Identification of Apolipoprotein A-I as a Retinoic Acid-binding Protein in the Eye*\textsuperscript{s}‡

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All-trans-retinoic acid may be an important molecular signal in the postnatal control of eye size. The goal of this study was to identify retinoic acid-binding proteins secreted by the choroid and sclera during visually guided ocular growth. Following photoaffinity labeling with all-trans-[11,12-\textsuperscript{3H}]retinoic acid, the most abundant labeled protein detected in the conditioned medium of choroid or sclera had an apparent \textit{Mr} of 27,000 Da. Following purification and mass spectrometry, the \textit{Mr} 27,000 band was identified as apolipoprotein A-I. Affinity capture of the radioactive \textit{Mr} 27,000 band by anti-chick apolipoprotein A-I antibodies confirmed its identity as apolipoprotein A-I. Photoaffinity labeling and fluorescence quenching experiments demonstrated that binding of retinoic acid to apolipoprotein A-I is 1) concentration-dependent, 2) selective for all-trans-retinoic acid, and 3) requires the presence of apolipoprotein A-I-associated lipids for retinoid binding. Expression of apolipoprotein A-I mRNA and protein synthesis were markedly upregulated in choroids of chick eyes during the recovery from induced myopia, and apolipoprotein A-I mRNA was significantly increased in choroids following retinoic acid treatment. Together, these data suggest that apolipoprotein A-I may participate in a regulatory feedback mechanism with retinoic acid to control the action of retinoic acid on ocular targets during postnatal ocular growth.

In humans and diurnal vertebrates, postnatal eye growth is tightly regulated by a vision-dependent “emmetropization” mechanism that minimizes refractive error through modulation of the axial length of the growing eye to match the refractive target (1). Failure of the emmetropization process can lead to abnormally long or short eyes, resulting in myopia or hyperopia, respectively. In humans, the incidence of myopia is reaching epidemic proportions (2), theorized to be largely due to reduced time spent outdoors (3, 4). It has been suggested that sustained near-work activities indoors such as reading, studying, or working on computers and smartphones may not provide the visual cues necessary for emmetropization to occur (5). It is well established that visually guided eye growth is controlled via a cascade of locally generated chemical events that are initiated in the retina and ultimately cause changes in scleral extracellular matrix remodeling to effect changes in eye size and refraction (6–8). Therefore, the molecular basis for visually induced changes in scleral remodeling is of great interest as understanding this process may allow the design of therapies to slow or prevent the progression of myopia.

All-trans-retinoic acid (atRA)\textsuperscript{2} may be an important molecular signal in the postnatal control of eye size (9–12). Choroidal synthesis of atRA is modulated during periods of visually induced changes in ocular growth and has pronounced effects on scleral extracellular matrix metabolism in chicks (9, 12), guinea pigs (11), and marmosets (10). Based on estimated endogenous concentrations of atRA in the choroid (\textgreek{~}4 \times 10^{-9} to \textgreek{~}3 \times 10^{-8} M) and the calculated half-maximal effective concentration (EC\textsubscript{50}) of atRA on inhibition of scleral proteoglycan synthesis (8 \times 10^{-9} M), we speculate that choroidally derived atRA is transported to the sclera where it inhibits proteoglycan synthesis, resulting in a slowing of ocular growth (13). However, direct evidence that choroidally generated atRA acts on the sclera to alter extracellular matrix remodeling has yet to be demonstrated. Moreover, the mechanism by which atRA could be transported from the choroid to the sclera has not been considered.

Retinoids associate \textit{in vivo} with soluble intracellular and extracellular retinoid-binding proteins, which are important in regulating their transport, availability, metabolism, and biological activity (14). We hypothesize that atRA-binding proteins are synthesized in the chick choroid to regulate the transport and activity of atRA. Mertz and Wallman (9) and our laboratory

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† This article contains supplemental Table 1.

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\textsuperscript{2} The abbreviations used are: atRA, all-trans-retinoic acid; RPE, retinal pigment epithelium; apoA-I, apolipoprotein A-I; p27\textsuperscript{*}, M\textsubscript{r} 27,000 \textit{[^3H]atRA}-binding protein; p27, M\textsubscript{r} 27,000 atRA-binding protein; L-apoA-I, lipid-associated native apoA-I; TFE, trifluoroethanol; CCM, choroid-conditioned medium; ABCA1, ATP-binding cassette transporter 1; Bis-Tris, 2-[bis (2-hydroxyethyl) amino]-2-[bis (hydroxymethyl)propane]-1,3-diol.
(15) have identified a secreted atRA-binding protein from choroid-and sclera-conditioned media with an apparent Mr of 27,000. This Mr 27,000 protein does not correspond in size to any of the previously identified atRA-binding proteins (14) and has yet to be identified. An atRA-binding protein with an apparent Mr of 28,000, previously described in bovine retinal pigment epithelium (RPE) cell lysates (16), may be similar to the chick Mr 27,000 protein; however, this Mr 28,000 protein has also not been identified. Consequently, the goal of the present study was to identify the Mr 27,000 atRA-binding protein in choroid-and sclera-conditioned media. Using a combination of ion exchange and gel filtration chromatography, mass spectrometry, and immunoprecipitation, we provide evidence that the Mr 27,000 binding protein is apolipoprotein A-I (apoA-I). ApoA-I has saturable binding for [3H]atRA, demonstrates binding selectivity for all-trans-retinoic acid, and requires the presence of apoA-I-associated lipids for retinoid binding. Furthermore, we found that apoA-I synthesis is coordinately up-regulated with atRA synthesis and that choroidal apoA-I is transcriptionally up-regulated by atRA. Considering that apoA-I can function as an atRA-binding protein, our results also suggest that transcriptional control of apoA-I gene expression in the choroid by endogenous atRA may provide an additional level of regulation of retinoic acid transport and activity in the eye.

**Results**

**Choroid- and Sclera-conditioned Media Contain a Novel [3H]Retinoic Acid-binding Protein**—Retinoic acid-binding proteins secreted by chick tissues were identified by direct photoaffinity labeling of proteins in conditioned medium with [3H]atRA (16), which covalently links [3H]atRA to atRA-binding sites on proteins. Following SDS-PAGE and autoradiography, three major bands with apparent Mr values of 60,000, 27,000, and <10,000 were apparent in choroid- and retina/RPE-conditioned media (Fig. 1A). In contrast, liver-conditioned medium contained numerous atRA-binding proteins between the 65- and 20-kDa markers with the Mr 27,000 protein band relatively less abundant as compared with the Mr 27,000 protein band in choroid and retina/RPE samples. The Mr 66,000 band present in N2 medium and in all samples is most likely due to the presence of serum albumin (Mr 66,500) in the N2 supplement in addition to that secreted from chick tissues into the medium as several studies have demonstrated that serum albumin specifically binds atRA (17) for transport in the general circulation (18, 19). The Mr 66,000 and 27,000 atRA-binding proteins were also detected in sclera-conditioned medium (Fig. 1B).

