Inhibition of RPE65 Retinol Isomerase Activity by Inhibitors of Lipid Metabolism

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RPE65 is the isomerase catalyzing conversion of all-trans-retinyl ester (atRE) into 11-cis-retinol in the retinal visual cycle. Crystal structures of RPE65 and site-directed mutagenesis reveal aspects of its catalytic mechanism, especially retinyl moiety isomerization, but other aspects remain to be determined. To investigate potential interactions between RPE65 and lipid metabolism enzymes, HEK293-F cells were transfected with expression vectors for visual cycle proteins and co-transfected with either fatty acyl-CoA ligases (ACSLs) 1, 3, or 6 or the SLC27A family fatty acyl-CoA synthase FATP2/SLCA27A2 to test their effect on isomerase activity. These experiments showed that RPE65 activity was reduced by co-expression of ACSLs or FATP2. Surprisingly, however, in attempting to relieve the ACSL-mediated inhibition, we discovered that triacsin C, an inhibitor of ACSLs, also potently inhibited RPE65 isomerase activity in cellulo. We found triacsin C to be a competitive inhibitor of RPE65 (IC$_{50}$ = 500 nM). We confirmed that triacsin C competes directly with atRE by incubating membranes prepared from chicken RPE65-transfected cells with liposomes containing 0–1 μM atRE. Other inhibitors of ACSLs had modest inhibitory effects compared with triacsin C. In conclusion, we have identified an inhibitor of ACSLs as a potent inhibitor of RPE65 that competes with the atRE substrate of RPE65 for binding. Triacsin C, with an alkenyl chain resembling but not identical to either acyl or retinyl chains, may compete with binding of the acyl moiety of atRE via the alkenyl moiety. Its inhibitory effect, however, may reside in its nitrosobhydrozaine/triazene moiety.

The process of vision begins in the photoreceptor outer segments of the retina with absorption of a photon of light by the 11-cis-retinylidene chromophore ligand of photoreceptor visual pigment opsins, such as rhodopsin. This event photoisomerizes the 11-cis-retinal to the all-trans-isomer, thereby activating the rhodopsin to metarhodopsin and initiating the phototransduction pathway that results in transmission of a signal, via retinal interneurons, for central processing in brain visual centers. Upon completion of the phototransduction cascade processes, the all-trans-retinal releases from its Schiff base linkage following decay of metarhodopsin and is reduced to all-trans-retinol and transported to the retinal pigment epithelium (RPE), a monolayer epithelium apposed to the photoreceptor outer segments. To regenerate the visual pigment, input of 11-cis-retinal is required. This is provided by enzymatic isomerization in the RPE of the esterified all-trans-retinol in a process termed the visual cycle. RPE65 is the key isomerase in the RPE visual cycle that catalyzes the conversion of all-trans-retinyl ester (atRE) into 11-cis-retinol (1–3). RPE65 is a member of a family of carotenoid oxygenases that has evolved to become a retinol isomerase in the vertebrate retinal visual cycle (4). Recent crystal structures of RPE65 (5–7) and site-directed mutagenesis studies (8, 9) have revealed aspects of its catalytic mechanism, especially with respect to retinyl moiety isomerization, but other aspects remain to be determined. RPE65 performs a concerted O-alkyl cleavage of an all-trans-retinyl ester into retinol and fatty acid along with isomerization of the retinyl moiety. This proceeds via a carbocation or, possibly, a radical cation mechanism. The latter mechanism is supported by the finding that RPE65 isomerase activity is inhibited by several lipophilic aromatic spin traps, including N-tert-butyl-α-phenylnitrone (10).

Identification of such modifiers or inhibitors of RPE65 retinol isomerase activity serve not only as potential therapeutic agents for modulation of the visual cycle in diseases where accumulation of bisretinoid byproducts is a factor (Stargardt disease and geographic atrophy form of age-related macular degeneration (11)) but also to illuminate aspects of the catalytic mechanism of RPE65. Co-crystallization of RPE65 with the non-retinoid inhibitor emixustat has yielded structural information on substrate orientation (12) though conflicting with a prior study (5).

