Published in final edited form as:

Exp Eye Res. 2008 February ; 86(2): 344–354. doi:10.1016/j.exer.2007.11.006.

RETINOID PROCESSING IN CONE AND MÜLLER CELL LINES

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Abstract

To determine whether cones and Müller cells in the rod dominated retina, cooperate to regenerate the 11-*cis* retinal chromophore via the retinoid cycle, two cell lines from the rod dominated retinas of Murine were used for this study, 661W a mouse cell line derived from cones and rMC-1, a rat Müller cell line. Retinoid cycle enzymes were analyzed by RT-PCR and their catalytic activity were detected by incubation with retinoids and analyzed by HPLC. We found that, 661W cells are capable of reducing all-trans retinal to all-trans retinol due to the presence of multiple dehydrogenases and generate minor amounts of retinyl-ester. The rMC-1 cells take up all-trans retinol and oxidizes it to all-trans retinal or esterify it to retinyl-ester, but are incapable of isomerizing all-trans retinoids to 11-*cis* retinoids. This could be a reflection of lack of necessary activities in Müller cells *in vivo* which suggests that Müller cells do not contribute to retinoid cycling by regenerating 11-*cis* retinoids. Alternatively, this could be due to the potential that rMC-1, as a transformed cell line, has stopped expressing the proteins needed for the regeneration of 11-*cis* retinoids.

Keywords

cone photoreceptors; retinoids; retinoid recycling; retinal cell culture

INTRODUCTION

Light stimulation of retinal photoreceptors isomerizes the chromophore, 11-*cis* retinal to all-*trans* retinal (ATR). The resulting ATR is released from opsin and recycled to 11-*cis* retinal through the biochemical pathway called the retinoid cycle. The first step in this pathway is the reduction of ATR to all-trans retinol (ATol) by the photoreceptor NADPH-dependent retinol dehydrogenases (RDHs) (Palczewski, Jager, Buczylo, Crouch, Bredberg, Hofmann, sson-Batres and Saari, 1994). In rod photoreceptors, ATol is released from the cell and delivered to

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the retinal pigment epithelium (RPE) where the enzyme lecithin: retinol acyl transferase (LRAT) converts it to retinyl-ester (Saari, Bredberg and Farrell, 1993). RPE65 (an isomerohydrolase enzyme) converts the ester to 11-*cis* retinol (Moiseyev, Chen, Takahashi, Wu and Ma, 2005). The 11-*cis* retinol is oxidized to 11-*cis* retinal in the RPE by NAD-dependent RDHs (Jang, Van Hooser, Kuksa, McBee, He, Janssen, Driessen and Palczewski, 2001). 11-*cis* retinal is then delivered to the rod photoreceptors to regenerate a functional pigment. Therefore, aside from the conversion of ATR to ATol, the retinoid cycle for rods is mostly carried out in the RPE (McBee, Palczewski, Baehr and Pepperberg, 2001).

An alternative visual cycle has been proposed for cone photoreceptors in the cone-dominated retinas of chicken and squirrel (Mata, Radu, Clemmons and Travis, 2002; Mata, Ruiz, Radu, Bui and Travis, 2005; Trevino, Villazana-Espinoza, Muniz and Tsin, 2005). As in rods, light causes the isomerization of 11-*cis* retinal to ATR, which is then dissociated from cone opsin, and reduced to ATol by NADPH-dependent RDHs and released to the outside of the cell. However, instead of delivery to the RPE, ATol is proposed to be taken up by the Müller cells where it is isomerized to 11-*cis* retinol by a novel enzyme called all-trans retinol isomerase. 11-*cis* retinol is then released by the Müller cells and delivered to the cone photoreceptor where it is oxidized to 11-*cis* retinal by a cone-specific NADP-dependent RDH (Mata, Radu, Clemmons and Travis, 2002; Mata, Ruiz, Radu, Bui and Travis, 2005; Trevino, Villazana-Espinoza, Muniz and Tsin, 2005). 11-*cis* retinol can also be esterified to 11-*cis* retinyl ester by acyl CoA retinol acyltransferase (ARAT) and stored in Müller cells (Mata, Ruiz, Radu, Bui and Travis, 2005).

Studies have suggested that the retinoid recycling in cones in the rod dominated retina may differ from that of their counterparts in the cone dominated retina. For example, there is no prescribed role for RPE65 in the retinoid cycle for cones in the cone dominated retina. However, knockout of this gene in the rod dominated retina of the mouse causes complete loss of cone function (Znoiko, Rohrer, Lu, Lohr, Crouch and Ma, 2005). Furthermore, it has also been shown that knocking out RPE65 in the 'all cone' *Nrl*^{-/-} retina and in the 'cone only' retina of the *Rho*^{-/-} mouse, causes cones to be functionless (Seeliger, Grimm, Stahlberg, Friedburg, Jaissle, Zrenner, Guo, Reme, Humphries, Hofmann, Biel, Fariss, Redmond and Wenzel, 2001; Wenzel, von, Oberhauser, Tanimoto, Grimm and Seeliger, 2007). This loss of function cannot be explained by the cone retinoid cycle as described for the cone dominated retina. An alternative pathway, dependent on RPE65 and somewhat different from that in ground squirrel and chicken retinas, must exist. Since RPE65 expression is suggested to occur predominantly in the RPE cells (Hemati, Feathers, Chrispell, Reed, Carlson and Thompson, 2005; Seeliger, Grimm, Stahlberg, Friedburg, Jaissle, Zrenner, Guo, Reme, Humphries, Hofmann, Biel, Fariss, Redmond and Wenzel, 2001), it is possible to assume that the cones require the RPE for the completion of their retinoid cycle.