**Purification of the Mr 27,000 [3H]atRA-binding Protein ("p27")—**Sclera-conditioned medium was used for subsequent column purification of the Mr 27,000 [3H]atRA-binding protein (“p27”) (Fig. 2). During anion exchange chromatography on Mono Q, p27* eluted as a single peak following the bulk of other proteins as determined by SDS-PAGE autoradiography (Fig. 2A). The peak fractions were pooled, concentrated, and applied to gel filtration on a Superdex 200 column. p27* eluted monodispersely at a position corresponding to an Mr of ~27,000 together with the majority of total protein (Fig. 2B). Unlabeled conditioned medium was separated on Mono Q and Superdex 200 based on conditions established previously for p27*, and a co-eluting band at the Mr 27,000 position, referred to as p27, was observed in the corresponding Superdex fractions (fractions 25–31) by SDS-PAGE after Coomassie Blue staining (Fig. 3).

**Identification of p27 as Apolipoprotein A-I—**To identify the protein content of this band, gel slices labeled “A”, “B”, “C”, “D”, and “E” were excised from the Coomassie-stained gel for Superdex fraction 28 and processed for mass spectrometry. Slices A, B, and C were taken from areas above p27; slice D was taken at the position of p27; and slice E was taken immediately below p27. Additionally, gel slices corresponding to the position of p27 (slice D) were excised from the gel containing fractions 26, 27, 28, 29, and 30. A similar gel slice was also excised at the position of Mr 66,000 from gel slice 27. The protein content of the gel bands was estimated after trypsinization and analysis of the recovered peptides by LC-MS/MS on a Thermo Orbitrap Elite mass spectrometer. Following analyses of mass spectra with Proteome Discoverer, proteins with at least three identified peptides were plotted in Fig. 4 as total chromatographic peak area of the three most abundant peptides for each protein.
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(see supplemental Table 1 for additional details on mass spectrometry and database search results). Chicken apoA-I (UniProt reference number P08250) was the most abundant protein in slices A–E of fraction 28 with highest concentration in gel slice D relative to the flanking positions (Fig. 4A). All other identified proteins were ≥100-fold lower in relative concentration. ApoA-I also co-eluted faithfully with the p27 band across Superdex fractions 26–30 (Fig. 4B). Additionally, albumin and triose-phosphate isomerase were identified in Superdex fractions 26–30; however, their relative apparent abundance was much lower than that of apoA-I, and their elution patterns did not correspond to that of p27. Thus, apoA-I became the top candidate for the identity of p27. The predicted Mr of mature chicken apoA-I (following cleavage of the 18-amino acid signal peptide and 6-amino acid propeptide), 27,964, is consistent with the assignment. Mass spectrometry analyses of the Mr 66,000 protein from gel slice 27 identified chicken serum albumin (UniProt reference number P19121) as the most abundant protein present. A relatively small amount of apoA-I (~7%) and trace amounts of heat shock cognate 71-kDa protein (HSPA8; UniProt reference number F1NW3) were also identified at the Mr 66,000 position.

A few of the predicted proteins at the Mr ~27,000 position were identified by mass spectrometry as proteins larger than Mr ~27,000 (e.g. serum albumin (Mr 69,900) and lamin-A (Mr 73,100)). Although both of these proteins are relatively abundant in the choroid, they were present in trace amounts at the Mr 27,000 position (0.1–0.01% of the apoA-I peak area) and are most likely degradation products of the mature proteins that occurred as a consequence of organ culture for 3 h at 37 °C in the absence of protease inhibitors.

To test whether p27* from choroid- and sclera-conditioned media is apoA-I, its susceptibility to immunocapture by antibodies specific for chicken apolipoprotein A-I (20) (generously supplied by Dr. Marcela Hermann, Medical University of Vienna) was examined. As shown in Fig. 5A, anti-apoA-I coupled to magnetic beads was able to capture the majority of both p27 and p27* from choroid-conditioned media. Capture was specific for anti-apoA-I as no p27 or p27* was seen using non-specific IgG beads. Anti-apoA-I also captured an unidentified protein with an apparent Mr of ~55,000, which was not photolabeled, and the Mr 8000 band, which may be a degradation product of apoA-I. The unlabeled and photolabeled Mr 66,000 proteins did not bind to anti-apoA-I-coupled beads and were present in flow-through and wash fractions. Similar results were observed for sclera-conditioned medium (Fig. 5B). These results strongly support the interpretation that p27 and p27* are the same protein and correspond to chicken apoA-I.

The Apolipoprotein A-I Interaction with atRA Requires ApoA-I-associated Lipids—The interaction of [3H]atRA with native and recombinant apoA-I was investigated in Fig. 6. Recombinant chicken apoA-I was generated from Escherichia coli, and native apoA-I (delipidated or lipid-associated) was purified from chicken serum. Following preincubation of [3H]atRA with 1 μg of lipid-associated native apoA-I (L-apoA-I) (Fig. 6A, lane 2), delipidated apoA-I (Fig. 6A, lane 3), recombinant apoA-I (Fig. 6A, lane 4), or total chicken serum (containing ~1 μL of L-apoA-I; Fig. 6A, lane 1), UV irradiation, SDS-PAGE, and autoradiography, radioactive bands at Mr 66,000 and 27,000 were clearly detectable in serum, and a single strong radioactive band at Mr 27,000 was detected in the L-apoA-I sample (Fig. 6A, Autorad, lanes 1 and 2). Little to no radioactive adduct was detected in delipidated and recombinant apoA-I samples (Fig. 6A, Autorad, lanes 3 and 4), suggesting that the atRA/apoA-I interaction requires apoA-I-associated lipids. This result also suggests that the apoA-I fractions purified from serum are either not already saturated with atRA or that binding is readily reversible.