We are particularly interested in interactions between the visual cycle and lipid metabolic processes in the RPE. For example, FATP1 and FATP4, fatty acyl-CoA synthetases/long chain fatty acid transport proteins (FATPs) of the solute carrier transporter protein family SLC27A, have been shown to inhibit RPE65 activity (13, 14). In this report we investigated whether long chain fatty acyl-CoA ligases (ACSLs), a family unrelated to FATPs, inhibit RPE65 and found that they do. In an effort to

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This article contains supplemental Methods.

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2 The abbreviations used are: RPE, retinal pigment epithelium; ACSL, fatty acyl-CoA ligases; atRE, all-trans-retinyl ester; CRALBP, retinaldehyde-binding protein 1; EDYA, 8,11-eicosadiynoic acid; FATPs, SLC27A family fatty acid transport proteins; 2-FPA, 2-fluoropalmitic acid; LRAT, lecithin-retinol acyltransferase.
negate this inhibition, we used inhibitors specific for ACSLs. Triacsin C, a fungal secondary metabolite, is an 11-carbon alkanyl-1-hydroxytriazene synthesized by Streptomyces sp. (15, 16). Although it has other effects (such as vasodilatory), it is best known as an inhibitor of ACSLs (17, 18). Importantly, it does not inhibit the FATP/SLC27A family of fatty acyl-CoA synthetases (19). We demonstrate here that it is a potent inhibitor of RPE65 and dissect its mode of inhibition.

In addition, to help distinguish between an alkanyl/acyl moiety-mediated effect and a nitrosohydrazone/triazene moiety-mediated effect in triacin C, we tested acyl-based inhibitors of ACSLs, the diacylenic fatty acid 20:2Δ8a,11a (8,11-eicosadiynoic acid (EDYA); Fig. 1B) and 2-fluoropalmitic acid (2-FPA; Fig. 1C). EDYA inhibits uptake of arachidonic acid by platelets and activities of arachidonyl and other ACSLs, decreases conversion of dihomo-γ-linolenic acid to arachidonic acid, and inhibits cyclooxygenase (20). 2-FPA is an inhibitor of ACSLs and of sphingosine biosynthesis (21). Given their inhibitory activities toward ACSLs, we sought to determine whether they also inhibited RPE65. We found that they do but only at significantly higher concentrations than does triacin C.

FIGURE 1. Structures of inhibitors tested. A, triacin C, 2E, 4E, 7E-undecatriene-1-triazene. Triacin C may exist as a tautomeric pair where the compound is capable of interconversion between a nitroso compound (upper) or a triazenol compound (lower) depending on the location of the proton. B, 20:2Δ8a,11a or EDYA is a di-acyclic fatty acid. C, 2-FPA.

Experimental Procedures

Transient Transfection and Cell Culture—Cell culture methods and transient transfection protocols were used as previously published (3, 9). In a typical experiment, 3 × 10⁷ HEK293-F (Invitrogen) cells were transfected with 30 μg of pVitro2 plasmid (Invivogen, San Diego, CA; containing dog or chicken RPE65 and bovine CRALBP open reading frames) and 30 μg of pVitro3 plasmid (Invivogen; containing bovine lecithin-retinol acyltransferase (LRAT) and retinol dehydrogenase 5 or ACSL1, ACSL3, ACSL6, or FATP2 open reading frames) in the presence of 40 μl of 293fectin transfection reagent (Invitrogen), all in a total volume of 30 ml. At 24 h after transfection, all-trans-retinol (Sigma-Aldrich) was added to a final concentration of 0.79–7.9 μM, and the cells were cultured for a further 7 h and then harvested for analysis. In experiments where inhibitors were used, triacsin C (Sigma-Aldrich), EDYA (Enzo Life Sciences, Farmingdale, NY), or 2-FPA (Cayman Chemical, Ann Arbor, MI) dissolved in dimethyl sulfoxide (Sigma-Aldrich) were added at the appropriate concentrations 24 h after transfection and just prior to addition of all-trans-retinol.

Retinoid Extractions and HPLC—Retinoids were extracted and saponified under dim red light as previously described (3) from cells harvested by centrifugation from 29-ml volumes of cultures of transfected 293-F cells or from 1-ml lipid reaction mixtures. Resultant isomeric retinols were analyzed on 5-μm particle Lichrospher (Alltech, Deerfield, IL) normal phase columns (2 × 250 mm) on a HPLC system equipped with a UV-visible diode array detector (Agilent 1100/1200 series; Agilent Technologies, New Castle, DE), following Landers and Olson (22) as earlier modified by us (3). The data were analyzed using ChemStation32 software (Agilent).