Studies on cone retinoid recycling in rod dominated retinas are hampered by the low number of cones (3–5%). For this purpose, we used two cell lines generated from rod dominated retinas: the 661W cell line, derived from a mouse retinal tumor and characterized as a cone specific cell line (Al-Ubaidi, Font, Quiambao, Keener, Liou, Overbeek and Baehr, 1992; Tan, Ding, Saadi, Agarwal, Naash and Al-Ubaidi, 2004), and the Müller cell line rMC-1, a rat derived cell line expressing GFAP and CRALBP, both markers for Müller cells (Sarthy, Brodjian, Dutt, Kennedy, French and Crabb, 1998).

We characterized the proteins involved in the retinoid cycle in both cells lines to determine if they complement each other to conclude the retinoid cycle. We show that 661W cells express five out of six photoreceptor NADPH-dependent RDHs (RDH11, RDH13, RDH14, RDH12 and the cone-specific retSDR1, but not RDH8) and can convert ATR to ATol. Although a small

portion of ATol is esterified, this esterification is not due to the presence of lecithin:retinol acyltransferase (LRAT).

We also show that the Müller cell line rMC-1 can convert ATol to ATR, 13-*cis* retinol, and retinyl-ester. Our characterization of the 2 cell lines shows that Müller cell line rMC-1 cannot recycle retinoids. This may be reflective of its true function *in-vivo* or that these transformed cell lines have stopped expressing the proteins needed for the regeneration of 11-*cis* retinoids.

MATERIALS AND METHODS

Animals

Nrl^{-/-} (a kind gift from Dr. Anand Swaroop at Kellogg Eye Center, Ann Arbor, Michigan) and C57BL/6J mice were maintained in a 12 dark: 12 light cycle with average luminance of 10–20 lux using fluorescent cool-white 40-watt lamps. All experiments involving animals were approved by the local Institutional Animal Care and Use Committees, and conformed to the guidelines of the National Institute of Health Guide for the Care and Use of Laboratory Animals and the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research.

Culture Conditions

The 661W cells were grown in DMEM media (Gibco, Carlsbad, California) supplemented with 10% FBS (Cellgro, Herndon, VA) and 1% antimycotic-antibiotic (Invitrogen) (Tan, Ding, Saadi, Agarwal, Naash and Al-Ubaidi, 2004). The Müller cell line rMC-1 was grown in DMEM media with 10% FBS, 2mM Glutamine and 1% antimycotic-antibiotic (Sarthy, Brodjian, Dutt, Kennedy, French and Crabb, 1998). All cultures were maintained in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

Uptake of retinoids into cells

661W or rMC-1 cells were incubated, for 4 hours in the dark, in media containing 10 µM of either 9-*cis* retinal (Sigma-Aldrich, St. Louis, MO), ATR (Sigma), ATol (Sigma) or 11-*cis* retinol (kind gift from Dr. Rosalie Crouch). All retinoids were dissolved in DMSO and then dispersed in the media. Afterwards, the cells were washed three times in Hank's salt solution, media devoid of retinoids was added, and cells were either exposed to either 15,000 or 30,000 Lux of light for 30 minutes or kept in the dark for the same duration and harvested to extract intracellular retinoids.

Light Source

The light box (Model MS1417-C-DIM-T83) was obtained from Aristo Lighting Technologies (Roslyn, NY). Three phosphors are used in manufacturing the light source in this model. They peak at 453 nm (red), 546 nm (blue), and 611 nm (green). UV light was blocked by a Plexiglas filter. To maintain temperature, the light box was fitted with two small fans.

HPLC analysis of retinoids

661W or rMC-1 cells were collected with a scraper, washed with PBS, and centrifuged at 500 × g for 5 min. The cell pellet was resuspended in 300 µl 50 mM MOPS in 50% ethanol and sonicated 2 times for 20 seconds each. Then, 20 µl of 1M hydroxylamine (in 0.2 M sodium phosphate buffer, pH 7.4) was added, samples were vortexed for 30 s and allowed to stand for 10 min at room temperature. The samples were then extracted twice with 600 µl of hexane. After centrifugation (16,000 × g for 5 min) the upper phases were collected and dried under argon. Samples were dissolved in HPLC mobile phase (85.4/11.2/2/1.4 of hexane/ethyl acetate/dioxane/octanol) and retinoids were separated by using either a Lichrosphere SI-60 (Alltech

Associates, Deerfield, IL) 5- μ m column of size 250 $\times$ 4.6 mm or 150 $\times$ 4.6 mm with a flow rate of 1 ml/min. The retinal peaks were identified by their retention time in comparison with pure retinoid isomeric standards and their corresponding absorption spectra. All procedures were performed under dim red light (Kodak filter GBX-2).