To determine whether delipidated apoA-I and recombinant apoA-I maintained correct folding in our [3H]atRA binding experiments, the structural properties of lipid-associated apoA-I, delipidated apoA-I, and recombinant apoA-I were evaluated spectroscopically (Fig. 6B). Far-UV circular dichroism (CD) analysis of apoA-I indicated that L-apoA-I and delipidated apoA-I demonstrated the characteristic high content of α-helical secondary structure. Addition of 50% trifluoroethanol (TFE), an agent known to induce and stabilize the α-helical structure in proteins (21), to delipidated apoA-I resulted in a...
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To determine whether apoA-I in choroid-conditioned medium was lipid-associated, choroid-conditioned medium, previously photoaffinity-labeled with [3H]atRA, was subjected to ultracentrifugation in the presence of 0.2 g/ml NaBr (Fig. 7). Following ultracentrifugation, upper (lane 2), middle (lane 3), and bottom (lane 4) regions of the gradient were subjected to SDS-PAGE and autoradiography. Comparison of the Coomassie Blue-stained gel (prior to autoradiography) with the autoradiogram indicates that the 3H-labeled apoA-I secreted from choroids is located in the upper 1/10 of the NaBr gradient (lane 2), whereas the photolabeled M, 66,000 protein albumin, which also binds [3H]atRA, segregates to the bottom of the NaBr gradient. These observations suggest that choroidally derived apoA-I is secreted from the choroids as part of lipoprotein particles or becomes associated with lipid shortly after its secretion, and it is this lipid-associated apoA-I that binds [3H]atRA.

Characterization of "Holo-apoA-I" by Absorption Spectroscopy—The UV/visible light absorption spectrum of L-apoA-I purified from chicken serum had one peak with a maximum at 280 nm (Fig. 8B). Following incubation of L-apoA-I with a 10-fold molar excess of atRA and removal of the excess atRA by centrifugal ultrafiltration, holo-apoA-I was generated (indicated by absorbance maxima at 280 and 346 nm). The additional peak with a λ_max of 346 nm was nearly identical to the spectra for atRA in ethanol (Fig. 8A), indicating the presence of atRA in association with apoA-I. Negative control samples prepared in the absence of apoA-I had undetectable levels of atRA. Thus, native apoA-I from serum was not substantially and stably loaded with atRA, and based on the extinction coefficients apoA-I appeared to bind 0.43 ± 0.01 mol of atRA/mol of apoA-I in a manner that was stable for at least 6 h at 4°C.

To determine whether atRA could be recovered from holo-apoA-I, we extracted atRA from holo-apoA-I and determined its solvent partition characteristics by monitoring absorption spectra between 250 and 450 nm (Fig. 8C). Following extraction with hexane under alkaline conditions, no retinoid-like absorbance peaks were detected in either of the neutral lipid extractions ("neutral lipid 1" and "neutral lipid 2"), indicating that none of the bound atRA had been reduced (e.g. to retinol or retinal). After acidification of the aqueous ethanol phase by addition of HCl and re-extraction with hexane, all atRA was recovered in the hexane phase ("acidic lipid"). The slight red shift of the absorbance maxima to 355 nm for atRA observed in the acidic lipid fraction is characteristic of atRA in acidified ethanol (23). Thus, atRA bound to apoA-I behaved as an acidic lipid as expected for atRA.

apoA-I Specifically Binds atRA in Vitro—Retinoic acid lacks intrinsic fluorescence but can quench inherent protein fluorescence due to energy transfer from tryptophan residues (24). We exploited this effect to measure the binding affinities of atRA and other retinoic acid derivatives toward lipid-associated native apoA-I using a fluorescence assay that monitored...
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The synthesis of apolipoprotein A-I (apoA-I) was examined in chick choroids and scleras cultured with retinoic acid (atRA) and retinol. The binding of apoA-I to atRA was assessed by monitoring atRA fluorescence emission spectra following 5 days of recovery from treated eyes of chicks. To test whether apoA-I associates with atRA, a peak of emission was recorded at 474 nm. When excited at 340 nm, a peak of emission was recorded at 528 nm in conditioned medium (CCM). A qualitatively similar spectrum was obtained for apoA-I previously incubated with 10 μM atRA. In contrast, lipid-associated native apoA-I in the absence of atRA displayed a maximum emission at 422 nm that gradually declined to background levels by 525 nm. atRA in the absence of protein lacked fluorescence.

ApoA-I Synthesis Is Up-regulated in Choroids during Recovery—TaqMan real-time quantitative PCR analyses indicated that choroidal gene expression of apoA-I was significantly increased in treated eyes following 4–15 days of recovery as compared with paired contralateral controls (*, p < 0.05; ***, p < 0.001, paired t test). ApoA-I gene expression was also elevated in 20-day recovering choroids compared with contralateral controls but did not reach statistical significance (p = 0.069, paired t test). No significant differences were detected between control and treated eyes in choroids following 10 days of form deprivation (0 days of recovery) or following 1 day of recovery (Fig. 11B). Additionally, analyses of all control groups in Fig. 11B indicated a significant difference in choroidal apoA-I gene expression in the 4- and 20-day control groups (p < 0.05, analysis of variance with Bonferroni correction). Comparisons of pairs of control groups (without Bonferroni correction) indicated that the 4- and 7-day control groups were significantly increased when each was compared with the 0-day control group (p < 0.001 and p < 0.01, respectively; Student’s t test). No significant differences were detected in retinas/RPE or scleras of control and treated eyes at any of the time points examined.

Immunoprecipitation analyses verified de novo apoA-I synthesis by chick choroids. ApoA-I was immunoprecipitated from 35S-labeled proteins synthesized and secreted in conditioned medium of chick retina/RPE, choroids, and sclera (Fig. 11D). Comparison of samples immunoprecipitated with non-immune IgG with the same sample immunoprecipitated with anti-apoA-I indicated the presence of a specific band in choroid culture medium but not in the middle or lower 1/10. The majority of apoA-I was secreted into the conditioned medium (Fig. 10A) and secreted apoA-I was also compared in choroid-conditioned medium (CCM) (Fig. 11E). Newly synthesized apoA-I was most apparent in conditioned medium of recovering choroids following immunoprecipitation with anti-apoA-I as a radiolabeled protein migrating at M, 27,000.
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FIGURE 6. [3H]atRA binding to apoA-I requires lipid. A, total chicken serum (containing ~1 μg of L-apoA-I; lane 1), L-apoA-I (1 μg; lane 2), delipidated apoA-I (1 μg; lane 3), and recombinant apoA-I (11 μg; lane 4) were photoaffinity-labeled with [3H]atRA and analyzed by SDS-PAGE (Coomassie) and autoradiography. [3H]atRA-labeled apoA-I is found exclusively at the top portion of the centrifuge tube (lane 2), indicating that it is lipid-associated.

