A2E, a Pigment of the Lipofuscin of Retinal Pigment Epithelial Cells, Is an Endogenous Ligand for Retinoic Acid Receptor*

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Lipofuscin contains fluorophores, which represent a biomarker for cellular aging. Although it remains unsubstantiated clinically, experimental results support that the accumulation of lipofuscin is related to an increased risk of choroidal neovascularization due to age-related macular degeneration, a leading cause of legal blindness. Here, we report that a major lipofuscin component, A2E, activates the retinoic acid receptor (RAR). In vitro experiments using luciferase reporter assay, competitive binding assay, analysis of target genes, and chromatin immunoprecipitation (ChIP) assay strongly suggest that A2E is a bona fide ligand for RAR and induces sustained activation of RAR target genes. A2E-induced vascular endothelial growth factor (VEGF) expression in a human retinal pigment epithelial cell line (ARPE-19) and RAR antagonist blocked the up-regulation of VEGF. The conditioned medium of A2E-treated ARPE-19 cells induced tube formation in human umbilical vascular endothelial cells, which was blocked by the RAR antagonist and anti-VEGF antibody. These results suggest that A2E accumulation results in the phenotypic alteration of retinal pigment epithelial cells, predisposing the environment to choroidal neovascularization development. This is mediated through the agonistic function of A2E, at least in part. The results of this study provide a novel potential therapeutic target for this incurable condition.

Retinal age pigments, or lipofuscin granula, contain the fluorophores that accumulate with age and that are thought to represent a biomarker for cellular aging (1). Lipofuscin results from an incomplete degradation of altered material trapped in lysosomes (2) and the accumulation of lipofuscin is related to an increased risk of choroidal neovascularization (CNV)2 due to age-related macular degeneration (AMD) (1, 2). AMD is a leading cause of legal blindness in developed countries (3), and even with the recent advent of several treatment options (4), treatment of AMD remains difficult (5). Thus, a better understanding of the pathogenesis is needed to pursue a novel potential pharmaceutical target.

Visual loss in AMD is caused by CNV, i.e. the neovascular vessels extending from the choroid below the sensory retina, and the subsequent atrophy of the RPE. The process preceding AMD, early age-related maculopathy (4), is pathologically characterized by age-related changes in the RPE, such as the accumulation of a deposit called drusen in the basement membrane of the RPE, i.e. Bruch’s membrane, and fluorescent lipofuscin granules in the RPE cells (6). It is generally considered that the age-related accumulation of these potentially toxic deposits affects normal RPE functions (4). Although it remains unsubstantiated whether the accumulation of lipofuscin is related to the development of exudative AMD, the fact that most abundant accretion is in the RPE cells under the central retina suggests that there may be a causal relationship between lipofuscin accumulation and exudative AMD (2). Laboratory studies also support that there may be a causal relationship between lipofuscin accumulation and the progression of exudative AMD (7, 8). There is a strong impetus to understand how the accumulation of these potentially toxic lipofuscin fluorophores contribute to AMD; however, little is known about the underlying molecular mechanism of CNV development.

RPE lipofuscin is a byproduct of the phagocytosis of lipid-rich photoreceptor outer segments and consists of a complex mixture of pigments. A major fluorophore is A2E (N-retinyle-din-N-retinylethanolamin) (9), which arises from a Schiff base reaction from ethanolamine and vitamin A-aldehyde (10). In vitro, A2E affects normal RPE functions by causing membrane permeabilization inhibiting lysosomal function (11), inhibiting cytochrome c oxygenase (12), acting as a detergent inhibiting the ATP-driven proton pump (13), and partly mediating light damage by acting as a photosensitizer (14), targeting DNA (15).

This study demonstrates that A2E is an endogenous ligand for retinoic acid receptor (RAR). The data suggest that A2E...
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accumulation results in the pro-angiogenic conversion of retinal pigment epithelial cell phenotype predisposing the environment to CNV development via RAR activation, at least in part.