In our column, it is not possible to separate the *-cis* and *-trans* isomers of retinyl-esters and therefore it is possible for the two esters to come as one peak.

Quantitative RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen). Genomic DNA contamination was prevented by treatment of the samples with RQ1 RNase free DNase (Promega, Madison WI) and verified by the absence of amplification in samples that were not reverse transcribed to cDNAs.

Reverse transcriptase reaction was performed using SuperScript III reverse transcriptase (Invitrogen). PCR primers were designed to the cDNA of RDHs and BLAST searches verified that these sequences were unique to the target RDH.

RT-PCR primers:

RDH8:	Forward primer AGGTCATTGCCAAGGTCATC Reverse primer GACCAAGGTTGAGGAGGTGA
RDH13:	Forward primer TGGTCCCTCCTTGTGAGTTC Reverse primer GTGTGGCCGACCTGTAATCT
RDH11:	Forward primer CCCTAACCAGGAGCATGAAA Reverse primer AGATGTTGGGACCACAAAGC
RetSDR1:	Forward primer CGAGAGAAGGTGGGTGACAT Reverse primer CAATATGGCCGTTCTGGAGT
RDH12:	Forward primer AGAATCTTCGGGAACCTGT Reverse primer TGTCCCTGTGAGCGTGTAAG
RDH14:	Forward primer GGCCACTTCCTACTCACCAA Reverse primer AGGATGCAGCACATTGACAG
HPRT:	Forward primer GGACCTCTCGAAGTGTGGAT Reverse primer GGACGCAGCAACTGACATT
LRAT1:	Forward primer GCAGTTGGGACTGACTCCAT Reverse primer CAGATTGCAGGAAGGGTCAT
RGR1:	Forward primer GGAACCTTGAGGTGCTGGCTA Reverse primer TGTCCCATGCCAACTGTCTA
RGR2:	Forward primer GGCTATCATCTGCCTTCTGG Reverse primer GGCAGGCACCATCTGTAGTT

Quantitative RT-PCR was performed on the cDNA in triplicates, using SYBR green (Invitrogen) and run on a real-time PCR detection system (iCycler; Bio-Rad Laboratories). The Δ cT values were calculated against the neuronal housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT), since it is commonly used in analysis of retinal gene expression (Zacks, Han, Zeng and Swaroop, 2006). HPRT was assigned an arbitrary expression level of 10,000, and relative gene expression values were calculated by the following equation: relative expression = $10,000/2^{\Delta$ cT, where Δ cT = (gene cT–Hprt cT) then plotted as relative expression of the gene of interest to HPRT.

Preparation of microsomal fraction

Cells were washed in PBS, resuspended in a sucrose buffer containing 0.25 M sucrose, 10 mM Tris-HCl, pH 7.2, 1 mM EDTA, and protease inhibitors (2.0 µg/ml aprotinin, 5 µg/ml pepstatin A, 10 µg/ml leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride, final concentrations), and disrupted using a Polytron homogenizer (Kinematica Inc. Newark, NJ). Cell homogenates were centrifuged at $800 \times g$ for 15 min at 4°C. The resulting supernatant was then centrifuged at $100,000 \times g$ for 30 min at 4°C, and microsomal pellets were resuspended in storage buffer containing 50 mM Tris-HCl, pH 7.2, 1 mM EDTA, 20% glycerol and the protease inhibitor mixture described above. Microsomal fractions were stored at -80°C after snap freezing in liquid nitrogen. Protein quantification was done by the Bradford Protein Assay Kit (Pierce Biotechnology, Rockford, IL).

Determination of RDH activity

Assays with ATR and ATol were carried out in 1 ml reaction buffer (100 mM Tris-HCl at pH 7.2, 200 mM NaCl, 1 mM dithiothreitol, 2 mg/ml bovine serum albumin, 1% glycerol, and 150 µM of the indicated coenzyme) containing 0 or 10 µg of proteins from the microsomal fraction, and varying amounts of substrate. After 4 hours incubation at room temperature, reactions were terminated by the addition of 1 volume of cold methanol. Retinoids were then extracted with 2 volumes of hexane, dried under argon and resuspended in mobile phase for HPLC analysis. Retinoids were quantified by comparing their peak areas to calibration curves of standards. Background values obtained in the absence of microsomal proteins were subtracted from measured values.

Detection of ARAT activity

250 µg of 661W microsomes or Bovine RPE microsomes were incubated in a buffer containing 10 mM BTP (bis-tris propane), 0.1M NaCl and 1% BSA at pH 8, either alone or with 10 µM ATol (with or without 100 µM Palmitoyl CoA) and left at 37°C for 1 hour. The retinoids were then extracted with 2 volumes of hexane and analyzed by HPLC as described above. The ester synthesized in Bovine microsomes without the addition of ATol, was subtracted from the ester synthesized in the presence of exogenous ATol (with or without Palmitoyl CoA) to calculate the ester synthesized from the exogenous ATol. For the radioactive detection method, radioactive ATol was purchased (PerkinElmer, Waltham, MA) and samples were incubated at 37°C overnight.