FIGURE 7. ApoA-I in choroid-conditioned medium is lipid-associated. Choroid-conditioned medium was photoaffinity-labeled with [3H]atRA, adjusted to 0.2 g/ml with NaBr, and centrifuged at 125,000 × g, for 22 h. Samples of the conditioned media prior to centrifugation (lane 1), the top 1/10 portion of the centrifuge tube (lane 2), the middle 1/10 of the tube (lane 3), and the bottom 1/10 of the centrifuge tube (lane 4) were subjected to SDS-PAGE and autoradiography. [3H]atRA-labeled apoA-I is found exclusively at the top of the NaBr gradient (Autorad, lane 2), indicating that it is lipid-associated.

synthesized apoA-I was also detected in choroid-conditioned medium of control eyes at substantially lower concentrations as a faint band at M₉ 27,000 following immunoprecipitation with anti-apoA-I. Based on autoradiogram band intensity, apoA-I protein synthesis was increased in 8-day recovering choroids by 6–10-fold over contralateral controls. Additionally, a radiolabeled protein was faintly present at M₉ 28,000–32,000 retinoic acid-binding protein in in vitro organ cultures. Furthermore, this protein represented the major, if not exclusive, extracellular retinoic acid-binding protein present in choroid cultures. The size of this choroidal protein did not agree with the sizes of previously described extracellular retinoic acid-binding proteins, including albumin (M₉ 66,000) (30), interphotoreceptor retinoid-binding protein (M₉ 135,000) (31), lipocalcin-type prostaglandin D synthase (β-trace; M₉ 21,000) (32), epididyimal retinoic acid-binding protein (M₉ 21,000) (33), and retinol-binding protein (M₉ 20,000) (34).

In the present study, atRA-binding proteins present in choroidal and sclera-conditioned media were labeled with [3H]atRA by photoaffinity labeling, and the major M₉ 27,000 [3H]atRA-binding protein was purified by anion exchange, gel filtration chromatography, and SDS-PAGE and identified using mass spectrometry. Results from mass spectrometry indicated that apoA-I was a probable candidate as the 240-amino acid mature protein (following cleavage of the 18-amino acid signal peptide and 6-amino acid propeptide) has an M₉ of 27,960 and was present at the expected relative concentrations in fractions 26–30 of Superdex 200 of the elution profile. We confirmed that apoA-I represented the M₉ 27,000 [3H]atRA-binding protein by showing that it was recognized by a previously described antibody directed against apoA-I using immunoprecipitation of [3H]atRA-labeled proteins from choroid- and sclera-conditioned media.

Biochemical studies indicate that the lipid-bound holoprotein is the active atRA-binding protein. Comparison of [3H]atRA binding using recombinant chicken apoA-I, native delipidated chicken apoA-I, and native lipid-associated chicken apoA-I indicated that the binding of [3H]atRA to apoA-I requires the presence of apoA-I-associated lipids. No binding was observed with delipidated native apoA-I or recombinant chicken apoA-I. CD spectroscopy indicated that although recombinant apoA-I lacked proper α-helical secondary structure delipidated native apoA-I displayed folding nearly identi-
cal to that of native lipid-bound chicken apoA-I. CD spectra analyses indicated that lipid-associated apoA-I and native delipidated apoA-I were primarily \( \alpha \)-helical, consisting of 61 ± 5\% \( \alpha \)-helix and 5 ± 2\% \( \beta \)-sheet, consistent with previous reports (22). Therefore, the differential binding of \( ^{3} \text{H} \)atRA to lipid-associated apoA-I, but not native delipidated apoA-I,
indicates a requirement for apoA-I-associated lipids rather than differences in apoA-I secondary structure in the lipid-bound and lipid-free states. Moreover, [3H]atRA-labeled apoA-I in choroid-conditioned medium demonstrated “floata- tion behavior” following ultracentrifugation with 20% NaBr, indicating that the choroid-derived apoA-I is secreted as part of a lipoprotein complex or becomes associated with lipids shortly after its secretion. It is unclear whether atRA directly interacts with apoA-I-associated lipids or whether the lipid-bound con- formation of apoA-I, irrespective of associated lipids, is required for atRA binding. Near-UV CD spectroscopic studies provide evidence that chicken apoA-I undergoes a major con- formalional change upon binding to lipid, resulting in an increase in α-helical content in the C terminus of apoA-I, and a rearrangement of helices in the N-terminal half to form a “double belt” around phospholipid bilayer disks or the circumference of spheroidal high density lipoprotein (HDL) without changing overall α-helix content (35). However, the finding that [3H]atRA was retained on apoA-I following chromatographic separation and denaturing SDS-PAGE, conditions that strip the lipids, suggests that [3H]atRA associates closely enough to the polypeptide backbone to become covalently linked following UV irradiation. Furthermore, the ability of atRA to quench inherent protein fluorescence due to energy transfer from tryptophan resides suggests that atRA is in very close proximity (≤3.5 nm) to one of the two tryptophan residues in mature apoA-I protein based on the distance calculated for energy transfer between tryptophan residues on retinol-
binding protein and retinol (36). This suggests that a mutational approach might be successful in separating atRA binding from binding of other lipids to allow a genetic analysis of the importance of atRA binding in vivo.

**ApoA-I Exhibits a Preference for Binding atRA Relative to Other Physiological Retinoids**—Using lipid-bound apoA-I purified from chicken serum together with absorption and fluorescence spectroscopy, we provide evidence that apoA-I demonstrates binding specificity for all-trans-retinoic acid with an apparent $K_d$ of 0.62 $\mu M$. Of the other retinoids tested, a $K_d$ value could only be determined for all-trans-retinaldehyde (2.3 $\mu M$). Although our estimated $K_d$ for the interaction between apoA-I and atRA is considerably larger than that described for the cellular retinoic acid-binding proteins I and II (6–60 nM) (37–39), the affinity of apoA-I for atRA is similar to that of other extracellular retinoid-binding proteins, including epidermal retinoic acid-binding protein ($K_d = 0.9$ $\mu M \pm 0.2$ $\mu M$) (40), interphotoreceptor retinoid-binding protein ($K_d = 3.5 \pm 0.1$ $\mu M$) (41), and retinol-binding protein ($K_d = 0.210$ $\mu M$) (24). Additionally, atRA could be extracted from holo-apoA-I (atRA-bound apoA-I) and partitioned as an acidic lipid, indicating that the ionic character of atRA was unaltered by its association with apoA-I. In an attempt to identify endogenous atRA in association with apoA-I, choroids, which were isolated from recovering eyes, were used to generate conditioned medium under conditions that maximize atRA synthesis (9, 25). Following purification of apoA-I from the medium, the presence of bound atRA was detected by a fluorescence emission spectrum qualitatively similar to that of apoA-I previously incubated with atRA, suggesting that atRA and apoA-I are both synthesized by the choroid and become associated prior to or shortly after their secretion into the medium. It is possible, however, that other retinoids, such as retinol (which was added to the medium) or retinaldehyde, a precursor in retinoic acid synthesis, may also be in association with endogenous apoA-I as their fluorescence emission spectra are similar to that of atRA (23). However, our ligand binding studies indicated that apoA-I has very low affinity for other retinoids at the concentrations likely to be present in the conditioned medium, and care was taken to remove unbound retinoids from apoA-I prior to fluorescence spectroscopy. Nevertheless, further studies are needed to confirm the identity of the endogenous retinoid(s) bound to choroidally derived apoA-I in vivo, particularly under visual conditions in which retinoic acid synthesis is modulated.