Liposome Preparation and Liposome Isomerase Reactions—Liposomes containing all-trans-retinyl palmitate were prepared by a modification of a published method (23). Phospholipids and the Mini-Extruder device were purchased from Avanti Polar Lipids (Alabaster, AL). Liposome generation was conducted under dim yellow or red lighting. Liposomes were made with 1,2-dioleoyl-sn-glycero-3-phosphocholine and 1,2-diheptanoyl-sn-glycero-3-phosphocholine and all-trans-retinyl palmitate (Sigma-Aldrich) at a phospholipid-retinyl palmitate ratio of 120:1. On the basis of pilot experiments, we used a maximal concentration of 1 μM all-trans-retinyl palmitate because concentrations higher than this destabilized the liposomes. For example, to make liposomes containing 1 μM all-trans-retinyl palmitate, 675 μl of 1 mg/ml 1,2-dioleoyl-sn-glycero-3-phosphocholine and 54 μl of 1 mg/ml 1,2-diheptanoyl-sn-glycero-3-phosphocholine were mixed with 12 μl of 10 mM all-trans-retinyl palmitate, and the mixture was dried under argon. When dry, the mixture was hydrated with 2 ml of 100 mM Bis-Tris propane buffer, pH 8, and placed in a 37 °C water bath for 60 min before being processed into large unilamellar vesicles in the mini extruder device, following the manufacturer’s instructions. For liposome-containing RPE65 activity reactions, cell pellets from 30-ml cultures of HEK293-F cells transfected with pVitro2 expressing chicken RPE65/CRALBP and pVitro3-LRAT/retinol dehydrogenase 5 were homogenized in 1.25 ml of buffer containing 10 mM Bis-Tris propane,
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100 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, EDTA-free protease inhibitor tablet (Roche), 0.5% (w/v) essentially fatty acid-free bovine serum albumin (Sigma), and 2.5% (w/v) dodecyl maltoside (Sigma) with 20 strokes in a Dounce tissue grinder. The homogenates of the transfected HEK293-F cells were centrifuged at 16,000 × g for 5 min, and the supernatants were removed and used immediately. Reaction mixtures were set up under dim red light, containing 900 µl of transfected HEK293-F supernatant, 100 µl of liposome preparation (containing 0.5, 0.75, or 1 µM all-trans-retinyl palmitate), and 2 µl of triacsin C dilutions (giving final concentrations of 0–2000 nM triacsin C per reaction), mixed, and incubated for 40 min at 37 °C. (The 40-min time point was chosen on the basis of pilot time course experiments that showed the reaction to be optimal at 40 min and that it reached a plateau after 40 min.) The reactions were then extracted, saponified, and analyzed for isomeric retinols, as above.

Docking and Modeling—Docking simulations were done using the AutoDock Vina program (24). The receptor “.pdbqt” file was made from the A chain of the RPE65 crystal structure 4F30 (7) as recommended by the program protocol. Three-dimensional ligand structures were obtained from the PubChem database where available or made using the PRODRG program (25) from templates submitted to the GlycoBioChem PRODRG2 server and processed to .pdbqt files as recommended by the program. All possible torsions were routinely allowed in these files; however, the program does not allow torsions around double bonds. Two series of dockings were done, one in which the grid box included the entire area of the protein above the seven-bladed propeller structure (“cap”) and a second in which the grid box was restricted to the substrate cleft (“cleft”). A file containing all these parameters and .pdbqt files employed is found in the supplemental Methods.