Photoisomerization assays

For the photoisomerization assays, the protocol described by Hao et al. (Hao and Fong, 1996) was used with the following modifications. rMC-1 cells were incubated with 10 µM ATR for 4 hours in the dark. One set of samples were then placed on a light box at 15,000 Lux (3 lumen/cm²/9 mW/cm²) for 10 minutes at room temperature and another set kept in the dark for the same time. Cells were then thoroughly washed and lysed and HPLC analysis of retinoids was done. Absorption maxima of the photopigment RGR opsin is 469 ± 2.4 nm, and our light box emits light at this wavelength and peaks at 453 nm.

RESULTS

Conversion of ATR to ATol and retinyl ester in 661W cells

Normally, the 661W cells lack the chromophore (Figure 1A) but when the cells are incubated in 10 µM 9-*cis* retinal, they internalize it (peak 1 and 2, Figure 1B). The choice of this specific concentration of the chromophore is based upon previous findings, where concentrations of 9-*cis* retinal up to 13 µM were determined to lack cytotoxic effects in the dark (Kanan, Moiseyev, Agarwal, Ma and Al-Ubaidi, 2007). Following internalization, and light treatment for 30

minutes, 661W cells converted 9-*cis* retinal to ATR (peaks 3 and 4, Figure 1C). When cells were left overnight in the dark following light treatment, ATR was converted to ATol (peak 7, Figure 1D) and then to retinyl-ester (peak 5, Figure 1D). No 11-*cis*-retinol was detected in the HPLC analysis, however we identified peak 6 in Figure 1D to be 13-*cis* retinol due its absorption maxima at 328 nm, which corresponds to the absorption maxima of 13-*cis* retinol. We have previously shown the absence of RPE65 protein in this cell line, (Tan, Ding, Saadi, Agarwal, Naash and Al-Ubaidi, 2004), The above two independent observations confirm the absence of RPE65 in this cell line. This observation is consistent with recent findings that RPE65 is not expressed in cone photoreceptors (Hemati, Feathers, Chrispell, Reed, Carlson and Thompson, 2005; Seeliger, Grimm, Stahlberg, Friedburg, Jaissle, Zrenner, Guo, Reme, Humphries, Hofmann, Biel, Fariss, Redmond and Wenzel, 2001; Wenzel, von, Oberhauser, Tanimoto, Grimm and Seeliger, 2007).

To identify the retinol dehydrogenases (RDHs) responsible for the conversion of ATR to ATol in 661W cells, quantitative RT-PCR was performed using primers against six mouse homologues for all 6 known human photoreceptor dehydrogenases because mouse specific antibodies do not exist for all 6 photoreceptor dehydrogenases. The presence of five dehydrogenases, RDH11, RDH12, RDH13, RDH14 and retSDR1 (Figure 2A) was confirmed by RT-PCR in 661W cells, but RDH8 was not present. RDH11, RDH13 and RDH14 are expressed to greater extents than retSDR1 and RDH12 (Figure 2A).

In agreement with our findings in the 661W cells, the *Nrl*^{-/-} retina expresses RDH11, RDH13, RDH14 and retSDR, but RDH8 expression was barely detectable (Figure 2B). The only difference is the expression of RDH12; it is high in *Nrl*^{-/-} retina and low in 661W cells. This could potentially be due to expression of RDH12 in other retinal cell types. Dehydrogenases RDH13 and 14 are the highest expressed transcripts and are comparably expressed in both models. RetSDR1, a dehydrogenase that has cone specific location (Haeseleer, Huang, Lebioda, Saari and Palczewski, 1998), is expressed to greater levels in 661W cells and *Nrl*^{-/-} retina compared to WT retina (Figure 2A, B & C). Overall, it is clear that 661W cell resembles the ‘all cone’ *Nrl*^{-/-} retina more than the rod dominated C57BL/6J retina.

RDH8 is detected, albeit at comparatively low levels, in the *Nrl*^{-/-} retina but not in the 661W cells (Figure 2A & B). RDH8 has been previously shown to be expressed in the outer segments of rod and cone photoreceptors in the rod dominated retina (Maeda, Maeda, Imanishi, Kuksa, Alekseev, Bronson, Zhang, Zhu, Sun, Saperstein, Rieke, Baehr and Palczewski, 2005). However, according to the q-RT-PCR results in the cone dominated *Nrl*^{-/-} retina, it is barely expressed in cones compared to rods (Figure 2B & C) and not expressed in 661W cells (Figure 2A). RDH12 has been shown to be expressed in rod photoreceptor inner segments (Maeda, Maeda, Imanishi, Sun, Jastrzebska, Hatala, Winkens, Hofmann, Janssen, Baehr, Driessen and Palczewski, 2006), and has a higher expression levels in wild type retina than in the *Nrl*^{-/-} retina, confirming that it is preferentially expressed in rods.