EXPERIMENTAL PROCEDURES

A2E Synthesis—A2E was prepared from all-trans-retinal and ethanolamine according to the method in literature (10). Typically, a mixture of all-trans-retinal (50 mg, 176 µmol) and ethanolamine (4.6 mg, 78 µmol) in ethanol (1.5 ml) was stirred in the presence of acetic acid (4.7 µl, 78 µmol) at room temperature under dark conditions for 2 days. The reaction mixture was evaporated and then purified by silica gel column chromatography. After elution with MeOH:CH₂Cl₂ (5:95) to remove less polar byproducts, further elution with MeOH:CH₂Cl₂ (5:95) including 0.1% trifluoroacetic acid gave A2E (21 mg, 38%).

The product was confirmed by NMR (JEOL GSX 270), UV-visible (JASCO-V550), matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Bruker model Reflex III), and high performance liquid chromatography (HPLC) (TSK Silica Gel 60) analysis. All spectral data were consistent with previous reports (9, 10). Also, the chromatogram of A2E shows a single sharp peak without any other retinoid contamination as shown in Fig. 2C.

Cell Culture—Human retinal pigment epithelial (ARPE19) cells and human embryonic kidney 293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM). Cells were cultured at 37 °C in a humidified atmosphere with 10% CO₂.

For uptake into cultured cells, A2E was delivered at 1, 10, and 100 nm concentrations into the culture media, and confluent cultures of the cells were incubated with A2E, all-trans-retinoic acid (atRA), or ethanol (control) for the indicated period. Preliminary experiments have demonstrated that after confluence for 6 h and for 24 h the cells showed similar responses to A2E exposure, the cells at 24 h after confluence were used for analysis. ELISA was used for determining VEGF in ARPE-19 cells.

For the ELISA experiment, ARPE-19 cells were plated in a 24-well plate in phenol red-free DMEM containing 10% fetal bovine serum, pretreated with dextran-coated charcoal, and incubated overnight. The spent medium was collected for the measurement of VEGF. The ELISA for VEGF was performed using the human VEGF immunoassay system (R&D Systems, Minneapolis, MN).

Luciferase Assay—For the transfection experiment, 2 × 10⁴ HEC-293T cells were plated in a 24-well plate in phenol red-free DMEM containing 10% fetal bovine serum, pretreated with dextran-coated charcoal, and incubated overnight. The MH100-tkLuc reporter plasmid (100 ng) was cotransfected with the internal control plasmid pRL-CMV (Promega, Madison, WI) and the expression plasmids, pCMX-Gal, p-CMX-Gal-RARα, p-CMX-Gal-RXRα, p-CMX-Gal-PNR, p-CMX-Gal-PPARγ, and p-CMX-Gal-FXR using the SuperFect® transfection reagent (Qiagen, Valencia, CA) following the manufacturer’s protocol. Luciferase reporter vector for the VEGF promoter was constructed as previously described (16). The amount of each transfected DNA sample was adjusted with carrier DNA to make a total of 200 ng. After transfection, ligands were added to the medium and cultured for 24 h. The cells were lysed in 100 µl/well of passive lysis buffer, and the luciferase assay was performed in accordance with the protocols of the Dual Luciferase Reporter Assay System, using a Lumat LB 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany).

Binding Assay—For expression of the ligand-binding domain (DEF) of RARα in Escherichia coli, the expression vector pGEX was used. GST fusion protein (GST–RARα–DEF) was expressed in E. coli M-15 after isopropyl β-D-thiogalactopyranoside induction and purified on gluthathione-Sepharose beads as described previously (17). The expression of the protein of the predicted size was monitored by SDS-PAGE. The GST–RARα protein (2–3 pmol) was incubated in 0.2 ml of binding buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 0.2% (v/v) Nonidet P-40, 0.1% (v/v) Triton X-100) containing 270 pm [3H]atRA, and various concentrations of atRA or A2E. After 3 h of incubation at room temperature, the tubes were centrifuged at 12,000 × g for 10 min and the supernatants were subjected to liquid scintillation counting. To determine the Kₐ, nonlinear regression analysis of the competition curves was carried out using Prism 4 (GraphPad).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)—RNA for RT-PCR was isolated using an SV Total RNA Isolation Kit (Promega, Madison, WI) in accordance with the manufacturer’s instructions.