Results

Inhibition of RPE65 Activity in Cellulo by ACSLs and by the ACSL Inhibitor Triacsin C—Previous work from other groups has shown that certain members of the SLC27A family—very long chain acyl-CoA synthetases—such as FATP1 and FATP4, inhibit RPE65 activity (13, 14). Whether the inhibition results from direct interaction of these proteins with RPE65 or is due to competition for substrates between RPE65 and FATP1 or 4 is not completely disentangled. To further explore potential interactions of RPE65 with RPE lipid metabolic enzymes, we investigated potential interactions between RPE65 and members of the long chain ACSL superfamily, another group of acyl-CoA synthetases that are unrelated to the SLC27A family but that also catalyze the ligation of CoA to long chain fatty acids. We co-transfected HEK293-F cells with RPE65 and LRAT, and ACSL1, ACSL3, ACSL6, or FATP2 (SLC27A2), all of which we have found to be expressed in bovine RPE,3 in the presence of 2.5 µM all-trans-retinol, the pro-substrate. FATP2 (SLC27A2) was chosen over FATP1 or FATP4, because it is the most abundant SLC27A family member in bovine or human RPE.3 We found that co-expression of RPE65 and LRAT with any of these fatty acyl synthetases reduces 11-cis-retinol production in transected HEK293F cells by ~80% (Fig. 2), compared with control RPE65 and LRAT transfection alone. To explore this effect further, we attempted to reverse the inhibitory effect of ACSLs on retinol isomerization by RPE65 by inhibition of ACSL activity with 10 µM triacsin C. Triacsin C (Fig. 1A) is a well known specific inhibitor of ACSLs at low micromolar levels (16, 17) but not of SLC27A family very long chain acyl-CoA synthetases (19). Surprisingly, however, when we co-transfected ACSL1 alone with RPE65, we observed a further reduction in 11-cis-retinol production in the presence of 10 µM triacsin C, from ~20% to less than 5% of control (Fig. 3). Furthermore, we found that 10 µM triacsin C alone, without co-transfection of ACSL1, also markedly reduced 11-cis-retinol production by RPE65. This finding indicated a direct effect of triacsin C on RPE65 enzymatic activity.

Characterization of Triacsin C Inhibition of RPE65 and Determination of IC₅₀—The experiments in Fig. 3 indicate that triacsin C inhibited RPE65 retinol isomerization activity at or close to micromolar levels. To delineate the functional range, we performed a concentration curve over a 4-order range of concentrations, 0–30 µM in DMSO, of triacsin C in HEK293-F cultures co-transfected with RPE65 + LRAT in the presence of the pro-substrate 2.5 µM all-trans-retinol. The results (Fig. 4A) indicated a biphasic aspect to the inhibition where ~50% inhibition was achieved by ~1 µM triacsin C, whereas it took to 30 µM triacsin C to reduce activity a further 40% to achieve 90% inhibition. To more precisely determine the in cellulo IC₅₀ of triacsin C, we incubated HEK293-F cultures co-transfected with RPE65 + LRAT in the presence of 0–2 µM of triacsin C and the pro-substrate 2.5 µM all-trans-retinol. Under these conditions, we found the IC₅₀ of triacsin C to be ~500 nM (Fig. 4B). Next we wished to determine the mode of inhibition (competitive, noncompetitive, or uncompetitive) of triacsin C on RPE65. We tested the effect of different concentrations of all-trans-retinol on inhibition of RPE65 using a range of 0–2000 nM triacsin C in DMSO. We used a 10-fold range of pro-substrate concentrations—0.79, 2.5, and 7.9 µM all-trans-retinol—for these experiments. The increasing

3 W. Samuel, T. Parikh, R. K. Kutty, T. Duncan, C. J. Jaworski, and T. M. Redmond, in preparation.

FIGURE 2. Inhibition of RPE65 retinol isomerase activity by fatty acyl-CoA ligases. 11-cis-Retinol production in HEK-293F cells co-transfected with pVitro2 vector co-expressing RPE65 and CRALBP and pVitro3 vectors co-expressing LRAT and ACSL1, ACSL3, ACSL6, or FATP2 and incubated with 2.5 µM all-trans-retinol. All these fatty acyl-CoA ligases and synthase suppressed RPE65 isomerase activity to ~20% of control. 11-cis-Retinol production values are expressed as percentages of control RPE65 alone (displayed as means ± standard deviations; n ≥ 5).
IC₅₀ value seen (Fig. 4C) as all-trans-retinol concentration is increased indicates a competitive mode of inhibition by triacsin C. That RPE65, and not LRAT, is specifically inhibited is shown by the finding that there was no apparent effect on total retinol accumulation in HEK293-F cells treated with 0–2000 nM triacsin C, because totals of all retinol isomers remained relatively constant for each pro-substrate concentration for 0.79–7.9 μM all-trans-retinol and in the presence or absence of 10 μM triacsin C. 11-cis-Retinol production values are expressed as percentages of RPE65 without ACSL1 + vehicle (DMSO) control (displayed as means ± standard deviations; n = 6).