Dehydrogenases in the 661W cells act as reductases rather than oxidases

Retinol dehydrogenases are theoretically assumed to act, as both reductases and oxidases, depending on the relative amounts of oxidized or reduced substrate and coenzyme available. However, many of the dehydrogenases are unidirectional *in vivo*. We analyzed the endogenous RDH activity in the 661W cells *in vitro*, using microsomal fractions from 661W cells (Figure 3A). We tested the following 4 reactions: reduction of 10 μM ATR in the presence of 150 μM NADPH, oxidation of 10 μM ATol in the presence of 150 μM NADP, and the same reactions with 150 μM NADH and NAD, respectively. As shown in Figure 3A, the overwhelmingly preferred activity displayed by the endogenous RDHs in 661W cells is the reduction of ATR in the presence of NADPH. Although the reverse reaction is also catalyzed but with at least 10-fold lower efficiency. Our findings are in agreement with previously published reports

whereby RDH11, RDH12, RDH14 and RetSDR1 are shown to specifically prefer the phosphorylated coenzyme, and catalyze the reduction over the oxidation reaction (Haeseleer, Huang, Lebioda, Saari and Palczewski, 1998; Rattner, Smallwood and Nathans, 2000). RDH13 has been shown before to completely lack catalytic activity towards retinoids (Haeseleer, Jang, Imanishi, Driessen, Matsumura, Nelson and Palczewski, 2002) therefore it is not expected to contribute to the total RDH activity measured here. Our results in Figure 3A also show a low NAD/NADH-dependent activity suggesting either the presence of other and unknown RDHs in the microsomal fraction of 661W cells or that the existing RDHs can also use NAD and NADH but at a much lower efficiency than NADP/NADPH. We then characterized the preferred RDH activity in 661W cells, varying the protein concentration, and the incubation time of the reaction (not shown). Figure 3B shows the substrate-saturation curve using 10 μ g protein per reaction and 4 hours incubation. The apparent K_m value for ATR is 3.3 μ M, which is consistent with previously published studies reporting a K_m value for RDH11 in the micromolar range, 1.3 μ M (Kasus-Jacobi, Ou, Bashmakov, Shelton, Richardson, Goldstein and Brown, 2003). However, since the K_m value represents the substrate concentration when half the active sites are occupied and since there are multiple dehydrogenases in the 661W cells, the apparent K_m value presented here represents the average of all K_m values for all RDHs present with the influence of their relative abundances at the saturating substrate concentrations used. The average K_m value points to the fact that the affinity of each individual RDH towards ATR is different, suggesting that some of these RDHs may utilize products other than ATR as their primary substrates.

The apparent V_{max} is ~ 140 pmol/min/mg protein which is about two orders of magnitude lower than the V_{max} obtained with microsomal preparations from mouse retina and tested in the same conditions (not shown). Since the V_{max} is the rate at which a product is formed at saturating substrate concentration, and since the apparent V_{max} for RDHs in 661W cells is lower than that for retinal microsomal fractions, it is safe to assume that some of the RDHs in 661W utilize substrates other than ATR more efficiently. Alternatively, the lower apparent V_{max} can be the result of lower RDHs protein levels in 661W.

Lack of a Retinyl-ester synthase in 661W

Figure 1D shows that exposing 661W cells to light and then incubating in the dark overnight led to the conversion of ATR to ATol and ester. To determine the identity of the enzyme responsible for the ester formation, RT-PCR was performed using primer pairs against the sequence for lecithin:retinol acyltransferase (LRAT) (Zolfaghari and Ross, 2000). While a product for LRAT is clearly detected in RT reactions from 1-month old mouse RPE (Figure 4), no amplification product for LRAT was observed from RNA obtained from 661W cells. Amplification of HPRT from cDNA from 661W cells shows that the cDNA was of good quality and conclusively proves that 661W cells do not express LRAT.

We also investigated the possibility that the ester synthase activity is due to the enzyme ARAT. This experiment cannot be done *in-vivo* as in Figure 1 because palmitoyl CoA does not enter cells, which is why we chose to do the experiment *in-vitro* with 661W microsomes using Bovine RPE microsomes as positive control according to the protocol by Muniz et al. (Muniz, Villazana-Espinoza, Thackeray and Tsin, 2006).

About 250 μ g of either 661W or Bovine RPE microsomes were incubated for 1 hour in buffer alone or buffer containing 10 μ M ATol in the presence or absence of 100 μ M palmitoyl CoA. 661W microsomes do not contain endogenous retinyl-esters (Figure 1A) while Bovine RPE microsomes contain limited amounts of endogenous retinyl-esters (peak 1, Supplementary Figure 1A). Ester formed in the presence of exogenously added ATol (with and without palmitoyl CoA) in Bovine RPE microsomes (peak 1, Supplementary Figure 1 B & C) was subtracted from amount of ester formed in Bovine RPE microsomes incubated in the buffer

alone (peak 1, Supplementary Figure 1A) and we calculated an average increase of 40% retinyl-ester in the presence of palmitoyl CoA for 1 hour in Bovine RPE microsomes from 2 independent experiments. However we could not detect ester in 661W microsomes or whole cell lysates even after 4 hours after the addition of exogenous ATol (data not shown).

To increase sensitivity of this method, we used radioactive ATol and were able to detect low levels of retinyl-ester in 661W microsomal fraction (peak 1, Figure 4B & C). However this ester synthesis was not stimulated with the addition of palmitoyl CoA, which suggests the absence of ARAT in the 661W cell line. Alternatively, the absence of ester synthase activity in 661W cells microsomal fractions or whole cell lysates could be due to the destruction of the activity during the preparation of samples.