cDNA was prepared using Superscript III for RT-PCR (Invitrogen). Each PCR was carried out in a 20-µl volume using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) to denature for 15 min at 95 °C, followed by 55 cycles at 95 °C for 30 s, and 60 °C for 1 min in Roche LightCycler. Values for each gene were normalized to expression levels of GAPDH. The sequences of the primers used for RT-PCR were as follows: human GAPDH, left, 5’-gagtcagcaggttggctgt-3’, right, 5’-ttgatttgggaggtctgc-3’; human VEGF, left 5’-atgattgcgtgtggtagtggg-3’, right, 5’-gtgtcgctgtcaccctc-3’; human RARβ2, left 5’-gtgtcctgctgctcacc-3’, right, 5’-ggcaagttgaccaacg-3’; human NORP5, left, 5’-caagacctccaaccctc-3’, right, 5’-aagcttcacccacac-3’; and human SCD, left, 5’-ctcttctgggaagggcctgc-3’, right, 5’-gatagacaggggacagcag-3’.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP analysis was performed using the ChIP assay kit (Upstate), according to the manufacturer’s instructions. ARPE-19 cells were cultured in the presence of A2E or atRA. Soluble chromatin prepared from 1×10⁶ cells was immunoprecipitated with an antibody against αACh4 (Upstate Biotechnology). A negative control experiment was performed using mouse IgG, which gave no positive signals.

HPLC—Samples of A2E were analyzed using normal phase HPLC on a silica column (TSK Silica Gel 60 Toso) using the mobile phase, dichloromethane:methanol:triethylamine (200:10:25, v/v) at 1.0 ml/min. The eluted peaks were analyzed with a UV detector (430 nm). Samples of atRA were analyzed using normal phase HPLC on a silica column (TSK Silica Gel 60 Toso) using the mobile phase, dichloromethane/trifluoroacetic acid (2000:1, v/v), at 1.0 ml/min. The eluted peaks were analyzed with a UV detector (340 nm).

In Vitro Tube Formation Assay—In vitro anti-angiogenesis activity was evaluated with the in vitro tube formation assay. Human umbilical vein endothelial cells (HUVEC) starved of

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GAL-4 DBD fused to the RARα ligand-binding domain with a luciferase reporter gene plasmid containing the GAL-4 DBD-binding site in a dose-dependent manner (Fig. 1, a and b). Similar results were obtained using full-length RARα with a reporter plasmid containing the consensus RARE sequence (DR1) (data not shown). A2E only transactivated RAR, and not other nuclear receptors including the retinoid X receptor (RXR) α, peroxisome proliferator activator receptor (PPAR) γ, or orphan nuclear receptors including the farnesoid X receptor (FXR) or the photoreceptor cell-specific receptor (PNR) (Fig. 1a). Based on the fact that the nuclear receptor recruits coactivators such SRC-1 (steroid receptor coactivator-1), TIF2 (transcriptional intermediary factor 2), AIB-1 (amplified in breast cancer-1) upon ligand binding, the present study examined A2E-induced interactions of RARα with coactivators in the mammalian two-hybrid system, which showed A2E-induced bindings of p160 coactivators (SRC-1, TIF2, and AIB-1) to RARα (data not shown). Next, to verify whether A2E acts as a cognate ligand for RARα, it was investigated as to whether A2E could compete for specific binding of [3H]all-trans-retinoic acid (atRA) to the RARα ligand binding domain-GST fusion protein (GST-RARα) in vitro. It was demonstrated that A2E bound to GST-RARα with a half-maximal inhibitory concentration (IC_{50}) of 8.74 nM, whereas that of atRA was 0.49 nM (Fig. 1c). The control vehicle alone did not interfere with the binding of [3H]atRA to the GST-RARα, confirming the specific binding of A2E.