FIGURE 3. The ACSL inhibitor triacsin C inhibits RPE65 retinol isomerase activity. 11-cis-Retinol production in HEK-293F cells co-transfected with pVitro2 vector co-expressing RPE65 and CRALBP and pVitro3 vector co-expressing LRAT and ACSL1 and incubated with 2.5 μM all-trans-retinol and in the presence of ACSL1 + vehicle (DMSO) control (displayed as means ± standard deviations; n = 6).

FIGURE 4. Triacsin C inhibits RPE65 retinol isomerase activity. A, biphasic mode of inhibition of RPE65 by triacsin C over 4 orders of magnitude concentrations (displayed as means ± standard deviations; n = 4). B, triacsin C inhibits LRAT activity with an IC₅₀ of 500 nM (displayed as means ± standard deviations; n ≥ 5). C, triacsin C is a competitive inhibitor of RPE65. IC₅₀ values for triacsin C increase as pro-substrate concentration increases, implying a competitive mode of inhibition. 11-cis-Retinol production in cells incubated with 0.79, 2.5, or 7.9 μM all-trans-retinol and in the presence of 0–2000 nM triacsin C (displayed as means ± standard deviations; n ≥ 4). All experiments measure 11-cis-retinol production in HEK-293F cell minimal in vitro visual cycle system incubated with all-trans-retinol (concentration(s) as denoted) and in the presence of triacsin C (concentrations as denoted). 11-cis-Retinol production values are expressed as percentages of vehicle (DMSO) control.
TABLE 1
Accumulation of retinol in HEK293-F cells treated with triacsin C

| TriC (nM) | Total retinol isomers from saponification of extracted retinoids from cells |
|----------|--------------------------------------------------|
|          | 0.79 μM | 2.5 μM | 7.9 μM |
| 0 nM     | 2.6 ± 0.3 | 6.6 ± 0.9 | 13.5 ± 2.4 |
| 125 nM   | 2.7 ± 0.2 | 7.5 ± 0.6 | 13.7 ± 2.4 |
| 250 nM   | 2.6 ± 0.2 | 6.7 ± 1.0 | 13.8 ± 3.0 |
| 500 nM   | 2.6 ± 0.1 | 7.0 ± 1.4 | 13.3 ± 2.6 |
| 1000 nM  | ND² | 6.8 ± 0.3 | 14.0 ± 1.5 |
| 2000 nM  | ND² | 6.4 ± 0.9 | ND² |

² ND, not done; means ± standard deviations (n = 4).

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We observed that the ties, whereas 2-FPA inhibits sphingosine biosynthesis and arachidonic acid uptake, and of arachidonyl and ACSL activities.

Chicken RPE65 activity was 136.2 ± 7.4%; n = 8. (Although it has been reported that chicken RPE65 has severalfold higher specific activity than mammalian RPE65s (26), we have not found that to be the case in these experiments.) Liposomes containing 0.5, 0.75, or 1 μM all-trans-retinyl palmitate were presented to the HEK293-F/chicken RPE65 membranes in the presence of different concentrations (0–2 μM) of triacsin C. We found that, in common with the prior experiments using all-trans-retinol, that increasing substrate concentration gave rise to increasing IC₅₀ (Fig. 5), at a value close to that seen for all-trans-retinol. When the data were graphed on a Cornish-Bowden plot (27), it yielded roughly parallel lines (Fig. 5, inset), indicating a competitive mode of inhibition, as with the in cellular experiments.