Uptake and metabolism of ATol in rMC-1

Since 661W cells make ATol, and it constitutes the majority of the retinoid that is exported out of the photoreceptor cells *in vivo*, it became necessary to determine whether Müller cells are capable of uptake and conversion of ATol. Müller cells (rMC-1) were incubated in presence of ATol in media (16 hours) and retinoid profile analysis revealed the presence of relatively small amounts of 13-*cis* retinol and ester (Figures 5B), with 13-*cis* retinol also observed in control samples (Figure 5A).

Since living cells cannot uptake the cofactors from media, total cell lysates were incubated with 10 μ M ATol and either of the 2 cofactors, NAD or NADP (150 μ M) for 8 hours at room temperature. The retinoid profile analysis revealed the presence of ATR and 13-*cis* retinol (Figures 6C & E). ATR is absent in the reactions that do not contain rMC-1 cell lysates (Figures 6B & D) suggesting that the reaction is enzyme driven. 13-*cis* retinol is present in the reactions that do not contain rMC-1 cell lysates, albeit at smaller amounts than in the reactions containing rMC-1 cell lysates (Figures 6B & D or 6C & E). Furthermore, both NAD (Figure 6C) and NADP (Figure 6E) were utilized as cofactors for this reaction. The small amount of ATR formed from ATol is likely due to RDH10 activity in these cells, as has been previously shown (Wu, Moiseyev, Chen, Rohrer, Crouch and Ma, 2004).

The presence of ester synthesis in living cells but not in whole cell lysates, may suggests that some critical factor responsible for ester synthesis is destroyed when cells are homogenized to create whole cell lysates.

The lack of isomerization to 11-*cis* retinol suggests that the unknown all-*trans* retinol isomerase described in the Müller cells of cone dominated retinas (Mata, Radu, Clemmons and Travis, 2002; Mata, Ruiz, Radu, Bui and Travis, 2005; Trevino, Villazana-Espinoza, Muniz and Tsin, 2005) is not present in rMC-1 Müller cell line. However, we see some enzyme-driven isomerization to 13-*cis* retinol, as confirmed by its absorption spectra. Peak 1 has an absorption maxima at 328 nm which is the absorption maxima of 13-*cis* retinol, while 11-*cis* retinol although elutes close to 13-*cis* retinol, has an absorption maxima at 318 nm. Hence this peak was identified as is 13-*cis* retinol rather than 11-*cis* retinol.

The significance of this isomerization is unknown, but 13-*cis* retinoids have been detected at various levels in retinas of human, mouse, and other species. Particularly, high levels of 13-*cis* retinoic acid (13-*cis* RA) were found in mouse retina (McCaffrery, Posch, Napoli, Gudas and Drager, 1993). The functions of 13-*cis* RA are not fully understood, but it is proposed to mediate retinal development (McCaffrery, Posch, Napoli, Gudas and Drager, 1993) and light-adaptive effects in cone horizontal cells (Pottet and Weiler, 2000). The functional significance of 13-*cis* retinol production in Müller cells could be to provide this isomer for the synthesis of 13-*cis* RA.

Absence of a photoisomerase in rMC-1

Since rMC-1 cells can synthesize ATR from ATol, we asked the question if this ATR is further converted to 11-*cis* retinal in the presence of light. It has been proposed that, Müller cells may possess the photoisomerase RGR which has the ability to convert ATR to 11-*cis* retinal in a light dependent fashion (Jiang, Pandey and Fong, 1993) (Tao, Shen, Pandey, Hao, Rich and Fong, 1998). It has been previously shown by immunohistochemistry that RGR is expressed in Müller and RPE cells in both bovine and human retinas (Jiang, Pandey and Fong, 1993) but not in Müller cells in the mouse retina (Tao, Shen, Pandey, Hao, Rich and Fong, 1998). Therefore, if indeed the Müller cell line, rMC-1, expresses the photoisomerase RGR, it would be capable of generating 11-*cis* retinal by light irradiation of ATR generated from RDH10, to serve as substrate for RGR. Since it is uncertain if rat Müller cells express the photoisomerase RGR, we looked for its presence in rMC-1 either by RT-PCR or enzyme activity as indicated by HPLC.

To verify whether RGR is expressed in rMC-1, we designed 2 sets of primer pairs (RGR1 forward and reverse and RGR2 forward and reverse) in exons that span multiple introns. The logic behind the use of intron flanking primers to RGR is because two splice variants of RGR exist (Lin, Kochounian, Moore, Lee, Rao and Fong, 2007). One splice variant has a deletion of exon 6 and a second variant results from the use of an alternative splice site in the second intron.