Furthermore, RT-PCR analysis demonstrated that A2E treatment transactivated the established RAR target genes, such as NORPEG (novel retinal pigment epithelial cell gene) (18), SCD (stearoyl-CoA desaturase) (19), and RARβ2 (20) in a human retinal pigment epithelial cell line, ARPE-19 cells (Fig. 2a). Specifically, the real time RT-PCR measurements indicated that whereas vehicle-treated cells showed no change in the expression of these genes, greater than 3-fold increases were observed in levels of NORPEG, SCD, and RARβ2 mRNA following atRA treatment for 24 and 48 h, as expected. Likewise, 2.5–3-fold increases in levels of NORPEG, SCD, and RARβ2 mRNA were observed at 24 and 48 h after A2E treatment. Together, these experiments demonstrate that A2E is a bona fide RAR ligand.

FIGURE 1. A2E acts as a ligand for RAR. a, A2E only transactivates RARα, not other nuclear receptors such as RXRα, PPARγ, PNR, and FXR. Luciferase assays were performed in HEC-293T cells transfected with GAL-4 DBD containing the MH100-tk-Luc reporter plasmid (400 ng), GAL-fused expression vectors (200 ng), and ligands (atRA, 10^{-8} M; troglitazone, 10^{-8} M; GW4046, 10^{-4} M; A2E, 10^{-8} M), b, A2E transactivates RARα in a dose-dependent manner, similar to atRA. The concentrations of atRA and A2E were 10^{-10}, 10^{-9}, and 10^{-8} M. Luciferase assays were performed in HEC-293T cells with the MH100-tk-Luc reporter plasmid and a GAL-fused RARα vector. Columns 2, 3, and 4 correspond to 10^{-10}, 10^{-9}, and 10^{-8} M atRA, respectively, and columns 5, 6, and 7 correspond to 10^{-10}, 10^{-9}, and 10^{-8} M A2E, respectively. a and b, data are plotted as means; error bars represent the mean ± S.E (n = 3). c, GST-RARα protein (2–3 p mol) was incubated with 270 pm [3H]atRA in the presence of various concentrations of A2E and atRA. All experiments were performed in triplicate, and each gave similar results. Representative data are shown in a–c.

RESULTS

A2E Is a Ligand for RAR—Given the lipid-soluble nature of A2E, the effects of A2E on the nuclear receptor function were investigated by means of a luciferase assay. Subsequently, it was found that A2E transactivates RAR in HEC-293T cells using serum for 4 h were seeded at a cell density of 40,000 cells per well in 24-well culture plates (Nalge Nunc International), pre-coated with 0.4 ml of low-growth factor synthetic matrix (Matrigel; BD Bioscience, San Jose, CA) and cultured with DMEM or the conditioned medium (DMEM) collected from ARPE19 cells exposed to A2E for 24 h, either in the presence or absence of Ro415253 or neutralizing antibody against VEGF. Tube formation was determined 16 h after cells were plated on Matrigel, by counting the number of connected cells in five randomly selected fields at ×200 magnification, and dividing that number by the total number of cells in the same field. Micrographs were taken under a phase-contrast light microscope (Olympus, Tokyo, Japan).
A2E Induces Sustained Activation of RAR Target Genes—Noteworthy was that in the ARPE-19 cells, at 72 and 96 h after atRA treatment, the expression levels of RAR target genes returned to normal levels, whereas they remained up-regulated after A2E treatment. Data are plotted as means; error bars represent the mean ± S.E. (n = 3). b, ChIP assay demonstrated increased acetylation of histone H4 (AcH4) at promoters of SCD and RARβ2 genes at 24 h after A2E (10^{-8} M) treatment, as well as atRA (10^{-8} M) treatment. When chromatin prepared from ARPE-19 cells at 96 h after A2E or atRA treatment was subjected to analysis, AcH4 on the promoter region was diminished in ARPE-19 cells treated with atRA, whereas the acetylation was sustained in cells treated with A2E. c and d, HPLC analysis showing atRA (c) and A2E (d) in the extracts of ARPE-19 cells at 24, 48, and 72 h after A2E and atRA treatments (10^{-8} M).