A Native Functional Relationship between ApoA-I and atRA Is Suggested by Their Coordinate Expression in the Choroid—The results of the present study indicate that apoA-I mRNA levels and protein synthesis are up-regulated in choroids following 4–15 days of recovery from induced myopia, indicating that a portion of apoA-I secreted into choroid-conditioned medium is derived from de novo choroidal synthesis in addition to serum-derived apoA-I if present. We have previously shown that also at this time endogenous concentrations of atRA are also increased ∼2-fold in recovering choroids (13). We therefore examined the effect of atRA on choroidal apoA-I gene expression as it has been shown to modulate apoA-I synthesis (up- and down-regulation) in liver hepatocytes in a number of different species (26–28). We found that choroidal apoA-I gene expression was significantly increased by atRA concentrations of 0.1–10.0 $\mu M$. In contrast, the peroxisome proliferator-activated receptor $\alpha$ agonist GW7647 had no effect on choroidal apoA-I gene expression, although it has been shown previously to increase apoA-I protein expression in the chick retina and fibrous sclera (29). Together, these data indicate that transcriptional regulation of apoA-I is tissue-specific. Interestingly, apoA-I gene expression increased in contralateral control eyes over the first 7 days of recovery compared with the day 0 control group and then gradually decreased by 15 days of recovery (Fig. 11B). We speculate that this increase in apoA-I gene expression in contralateral control eyes may be the result of extraneous atRA produced by the right (recovering) eye that passed to the choroid of the left (control) eye as the posterior poles of chick eyes are in unusually close proximity, separated by only a thin interorbital septum (42).

**Potential Roles of ApoA-I in atRA Transport**—ApoA-I is the major protein component of plasma HDL and exists in alternate lipid-free and lipid-bound states. Lipid-poor apoA-I mediates removal of cholesterol from peripheral tissues, facilitating its return to the liver via the reverse cholesterol transport pathway (43–45). This pathway is believed to be responsible for the inverse correlation between HDL levels and risk of cardiovascular disease (45).

In addition to its role in cholesterol transport, results from the present study indicate, for the first time, that apoA-I also functions as an atRA-binding protein. As with other atRA-binding proteins, we speculate that apoA-I is synthesized and distributed where atRA is required. Mertz and Wallman (9) and Rada et al. (13) have demonstrated that atRA is synthesized by the chick ocular choroid during periods of slowed or decelerated ocular growth, due largely to reduced synthesis of scleral proteoglycans. We speculate that choroidally derived atRA is transported to the sclera where it inhibits proteoglycan synthesis, resulting in a slowing of ocular growth. A long standing
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question has remained unanswered: how can atRA be transported from its site of synthesis within the choroid across the highly vascularized choroidal stroma (up to 1000 \( \mu \text{M} \) in thickness depending on the visual condition (46)) to be delivered to the sclera in a relatively rapid and highly localized manner? The identification of apoA-I as the major choroidally derived atRA-binding protein may provide some mechanistic insight into this process. The concentration of endogenous atRA in the chick choroid has been estimated to be between 4 and 14 nM depending on the visual condition (13); this concentration is very similar to the range of atRA concentrations measured in mouse tissues (47) and in human plasma (48). Although endogenous concentrations of apoA-I in the choroid and sclera were not determined, apoA-I concentration in chick serum has been estimated to be 100–300 mg/100 ml (\( \approx \) 35–107 \( \mu \text{M} \)) depending on the age of the bird (49). Because the results of the present study indicate that atRA binds to apoA-I in a \( \approx 0.43:1 \) ratio, the concentration of apoA-I in ocular tissues is anticipated to far exceed that necessary for atRA transport. Moreover, the high concentration of apoA-I relative to atRA underscores the fact that atRA binding is only one of the multiple functions of apoA-I. Furthermore, the high apoA-I concentration and relatively low affinity for atRA would allow for high capacity with exchangeability where needed. For example, apoA-I might act as a buffer for atRA, binding excess amounts of atRA while allowing the presence of smaller amounts of free atRA similar to the function proposed for cellular retinoic acid-binding protein II (37). This theory might help to explain why choroidal atRA concentrations, as measured \( \text{in vitro} \), remained elevated beyond 15 days of recovery (13), long after scleral proteoglycan synthesis has returned to control levels (by 8 days).

We propose that apoA-I functions to transport atRA from its site of synthesis in choroidal cells to the extracellular space by a process similar to that described for its role in reverse cholesterol transport. During reverse cholesterol transport, extracellular lipid-free apoA-I stimulates the efflux of intracellular free cholesterol through direct interactions with the cAMP-inducible plasma membrane receptor, ATP-binding cassette transporter 1 (ABCA1) (50, 51). ABCA1 is highly expressed in a variety of tissues, including liver, adrenal gland, small intestine, fetal tissues, placenta, and brain (52). Interestingly, ABCA1 has also been shown to mediate basolateral secretion of free unesterified retinol in an intestinal cell line following absorption of vitamin A (53) similar to that described for basolateral secretion of unesterified cholesterol (54). Furthermore, ABCA1 shows greatest homology to the Rim protein (also known as ATP-dependent transport of light-bleached chromatophore, ABCA4, or adenosine triphosphate (ATP)-binding cassette rim (ABCR)) located in the disc membranes of photoreceptors where it functions to transport retinoids from the disc lumen into the cytosol (55, 56). Taken together, these data suggest that, in some cases, retinoid and cholesterol efflux may be regulated by similar receptor-mediated mechanisms involving apoA-I.