Primer RGR1forward was made in exon 1 and primer RGR1reverse was made in exon 4. There are 3 introns between these exons of sizes 2 kb between exons 1 and 2, 1 kb between exons 2 and 3, and a 3 kb intron between exons 3 and exon 4. Primer RGR2forward was designed in exon 4 and RGR2reverse sequence was split between exons 6 and 7. There is a 2 kb intron between exons 4 and 5, a 4.8 kb intron between exons 5 and 6 and a 0.4 kb intron sequence between exons 6 and 7 kb. Both primer pairs amplified products of about 350 bp from cDNA templates obtained from RNA from rat retinaless-eyecups (Figure 7A). The absence of RGR sequence in rMC-1 cells and its presence in rat RPE cells, demonstrate that RGR is not expressed in rMC-1. To verify if rMC-1 cells have photoisomerase activity that is not RGR dependent, rMC-1 cells were incubated with 10 μ M ATR for 4 hours in the dark, then one set of cells were exposed to light for 10 minutes at 15,000 Lux. A second set of cells were kept in the dark for the same time. Analysis of retinoids revealed the presence of 11-*cis* retinal in the cells and media exposed to light (peak 5, Figure 7C & D), but not in the cells kept in the dark (Figure 7B). This suggests that the photoisomerization observed under light conditions is non enzymatic in nature. The lack of any photoisomerase activity suggests that rMC-1 cells are incapable of converting ATR to 11-*cis* retinal in the presence of light.

DISCUSSION

The cone retinoid cycle in a cone dominated retina requires the cone photoreceptor and Müller cells to complete the cycle (Mata, Radu, Clemmons and Travis, 2002; Mata, Ruiz, Radu, Bui and Travis, 2005; Trevino, Villazana-Espinoza, Muniz and Tsin, 2005). However there is doubt whether this cycle is used by cones in the rod dominated retina. The uncertainty arises since according to the cone dominated retina model there is no prescribed role for RPE65 protein in the cycle. However when this gene is knocked out in the wild type retina, in the all cone the *Nrl*^{-/-} retina, or in the 'cone only' retina of the *Rho*^{-/-} mouse, the cones are functionless (Seeliger, Grimm, Stahlberg, Friedburg, Jaissle, Zrenner, Guo, Reme, Humphries, Hofmann, Biel, Fariss, Redmond and Wenzel, 2001; Wenzel, von, Oberhauser, Tanimoto, Grimm and Seeliger, 2007). This suggests that the cones in a rod dominated retina use an alternative pathway from that in the cone dominated retinas of ground squirrel and chicken. To investigate the role of Müller cells in the cone retinoid cycle in the rod dominated retina, we utilized the

661W cells, a cone cell line derived from mouse rod dominated retina, and rMC-1, a Müller cell line derived from rat rod dominated retina.

The data presented demonstrate that 661W cells can reduce ATR to ATol through a battery of RDHs providing further evidence to the cone-like nature of these cells with respect to the identity of the RDHs expressed. Data presented also show that 661W cells can esterify ATol to retinyl-ester, however this activity is independent of the enzyme LRAT and ARAT and may be catalyzed by an unknown synthase. The retinyl ester synthase activity of the 661W cells is limited suggesting that most if not all of the ATol is exported out of the cones, similarly to rod photoreceptors.

We found that the Müller cell line rMC-1 can take up ATol but does not isomerize it to 11-*cis* retinol. These cells however can convert a small portion of ATol to ATR by endogenous RDH10, but cannot further isomerize it to 11-*cis* retinal and the cells do not express the photoisomerase RGR. Furthermore, we investigated whether either cell type can generate 11-*cis* retinal from 11-*cis* retinol, especially since rMC-1 expresses CRALBP, a protein crucial for the generation of 11-*cis* retinal (Sarthy, Brodjian, Dutt, Kennedy, French and Crabb, 1998). We also found out that both cell lines are incapable of synthesizing 11-*cis* retinal and do not utilize the 11-*cis* retinol at all (Supplemental Figure 2).

From our *in-vitro* studies, the Müller cell line, rMC-1 does not provide the necessary enzymatic activity to finish the retinoid cycle as proposed for the cone-dominated retina. This could be reflective of *in-vivo* function of these cells or that this cell line had lost expression of certain proteins needed for the retinoid cycle. If the former is true then it is safe to propose that in the rod dominated retina, the cones convert ATR to ATol and Müller cells can convert ATol to ATR but the cycle cannot be completed because neither cell type can isomerize all-*trans* retinoids to 11-*cis* retinoids. Hence, we propose that cones, like rods, depend on the RPE to re-generate their chromophore. This proposal is supported by the findings that RPE65 is predominately expressed in RPE and not in Müller cells. The absence of cone function in *Rpe65*^{-/-} mouse models and in human patients with RPE65 mutations (Felius, et al. 2002) strongly suggest that the retinoid cycle as described for the cone dominated retinas of chicken and squirrel is not applicable to the cone retinoid recycling in the rod dominated retina.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The project described was supported by grants from the National Center For Research Resources (P20RR017703) and the National Eye Institute (P30EY12190 and R01EY EY14052 (MRA) and R01EY012231 and EY015650 (JXM)). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center For Research Resources, the National Eye Institute, or the National Institutes of Health. Further support was provided by the Knights Templar Eye Foundation, Inc. (YK).

The authors are grateful to Drs. Anand Swaroop (Kellogg Eye Center, Ann Arbor, Michigan) for providing the *Nrl*^{-/-} mice and Dr. Rosalie Crouch (Medical University of South Carolina, Charleston, South Carolina) for providing 11-*cis* retinol.