A2E Induces VEGF through the Activation of RAR—VEGF production is also up-regulated by atRA in some cell lines (16), raising the possibility that VEGF is up-regulated by A2E in RPE cells. Thus, following treatment with A2E, the changes in expression of the VEGF protein were investigated. The cells expressed higher levels of VEGF protein after treatment with A2E as well as atRA (Fig. 3a). The real time PCR measurements indicated a 3-fold increase in VEGF mRNA as a result of treatment by A2E (Fig. 3a). Luciferase assays demonstrated that as well as atRA, A2E increased the luciferase levels ~3–5-fold in HEC-293 cells transfected with the luciferase plasmid containing the VEGF promoter, suggesting that A2E regulated these expressions at the transcriptional level (Fig. 3c). Up-regulated expression of VEGF mRNA by A2E treatment was diminished by siRNA against RARα, but not by scramble RNA and the addition of Ro415253, a potent RARα antagonist. But it was not diminished by treatment of specific inhibitors for mitogen-activated protein kinase (MEK), U0126, or phosphoinositide 3-kinase inhibitor, wortmannin, or in the presence of the antioxidants, N-acetyl-L-cysteine, or pyrrolidinedithiocarbamate (Fig. 3b). Moreover, VEGF luciferase reporter activation induced by A2E was diminished by siRNA against RARα, but not by scramble siRNA (Fig. 3c), suggesting that A2E stimulates VEGF expression by RARα-mediated transactivation. Furthermore, the luciferase assay demonstrated that the effects of both atRA and A2E on the VEGF promoter were abrogated using plasmids containing mutations of four Sp-1 binding sites, previously shown to be indispensable for RAR-mediated VEGF transactivation (16), suggesting that the binding sites were necessary for VEGF induction by A2E, as well as atRA (Fig. 3c).

To examine whether A2E stimulates VEGF expression and promotes angiogenesis, it was examined whether HUVECs...
form capillary-like structures in response to secreted factors from the ARPE19 cells exposed to A2E. The results demonstrated that HUVECs cultured with the conditioned medium of ARPE-19 after A2E treatment induced capillary-like tube formation, suggesting that ARPE19 cells secrete factor(s) to promote angiogenesis after exposure to A2E. To examine whether activation of RARs and VEGF expression are involved, the in vitro tube formation assay was performed by coinoculation with either Ro415253 or human VEGF antibody, which demonstrated that tube formation was inhibited when Ro415253 or human VEGF antibody was present (Fig. 4), suggesting that inactivation of either RARα or VEGF leads to decreased angiogenesis activity. In conclusion, these data support that A2E stimulates VEGF expression and promotes angiogenesis presumably by activating RARα in cells.

**DISCUSSION**

It was demonstrated that A2E is an endogenous ligand for RARα. A2E binds RARα in vitro, and transactivates RARα. Furthermore, A2E treatment transactivated established RAR target genes, such as NORPEG, SCD, and RARβ2 in ARPE-19 cells, and the ChIP assay revealed that A2E treatment induces acetylation of histone H4 associated with SCD and RARβ2 promoters. These results support that A2E acts as a bona fide ligand for RAR. Previous investigations have demonstrated several examples where biological metabolites act as ligands for nuclear receptors (22). For example, bile acid, which is the biological metabolite of cholesterol is well known to act as the ligand for FXR and negatively regulate its own biosynthesis through FXR (22). Similarly, cholesterol metabolites produced from squalene by a shunt in the classical cholesterol biosynthesis pathway acts as natural LXR ligand. Pregnane X receptor (PXR) is activated by a wide variety of compounds including natural and synthetic steroids (22). The results of this study, in conjunction with those of these previous investigations, support the importance of biological metabolites on the nuclear receptor functions.