Extracellular atRA, in association with apoA-I and apoA-I-associated lipids, is likely to be stabilized in a protonated, uncharged state similar to that described for atRA in lipid bilayers (57). Upon delivery to the target tissue (\( \text{i.e.} \) the sclera), it would be expected that uncharged atRA could move spontaneously across cellular membranes to rapidly enter the cell. Additionally, the presence of apoA-I-associated lipids may regulate retinoid binding specificity and diffusion of atRA into cells similarly to the proposed role of the fatty acid docosahexaenoic acid in association with interphotoreceptor retinoid-binding protein (58).

Mertz and Wallman (9) noted that the sclera had a high affinity for atRA, effectively concentrating 50% of newly synthesized atRA into a piece of sclera representing \( \approx 1\% \) of the total volume of the culture. Our finding in the present study that the sclera also releases significant amounts of apoA-I into culture medium despite undetectable \( \text{de novo} \) protein synthesis suggests that choroidally derived apoA-I accumulates in the sclera, presumably as a consequence of retinoid transport. The specific up-regulation of choroidal apoA-I by atRA observed in the present study suggests that apoA-I may participate in a novel regulatory feedback mechanism to control the action of atRA on ocular targets during visually guided ocular growth. A very large body of evidence indicates that the mechanisms that regulate eye growth have been highly conserved across vertebrate species from fish to primates (59). Despite significant anatomical differences, changes in ocular refraction in response to visual stimuli are mediated in all species examined by changes in the axial length of the eye through modulation of vitreous chamber growth rates. Considering the similarities by which diverse animal species maintain emmetropia, it is likely that apoA-I functions as an atRA-binding protein in eyes of many vertebrates for the control of postnatal ocular growth and refraction.

Experimental Procedures

Animals—White Leghorn male chicks (Gallus gallus) were obtained as 2-day-old hatchlings from Ideal Breeding Poultry Farms (Cameron, TX). Chicks were housed in temperature-controlled brooders with a 12-h light/dark cycle and were given food and water \( \text{ad libitum} \). For some studies, form deprivation myopia was induced in 3-day-old chicks by applying translucent plastic goggles to one eye as described previously (6). The contralateral eyes (left eyes) of all chicks were never goggled and used as controls. Chicks were checked daily for the condition of the goggles. Goggles remained in place for 10 days after which time the chicks were either euthanized for isolation of ocular tissues from control and form-deprived eyes (=time 0), or the goggles were removed, and chicks were allowed to experience unrestricted vision (recover) for up to 20 days. Chicks were managed in accordance with the Animal Welfare Act and with the National Institutes of Health Guidelines. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center.

Tissue Isolation—To prepare ocular tissues for organ culture, chicks were euthanized by an overdose of isoflurane inhalant anesthetic (IsoThesia, Vetus Animal Health, Rockville Center, NY) at 10 days of age (normal, untreated chicks) or at various time points during recovery, and eyes were enucleated. Eyes were cut along the equator to separate the anterior segment and posterior eye cup. Anterior tissues were discarded, and the vit-
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Photoaffinity Labeling—Following organ culture (described above), medium was collected, and tissue debris was removed by centrifugation at 15,000 × g for 15 min. Following centrifugation, the supernatant, now referred to as “conditioned medium,” was collected and stored at −20 °C. In some cases, protein in conditioned medium was concentrated using 10-kDa centrifugal filter units (Microcon™.10, EMD Millipore, Billerica, MA). atRA-binding protein candidates were identified by photoaffinity labeling with all-trans-[11,12-3H]retinoic acid as described previously (16). Briefly, under red safe light illumination, 10 μCi of all-trans-[11,12-3H]retinoic acid in 10 μl of ethanol (48.4–50.0 Ci/mmol; PerkinElmer Life Sciences) was added to amber 1.5-ml microcentrifuge tubes. After the ethanol had been removed under vacuum, conditioned medium containing 200–400 μg of protein was then added to each tube, and the final volume was adjusted to 100 μl with 20 mM Tris buffer (pH 7.5) for a final [3H]atRA concentration of 2.0–2.7 μM. Samples were incubated with agitation at 23 °C for 1 h. The caps of the tubes were opened, and samples were placed on ice and exposed to an intense 365 nm UV light source (Spectroline model B–100X, Spectronics, Westbury, NY) suspended 6 cm above the surface of the liquid for 15 min. 3H-Labeled proteins in the medium were identified by SDS-PAGE followed by autoradiography of the dried gel. Specifically, proteins were separated on a 10% Bis-Tris SDS-polyacrylamide gel (NuPAGE gels, Novex/Life Technologies) and stained using SimplyBlue Safestain (Life Technologies). Gels were then treated with the autoradiography enhancer Fluoro-Hance (Research Products International, Mount Prospect, IL) for 5 min at room temperature, dried on Whatman 3 MM chromatography paper, and placed against film (Classic Blue autoradiography film BX, MIDSCI, St. Louis, MO) at −80 °C for 2–14 days.