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Figure 1.

HPLC analysis of retinoids extracted from 661W cell. (A) Endogenous retinoids in 661W cells. (B) 661W cells incubated with 9-*cis* retinal in the dark for 4 hours. (C) 661W cells incubated with 9-*cis* retinal in the dark for 4 hours, media replaced and then exposed to 30,000 lux light for 30 minutes. (D) 661W cells incubated with 9-*cis* retinal in the dark for 4 hours, media replaced and then exposed to 30,000 lux light for 30 minutes and then left in the dark overnight (16 hours). Identity of the peaks is as follows: 1. syn-9-*cis* retinal oxime; 2. anti-9-*cis* retinal oxime; 3. syn-all *trans* retinal oxime; 4. anti-all-*trans* retinal oxime; 5. retinyl-ester; 6. 13-*cis* retinol; 7. all-*trans* retinol (AToL)

Figure 2. Quantitative real time RT-PCR (qRT-PCR) for photoreceptor dehydrogenases. (A) in 661W; (B) in P30 *Nrl*^{-/-} retina; (C) in P30 C57BL/6J mice. Data was plotted using the software Prism (GraphPad Software). Each value was determined from triplicates on at least two different samples.

Figure 3.

Dehydrogenase activity in 661W cells. **(A)** Microsomal fractions were prepared from 661W cells and reactions were set up with 10 μ g microsomal protein in presence of 10 μ M indicated substrate and 150 μ M coenzyme. Two independent microsomal fractions were tested and for each fraction, the experiment was run in triplicate. **(B)** The same microsomal fractions were used in presence of increasing concentrations of ATR and 150 μ M NADPH. Data presented was obtained from one experiment.

Figure 4.

Analysis of ester synthase in 661W cells. **(A)** RT-PCR for LRAT and HPRT from 661W and P30 C57BL/6J mouse RPE. PCR was performed in duplicate and RNA not reverse transcribed (RT-) controls were used to exclude genomic DNA contamination. **(B)** HPLC analysis of retinoids extracted from 661W microsomes after overnight incubation with radioactive 10 μ M ATol in the absence of 100 μ M palmitoyl CoA. Retinyl ester standard is overlaid on the graph (gray trace). **(C)** HPLC analysis of retinoids extracted from 661W microsomes after overnight incubation with radioactive 10 μ M ATol in the presence of 100 μ M palmitoyl CoA. Retinyl ester standard is overlaid on the graph (gray trace). Identity of the peaks is as follows: 1. Retinyl-ester; 2. Unknown peak; 3. ATR; 4. all-*trans* retinoic acid; 5. 13-*cis* retinol; 6. Unknown peak; 7.

7. ATol. The experiments were performed twice with similar results. Only one representative result is shown.

Figure 5. HPLC analysis of retinoids extracted from rMC-1 cells after incubation with 10 μ M ATol. **(A)** Retinoid analysis of media alone incubated in presence of 10 μ M ATol. **(B)** Retinoid analysis of rMC-1 cells incubated with 10 μ M ATol. The experiment was performed two times and since results were similar, a representative is shown.

Figure 6.

HPLC analysis of retinoids extracted from rMC-1 total cell lysate after incubation overnight at 37°C (~16 hours) with 10 μ M ATol and 100 μ M NAD or NADP. **(A)** Endogenous retinoid profile of rMC-1 cells. **(B)** Buffer alone incubated overnight with 10 μ M ATol and 100 μ M NAD. **(C)** rMC-1 total cell lysate (250 μ g) incubated with 10 μ M ATol and 100 μ M NAD. **(D)** Buffer incubated overnight with 10 μ M ATol and 100 μ M NADP. **(E)** rMC-1 total cell lysate (250 μ g) incubated with 10 μ M ATol and 100 μ M NADP. The experiment was performed twice and only a representative of the similar results is shown. Identity of the peaks is as follows: 1. 13-*cis* retinol; 2. all-*trans* retinol; 3. syn-all-*trans* retinal oxime; 4. syn-9-*cis* retinal oxime.

Figure 7.

Expression of photoisomerase in rMC-1 cells. **(A)** RT-PCR amplification was performed on cDNA reverse transcribed from total RNA extracted from rMC-1 cells and P60 rat retinaless-eye cups (2 eye cups) by using two RGR primers (RGR1 and RGR2). HPRT was also amplified from the two sources as a control for the quality of cDNA. PCR was performed in duplicates for each primer. **(B)** HPLC analysis of retinoids extracted from rMC-1 cells after incubation with 10 μ M ATR for 4 hours in the dark **(C)** rMC-1 cells incubated with 10 μ M ATR for 4 hours in the dark followed by 10 minutes at 15,000 lux. **(D)** Media incubated with 10 μ M ATR for 4 hours in the dark followed by 10 minutes at 15,000 lux. Identity of peaks are as follows:

1. syn-all-*trans* retinal oxime; 2. syn-9-*cis* retinal oxime; 3. anti-13-*cis* retinal oxime; 4. anti-all-*trans* retinal oxime; 5. syn-11-*cis* retinal oxime.