Inhibition of RPE65 by EDYA and 2-FPA—To determine whether there was a common mechanism of RPE65 inhibition by inhibitors of ACSLs, we tested the diacetylenic fatty acid 20:2Δ8,11a (8,11-eicosadienoic acid, EDYA; Fig. 1B), and 2-fluoropalmitic acid (2-FPA; Fig. 1C). EDYA is an inhibitor of arachidonic acid uptake, and of arachidonoyl and ACSL activities, whereas 2-FPA inhibits sphingosine biosynthesis and ACSLs. We observed that the in cellular IC₅₀ of EDYA is ~30 μM, using a substrate concentration of 2.5 μM all-trans-retinol (Fig. 6A), whereas that for 2-FPA under the same conditions is ~70 μM (Fig. 6B). The IC₅₀ for EDYA is thus more than an order of magnitude higher than that observed for triacsin C, and that of 2-FPA is ~2 orders of magnitude higher. Interestingly, at concentrations of 10 μM, or lower, EDYA modestly enhanced 11-cis-retinol production, perhaps by more selectively inhibiting a competing enzyme activity (possibly ACSLs). This was not observed for 2-FPA, nor, parenthetically, for triacsin C. We conclude that neither EDYA nor 2-FPA are either selective or potent inhibitor of RPE65, and no further assessment of EDYA and 2-FPA (inhibition mode, etc.) was made.

Docking Models of Inhibitors—To visualize how triacsin C, EDYA, and 2-FPA may be interacting with the substrate-binding cleft of RPE65, we performed docking simulations using AutoDock Vina (Fig. 7). In general, the ligands tend to dock in the central region of the RPE65 substrate-binding cleft over the iron atom. No docking of either inhibitor was seen in the pocket above Phe-418 in these simulations. Docking of 2-FPA is not shown but resembles that of EDYA. Docking of triacsin C was seen to occur in either orientation with similar affinity, but that of the depicted orientation was slightly higher. Residues Thr-147 and Phe-418 are denoted as stick figures on the cartoon to mark the orientation of the cleft. 11-cis-Retinyl palmitate is included in the cleft model for reference and docks as previously found (8). We found that no substantial difference was seen in the docking positions or affinities of the ligands tested in the cap versus the cleft series (Table 2). These models indicate that interaction of triacsin C with the RPE65 binding site is consistent with that of the acyl moiety of 11-cis-retinyl palmitate and consistent with the presence in triacsin C of an alkenyl moiety that is somewhat analogous to that of an unsaturated fatty acyl chain or a retinyl chain. Comparison of docking of the two tautomers of triacsin C (Fig. 1A) indicates that their affinities are similar, with that of the depicted (Fig. 7) nitrosohydrazone tautomer being slightly higher. Similarly, docking of EDYA and 2-FPA are consistent with their acyl nature. It must be noted that AutoDock Vina does not recognize charges on the iron but only takes account of the atomic size, so that any possible interactions of the ligands to the iron that may be specifically dependent on its charge (ferrous versus ferric) will not be considered by the program. Thus, putting a charge on the iron when making the .pdbqt file of the protein has no effect.

Discussion

Identification of small molecule inhibitors of RPE65 retinol isomerase enzyme activity yields insights into the mechanism of RPE65 and also provides lead compounds for potential therapeutic use. Already several different families of inhibitors have been identified, each illuminating important aspects of the catalytic mechanism, including retinylamines (28), the non-reti-
noid emixustat and its derivatives (6, 12), and lipophilic aromatic spin traps (10). In this paper, we show that triacsin C, well known to be a specific inhibitor of long chain ACSLs, is also a potent inhibitor of RPE65 retinol isomerase. Because this compound has an alkenyl chain resembling, but not identical to, either acyl or retinyl chains, it might compete with binding of the acyl moiety of the all-trans-retinyl ester substrate of RPE65 via the alkenyl moiety, but be inhibitory via its nitrosohydrazone/triazene moiety. The majority of RPE65 inhibitors are thought to interfere with the interaction of the retinyl moiety with RPE65, and thus triacsin C appears to be the first compound that may act, at least in part, via competition with all-trans-retinyl ester acyl moiety binding.