Accumulation of A2E in RPE cells induces the sustained activation of RAR and stimulates the expression of a potent pro-angiogenic factor, VEGF. Several groups have previously reported the effects of atRA on angiogenesis, although the results obtained are controversial (23–25). Some groups have reported an anti-angiogenic effect (23, 24). For example, Majewski et al. (23) reported that atRA functions to inhibit angiogenesis using in vivo tumor cell-induced angiogenesis assay. Similarly, Pepper et al. (24) reported the anti-angiogenic effects of atRA using an in vitro three-dimensional collagen gel assay. On the other hand, pro-angiogenic effects have been reported by a in vitro tube formation assay using HUVECs and normal human dermal fibroblast (25). It has also been demon-
strated that atRA and the RARα ligand AM80 up-regulate VEGF production in fibroblast cells (26). The conflicting reports may be attributed, at least in part, to the fact that nuclear receptor ligands have different actions depending on cellular context. The difference in the response to the same ligand may be due to the quantity of cofactor or other regulatory factors in cells (27). The data in the present study support that A2E, as well as atRA, stimulate VEGF expression by activating RAR in RPE cells. The molecular mechanism that leads to different angiogenic response in different cells with the same ligand is an important issue for future research.

The age-related maculopathy is pathologically characterized by age-related changes in the RPE, such as accumulation of lipofuscin in Bruch’s membrane (4). Although it is not substantiated whether accumulation of lipofuscin is related to the development of exudative AMD clinically, some research results suggest that there may be a causal relationship between lipofuscin accumulation and exudative AMD (2, 7, 8). The data in the present study support that the accumulation of A2E induces the enhancement of VEGF expression, suggesting a role of A2E in the progression of exudative AMD. Anti-VEGF therapy is currently showing favorable results in the treatment of CNV (28), suggesting VEGF plays a pivotal role in the progression of CNV. However, the overexpression of VEGF in RPE cells is not sufficient to induce CNV. Recent studies have demonstrated that loss of integrity of Bruch’s membrane, as well as overexpression of VEGF in RPE cells, is required to induce CNV (29, 30). Schwesinger et al. (29) reported that in transgenic mice that overexpress VEGF in the RPE an intact Bruch’s membrane prevents CNV from penetrating into the subretinal space. Yoshida et al. (30) also reported that the expression of VEGF is increased in the neprylisin gene-disrupted mice but that no CNV was observed. Clinically, it is well known that the accumulation of A2E is seen in Stargardt’s disease, but the occurrence of CNV is rare (31). These findings suggest that the loss of integrity of Bruch’s membrane, in combination with the accumulation of A2E, is needed for the occurrence of CNV.

Photodynamic therapy with verteporfin (PDT) has been shown to reduce the overall risk of moderate visual loss and lesion growth, and has been the standard therapy for patients with exudative AMD (5). However, histopathological studies have shown that the effect of PDT is limited, and clinical studies have demonstrated that in most cases, repeated treatment is needed every three months. Recently, it has been demonstrated that intravitreous injection of anti-VEGF antibody is effective for patients with neovascular AMD, improving visual acuity and reducing retinal edema, but the inhibition of VEGF signaling is considered to elevate the risk of adverse systemic effects. For example, Lee et al. (32) demonstrated that genetic deletion of VEGF specifically in the endothelial lineage lead to progressive endothelial degeneration and sudden death in mutant mice. Another group demonstrated that the intravitreous injection of the anti-VEGF antibody fraction, ranibizumab, may tend to increase the risk of myocardial infarction and cerebral infarction in a dose-dependent manner (33, 34). Thus, to pursue a novel therapeutic target is mandatory.

Much effort has been directed to understanding the mechanism of lipofuscin toxicity on RPE cells. The current investigation suggests that the endogenous accumulation of A2E both induces sustained RAR activation in RPE cells and activates the expression of pro-angiogenic factors. The induction and activation contribute to the phenotypic alteration of RPE to the chronic pro-angiogenic state observed in AMD and predisposes eyes to CNV formation. The pro-angiogenic effect of A2E is diminished by the RAR antagonist and siRNA against RARα, suggesting that RAR may be a new potential pharmaceutical target for the treatment of AMD. As a variety of important biological functions are regulated through RAR, further investigations will be needed for the therapeutic application of these findings. Additionally, further identification of lipofuscin components and their actions on nuclear receptors might contribute to a better understanding of the pathogenesis of AMD.

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