Protein Purification—Following photoaffinity labeling, sclera-conditioned medium containing [3H]atRA-labeled proteins (100 μl) was centrifuged at 15,000 × g for 20 min, and 50 μl of the supernatant containing ~200 μg of total protein was brought to 0.2% (w/v) n-octyl β-D-thioglucoide and loaded onto a 1-ml HiTrap FastFlow Mono Q anion exchange column (5 × 50 mm; GE Healthcare) pre-equilibrated in 0.2% n-octyl β-D-thioglucoide, 50 mM NH₄HCO₃ and eluted in 20 volumes of a gradient of 50–1000 mM NH₄HCO₃ containing 0.2% n-octyl β-D-thioglucoide at a flow rate of 1 ml/min. Total protein was monitored using A₂₈₀ and NH₄HCO₃ was monitored as conductivity (millisiemens/cm). Fractions containing the Mr = 27,000 or 66,000 [3H]atRA-binding proteins were identified by SDS-PAGE and autoradiography, pooled, and separated on a Superdex 200 column (10 × 300 mm GL, GE Healthcare) equilibrated in 0.2% n-octyl β-D-thioglucoide in 50 mM NH₄HCO₃ (pH 7.8) at a flow rate of 80 μl/min. Protein elution was monitored by A₂₈₀, SDS-PAGE, and autoradiography. In some trials, unlabeled conditioned media (50 μl) were processed in parallel, and fractions corresponding to those containing the desired atRA-binding protein were separated on Mono Q and Superdex 200 as described for the [3H]atRA-binding protein. Superdex 200 fractions containing the unlabeled Mr = 27,000 or 66,000 protein were separated by SDS-PAGE (10% Bis-Tris NuPAGE gel) followed by staining with SimplyBlue Safestain. Gel slices were excised and processed for mass spectrometry.
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Mass Spectrometry—Proteins in gel slices were digested using trypsin as described (60). Briefly, slices were excised from a Coomassie Blue-stained gel, washed three times with 50% acetonitrile, 25 mM NH₄HCO₃ and treated with 10 mM DTT in 25 mM NH₄HCO₃ at 37 °C. After 1 h, the solution was replaced with 55 mM iodoacetamide in 25 mM NH₄HCO₃. After 45 min in the dark, gel slices were washed with 25 mM NH₄HCO₃, dehydrated in 50% acetonitrile, 50% 25 mM NH₄HCO₃, followed by brief vacuum centrifugation; and rehydrated in 12.5 ng of trypsin in 25 μl of 25 mM NH₄HCO₃. After incubation overnight at 37 °C, the supernatant was replaced with 50% acetonitrile, 5% formic acid in H₂O and sonicated. The solution was replaced, sonicated, pooled with the first sonicate, and taken to dryness. Samples were reconstituted in 50 μl of 1% acetic acid, and 5 μl was separated by an Easy nLC-1000 pump (2–30% acetonitrile gradient over 15 min and 30–98% in 5 min at 300 nl/min) with a 25-cm × 75-μm C₁₈ column (2-μm particles) and analyzed using an Orbitrap Elite Mass spectrometer (Thermo Scientific, Waltham, MA) operated in data-dependent mode acquiring a 60,000 resolution MS1 scan in the Orbitrap followed by 10 dependent collision-induced dissociation MS2 (dynamic exclusion, 30 s; isolation width, 2.0 thomson; activation Q, 0.25; normalized collision energy, 35%) in the ion trap. Mass spectral data were analyzed using Proteome Discoverer 2.1 (Thermo Scientific, Bremen, Germany) with the SEQUEST-HT search engine against the UniProt chicken database. Parameters for the Sequest search allowed for two missed tryptic cleavages, 10 ppm mass tolerance for precursor masses and 0.6 Da for fragment ions, static modifications including carboxymethylmethylation of Cys, and dynamic modifications including oxidation of Met and deamidation of Asn and Gln. Peptide identifications were accepted with criteria of >95% probability by Percolator (61) algorithm and at least three identified peptides. Relative protein abundance was calculated from the chromatographic peak areas of the three most abundant peptides for each protein (62). Biological replicates were also analyzed by Eksigent nanoLC and LTQ mass spectrometer.

Immunoprecipitation—The M₄₁ 27,000 [³H]atRA-binding protein was immunoprecipitated from [³H]atRA-labeled choroid- and sclera-conditioned media using protein A magnetic beads (Miltenyi Biotec, Auburn, CA). Rabbit antiserum against chicken apoA-I (generously provided by Dr. Hermann, Medical University of Vienna) was prepared as described previously (20). Briefly, for the polyclonal anti-apoA-I antibodies, the HDL fraction was isolated from rooster plasma, and the intact lipoprotein particles were used for immunization in adult female New Zealand White rabbits. The animal procedures were approved by the Intramural Committee for Animal Experimentation of the Medical University of Vienna and Federal Ministry for Science and Research (BMWF) Austria. Antiserum against chicken apoA-I or non-immune rabbit IgG was conjugated to protein A magnetic beads (Miltenyi Biotec, Auburn, CA). Rabbit antiserum against chicken apoA-I or non-immune rabbit IgG was conjugated to protein A magnetic beads (Miltenyi Biotec, Auburn, CA). Rabbit antiserum against chicken apoA-I or non-immune rabbit IgG was conjugated to protein A magnetic beads (Miltenyi Biotec, Auburn, CA). Rabbit antiserum against chicken apoA-I or non-immune rabbit IgG was conjugated to protein A magnetic beads (Miltenyi Biotec, Auburn, CA). Rabbit antiserum against chicken apoA-I, the supernatant was collected and adjusted to 0.2 g/ml NaBr (density, 1.14) by addition of the solid salt. Samples were centrifuged at 125,000 × g (at maximum radius) in a Beckman TLA 100.3 rotor for 6 h at 12 °C. The top ⅓ (≈800 μl from each 3-ml tube) volume of the tubes was pooled, and the preparation was diluted with 2 volumes of 10 mm Tris·HCl (pH 7.5) that contained 0.2 g/ml NaBr. Samples were centrifuged as before, and the upper portions of each tube containing the intensely yellow lipoprotein fraction (≈150 μl from each 3-ml tube) were pooled. In some cases, the lipoprotein fraction was delipidated using the ethanol/ether extraction method (64). Briefly, the lipoprotein fraction was extracted with 20 vol-
umes of ethanol/ether (3:1, v/v), incubated at −20 °C (2 h), and centrifuged at 3100 × g for 15 min at 4 °C. The supernatant (which contained the lipids) was discarded, and the remaining protein pellet was suspended with 15 ml ice-cold ethanol/diethyl ether (3:2, v/v) and incubated at −20 °C (1 h) before centrifugation at 8600 × g at 4 °C for 15 min. The remaining protein pellet was suspended in 15 ml of ice-cold ether and centrifuged at 8600 × g at 4 °C for 15 min. The protein pellet was then air-dried and resuspended in PBS buffer. Protein concentrations were determined by a Bradford assay (Bio-Rad) based on a standard curve of BSA (0−2000 μg/ml).

CD Spectroscopy—CD spectroscopy experiments were performed using a Jasco J-715 spectropolarimeter with a PTC-348WI Peltier temperature controller (Jasco Corp., Tokyo, Japan) in the University of Oklahoma Health Sciences Center Physical Biochemistry Equipment Core Facility. The spectral parameters used were as follows: 300−190-nm wavelength range, 0.1-cm cuvette path length, and three accumulations per spectrum. Spectra were acquired at 20 °C in a buffer consisting of 20 mM Tris-HCl (pH 7.5) or 50% (v/v) TFE in the same buffer. Lipid-associated apoA-I, delipidated apoA-I, and recombinant apoA-I were prepared at a concentration of 80 μg/ml in either 20 mM Tris-HCl (pH 7.5) or 50% (v/v) TFE. Protein secondary structural content was predicted using the CDPro software package as described previously (65). Data presented represent the average of results generated by the SELCON3, CDSSTR, and CONTINLL programs using protein reference set 4.

Retinoid Absorbance Studies—Absorption spectra of retinol, all-trans-retinoic acid, 9-cis-retinoic acid, 13-cis-retinoic acid, and retinaldehyde were recorded on a Nanodrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) in UV/visible mode with a path length of 0.1 cm. Concentrations of retinoids were calculated using Beer’s law at the absorbance maximum for each retinoid (24).