Triacsin C is one of four related compounds (triacsins A, B, C, and D) originally isolated from culture media of Streptomyces sp. strain SK-1894 (15, 29, 30). All are 11-carbon alkenyl-1-hydroxytriazenes (Fig. 1A) and are the only known naturally occurring triazene-containing compounds. Triacsin C is the only one of the four that is readily commercially available. Originally, triacsin C and D were identified as vasodilators (29, 30), but subsequently all were found to inhibit ACSLs, with triacsin A and C being the most potent (16). This inhibition was found to be competitive with respect to the fatty acid but noncompetitive with respect to CoA and ATP (16). In contrast, triacsin C does not inhibit FATP fatty acyl-CoA synthetases belonging to the SLC27A solute carrier transporter protein family (19, 30). Parenthetically, this provides a convenient discriminatory tool between the two classes of acyl-CoA ligases. Triacsin C strongly inhibits both microsomal and mitochondrial ACSLs (with activity toward palmitate or longer acyl chains) but inhibits medium chain (with activity toward hexanoate, etc.) and short chain (with activity toward propionate and butyrate) poorly or not at all, respectively (18). Triacsin C has been suggested to be of potential therapeutic use. However, there are no human clinical trials known to have been conducted or to be underway. Because triacsin C inhibits ACSLs, suppressing synthesis of triglycerides and cholesteryl esters and thereby suppressing lipid droplet formation, it has been suggested to be a potential anti-atherogenic agent and, to this end, has been tested in a mouse model of atherosclerosis (31). Also, triacsin C and analogs of triacsin C have been suggested to be of possible utility as antivirals against rotavirus by suppressing cellular triglyceride levels, crucial for rotavirus replication; this has only been inves-
tigated in cell culture (32). Potential use of triacsin C as therapy for these or other human pathologies would need to take account of its inhibitory effect toward RPE65 that might result in night blindness as a side effect. Conversely, local use of triacsin C as a visual cycle inhibitor might have adverse effects toward important ACSL-mediated roles in the eye.

Acetylenic fatty acids are known to inhibit prostaglandin synthesis by their inhibition of cyclooxygenases (33). However, diacetylenic fatty acids may also inhibit eicosanoid synthesis by impeding arachidonate uptake by cells and inhibiting arachidonic acid activation by arachidonate-specific and non-arachidonate-specific ACSLs (20). Nothing is known about their possible effects on SLC27A/FATP fatty acyl-CoA synthetases. Our data show that the level of inhibition of RPE65 by EDYA is \(\sim 2\) orders of magnitude less than that of triacsin C or an order of magnitude greater than substrate concentration, so we do not consider it to be a particularly potent inhibitor of RPE65. Given its apparently weak stimulatory effect at lower concentrations and an inhibitory effect at higher concentrations, it is not a specific inhibitor of RPE65 either. Similar to the situation for EDYA, 2-FPA (21) inhibits only at far higher concentrations than triacsin C does. Also, nothing is known about the possible effects of 2-FPA on SLC27A/FATP fatty acyl-CoA synthetases.

To properly determine the mode of inhibition of triacsin C, especially because it is an alkanyl compound that might be capable of competing with acyl compounds, it was necessary to directly evaluate its effect using all-trans-retinyl palmitate as substrate rather than the pro-substrate all-trans-retinol. There was a modest difference between the \(IC_{50}\) values calculated for triacsin C for all-trans-retinol and all-trans-retinyl palmitate substrates. It is most likely that the difference is accounted for by the methodological differences between the two assays employed: the one an in cellulo assay and the other a liposome-based in vitro assay. In addition, the in cellulo assay employed dog RPE65, whereas the liposome assay used the higher activity chicken RPE65. Our results show a direct competition of triacsin C toward the all-trans-retinyl palmitate substrate. The substrate range and triacsin C concentration used were necessarily restricted because higher concentrations of these components were found to destabilize the liposomes.

In conclusion, we show that triacsin C, a known inhibitor of ACSLs, inhibits RPE65. Although the major player in its mode of inhibition is likely its nitrosohydrazone/triazene moiety, its alkanyl moiety may facilitate its binding and interfere with specific interactions of the acyl moiety of the retinyl ester substrate in the substrate-binding cleft of RPE65. This advances the notion that the retinyl ester acyl moiety plays some role in substrate docking in the process of RPE65 catalysis. The most recent crystal structure of RPE65 reveals that the palmitate product of the concerted retinyl moiety isomerization and O-alkyl cleavage of all-trans-retinyl palmitate tends to remain longer, liganded to the iron center, than the 11-cis-retinol product (6). This fact and our current findings suggest that a better understanding of the interactions of RPE65 with the acyl moiety (substrate and product) is warranted.

**Author Contributions**—All authors contributed to study design. A. E. and T. M. R. conducted most of the experiments, analyzed most of the results, and wrote most of the paper. E. P. conducted some of the experiments, analyzed some of the results, and wrote some of the paper. S. G. analyzed some of the results, performed modeling and docking analyses and wrote some of the paper. All authors reviewed and approved the manuscript.

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