, 51, 52). T_3 has also been demonstrated to increase the expression of apoE in HepG2 cells (33). Cultured human RPE cells are regulated in a similar manner. Both basal (Fig. 9) and apical (data not shown) secretion of apoE were increased after the addition of LXR, RXR, and T_3 ligands. Maximally stimulated secretion was achieved with the combination of LXR/RXR and T_3/LXR ligands. In macrophages, secreted apoE is increased by intracellular cholesterol, probably secondary to the generation of oxysterols that bind to LXRs. LXR and RXR also are known to upregulate ABCA1 expression and increase reverse cholesterol transport in macrophages. Interestingly, we have shown that cultured RPE cells express ABCA1 (53), suggesting that, as in macrophages, this ATP binding cassette transporter also may be under the regulatory control of LXR and RXR ligands.

Although apoE-facilitated reverse cholesterol transport is thought to be atheroprotective (20–25), its role in AMD is not yet understood. The accumulation of lipid deposits in RPE and Bruch's membrane is implicated in the progression of AMD. The diffusion barrier created by lipid deposition at the junction of Bruch's membrane and the RPE has been well characterized morphologically and physiologically (3–9). It is tempting to speculate that increasing levels of apoE secretion and binding to HDL might facilitate the efflux of lipids from the RPE and the transit of lipids across Bruch's membrane, thus reducing RPE and Bruch's membrane lipid levels. The present demonstration that the secretion of apoE is regulated via nuclear hormone receptor ligands is intriguing, and we are currently pursuing the therapeutic implications of these findings.

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