Preparation of Holo-apoA-I—Purified native lipid-associated apoA-I (150 μg in 0.5 ml of 20 mM Tris-HCl (pH 7.5)) was incubated with a 10-fold molar excess of atRA (5.36 μM) of a 10 mM stock solution in EtOH dried under vacuum prior to addition of buffer and apoA-I) at room temperature for 1 h. To remove excess unbound atRA, the mixture was passed through a 10-kDa centrifugal filter unit (Microcon-10) followed by two washes with 0.5 ml of 20 mM Tris-HCl (pH 7.5). A negative control sample was prepared in parallel with holo-apoA-I that contained the same concentration of atRA in the same buffer without apoA-I. The retentate was collected in a 20-μl volume of 20 mM Tris-HCl (pH 7.5), and the presence of holo-apoA-I in the concentrate was determined by obtaining the absorbance spectra between 250 and 450 nm.

Extraction of Lipids from Holo-apoA-I—After preparation of holo-apoA-I, the ligand associated with 150 μg of apoA-I was extracted using a two-step acid-base extraction method (66). To 500 μl of holo-apoA-I in 20 mM Tris-HCl (pH 7.5), 5 μl of 1.7 M NaOH was added followed by 600 μl of hexane, and the sample was vortexed. Samples were centrifuged for 5 min at 10,000 × g to facilitate phase separation, and the upper layer was collected (neutral lipid 1). The lower layer was re-extracted with 600 μl of hexane, and the upper layer was collected (neutral lipid 2). The lower layer was acidified by the addition of 17 μl of 5 M HCl followed by extraction with 600 μl of hexane. The upper layer was collected as acidic lipid. All three fractions were evaporated to dryness under argon and reconstituted in 20 μl of ethanol, and absorbance spectra between 250 and 450 nm were recorded.

Ligand Binding Assays—A PC1 photon-counting spectrophotometer (ISS Inc., Champaign, IL) was used with an excitation slit width of 0.6 mm and an emission slit width of 1.0 mm to measure retinoid binding. Under dim red light, retinoids were added in ethanol to a 1.24 μM solution of native lipid-associated apoA-I in 20 mM Tris-HCl (pH 7.5) such that the final concentration of ethanol did not exceed 1% (v/v). Samples (in a final volume of 0.5 ml in amber microcentrifuge tubes) were incubated at room temperature for 1 h with gentle agitation. Following incubation, samples were briefly centrifuged (2000 × g for 10 s), and protein quenching was monitored at 290-nm excitation and 340-nm emission. Each fluorescence measurement was the average of 10 readings over a 15-s time period. At least 12 concentrations were used for each retinoid in ligand binding assays. Data were analyzed using GraphPad Prism 5.0.

Determination of Endogenous Choroidal ApoA-I/atRA Interactions—Choroids were isolated from treated eyes of chicks following 5 days of recovery from induced myopia (n = 20) under dim red light, placed in 10 ml of culture medium (DMEM/Ham’s F-12 1:1) containing 8 μM retinol (diluted from a 10 mM stock in EtOH) and ketoconazole (diluted from a 12 mM stock in DMSO), and incubated for 3 h at 37 °C protected from light. Following incubation, the conditioned medium was passed through an Amicon Ultra-15 Centrifugal Filter Unit (EMD Millipore), diluted, and reconcentrated two times with 15 ml each of culture medium to reduce unbound retinoids for a predicted dilution factor of conditioned medium constituents <M, 10,000 of ×0.00018. Samples were brought to a final volume of 1 ml and stored at −80 °C protected from light. The choroid tissues were homogenized in 15 ml of culture medium using a 2-cm rotor-stator homogenizer (Virtis Co., Gardiner, NY) and centrifuged at 10,000 × g. Supernatants were concentrated and rinsed using an Amicon Ultra-15 Centrifugal Filter Unit as described above. For purification of apoA-I, solid NaBr was added to the conditioned medium and choroid homogenate samples to a final concentration of 0.2 g/ml (1-ml final volume) as described above under “Purification of Native Chicken Apolipoprotein from Chicken Serum.” Samples were centrifuged at 125,000 × gmax in a Beckman TLA 100.3 rotor fitted with 1.5-ml microcentrifuge tube adaptors for 6 h at 12 °C. The top ½g, middle ½g, and lower ½g volume of each sample (~100 μl each) was collected. Samples were desalted and buffer-exchanged to 20 mM Tris-HCl (pH 7.5) using 10-kDa centrifugal filter units (Microcon-10). Sample volumes were adjusted to 250 μl with 20 mM Tris-HCl (pH 7.5). Fluorescence emission spectra (400–600 nm) were collected on a PC1 photon-counting spectrophotometer at 350-nm excitation with an excitation slit width of 0.6 mm and an emission slit width of 1.0 mm.

TagMan Quantitative PCR (RT-Quantitative PCR)—Retinas/RPE, choroids, and sclera were isolated from individual pairs of control and treated eyes following 0–20 days of days of recovery or following organ culture with atRA or GW7647 and
snip frozen in liquid nitrogen. Total RNA was isolated using TRIzol reagent (Invitrogen) followed by DNase treatment (DNA-free, Ambion, Foster City, CA) as described previously (13). RNA concentration and purity were determined via the optical density ratio of 260/280 using a Nanodrop ND-1000 spectrophotometer and stored at ~80 °C until use. cDNA was generated from DNase-treated RNA using a High Capacity RNA to cDNA kit. Real-time PCR was carried out using a Bio-Rad CFX 96. 20-μl reactions were set up containing 10 μl of TaqMan 2× Universal Master Mix (Applied Biosystems), 1 μl 20× 6-carboxyfluorescein (FAM)-labeled Assay Mix (Applied Biosystems), and 9 μl of cDNA. Each sample was set up in duplicate with specific primers and probe for apoA-I (assay ID number Gg03372342_s1) and the reference gene chicken GAPDH (assay ID number Gg03346982_m1). The PCR cycle parameters were an initial denaturing step at 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Normalized gene expression was determined by the ΔΔct method (67) using Bio-Rad CFX Manager™ version 3.1.

**Author Contributions**—J. A. S. conceived the idea for the project, prepared starting materials for purification, conducted most of the experiments, and wrote the paper. A. R. H. prepared and purified recombinant apoA-I and prepared samples for mass spectrometry. C. L. F. and H. V. carried out HPLC and mass spectrometry analyses. J. N. B. performed circular dichroism experiments. M. H. provided antibodies and provided scientific input. C. M. W. designed and implemented the strategy for HPLC purification and mass spectrometry analyses, provided scientific input, analyzed the results, and helped edit the manuscript.

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