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Characterization of a Dehydrogenase Activity Responsible for Oxidation of 11-*cis*-Retinol in the Retinal Pigment Epithelium of Mice with a Disrupted *RDH5* Gene:

A MODEL FOR THE HUMAN HEREDITARY DISEASE FUNDUS ALBIPUNCTATUS*

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Abstract

In the vertebrate retina, the final step of visual chromophore production is the oxidation of 11-*cis*-retinol to 11-*cis*-retinal. This reaction is catalyzed by 11-*cis*-retinol dehydrogenases (11-*cis*-RDHs), prior to the chromophore rejoining with the visual pigment apo-proteins. The *RDH5* gene encodes a dehydrogenase that is responsible for the majority of RDH activity. In humans, mutations in this gene are associated with fundus albipunctatus, a disease expressed by delayed dark adaptation of both cones and rods. In this report, an animal model for this disease, *11-cis-rdh*−/− mice, was used to investigate the flow of retinoids after a bleach, and microsomal membranes from the retinal pigment epithelium of these mice were employed to characterize remaining enzymatic activities oxidizing 11-*cis*-retinol. Lack of 11-*cis*-RDH leads to an accumulation of *cis*-retinoids, particularly 13-*cis*-isomers. The analysis of *11-cis-rdh*−/− mice showed that the RDH(s) responsible for the production of 11-*cis*-retinal displays NADP-dependent specificity toward 9-*cis*- and 11-*cis*-retinal but not 13-*cis*-retinal. The lack of 13-*cis*-RDH activity could be a reason why 13-*cis*-isomers accumulate in the retinal pigment epithelium of *11-cis-rdh*−/− mice. Furthermore, our results provide detailed characterization of a mouse model for the human disease fundus albipunctatus and emphasize the importance of 11-*cis*-RDH in keeping the balance between different components of the retinoid cycle.

The production of 11-*cis*-retinal allows the regeneration of visual pigments in the vertebrate retina (1), making it critical for sustaining vision. One pathway involved in enzymatic production of 11-*cis*-retinal, the retinoid cycle, is still not well understood (2). What is known is that in this cycle, photoisomerized all-*trans*-retinal is reduced to all-*trans*-retinol by an NADPH-specific all-*trans*-retinol dehydrogenase (all-*trans*-RDH).[1] After transport of all-

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trans-retinol from the rod outer segments (ROS) to the retinal pigment epithelium (RPE), isomerization to 11-*cis*-retinol occurs through either a mechanism termed isomerohydrolase (3), a carbocation intermediate (4), or an as yet unknown mechanism (reviewed in Ref. 2). In the last step, the 11-*cis*-retinol product is oxidized to 11-*cis*-retinal in a reaction catalyzed by 11-*cis*-RDH (Scheme 1) (5).

11-*cis*-RDH activity was reported to be membrane-associated (6–8), and the main enzyme responsible for this reaction (11-*cis*-RDH encoded by the *RDH5* gene) was isolated by coimmunoprecipitation with RPE65, an abundant RPE protein (9), believed to be essential for isomerization of all-*trans*-retinol to 11-*cis*-retinol (10,11). Independently, 11-*cis*-RDH has been cloned using antibodies against RPE proteins by Driessen *et al.* (12). 11-*cis*-RDH has been identified as a member of the short-chain alcohol dehydrogenase family. Those enzymes are involved in redox reactions of such hydrophobic substances as steroids, retinoids, and prostaglandins (13,14). Members of this family are characterized by molecular mass (~300 amino acids), a co-factor binding site (G*XXX*G*X*G), and a substrate binding site (Y*XXX*K) (13). They are often found as a dimer or tetramer and usually contain hydrophobic N-terminal tails thought to be involved in membrane anchoring. 11-*cis*-RDH is largely found associated with smooth endoplasmic reticulum, although some amount has also been found in the rough endoplasmic reticulum (15–17). The stereospecificity of 11-*cis*-RDH has been shown to be *pro-S*-specific to both 11-*cis*-retinol and NADH (18) and has the highest specificity for 11-*cis*-retinol, followed by 13-*cis*-retinol and 9-*cis*-retinol (18,19).

However, the question of additional functions of this enzyme in the retinoid cycle has recently been raised. Mutations in the *RDH5* gene have been found in patients with fundus albipunctatus, a disease characterized by profound delayed dark adaptation of cone and rod cells after a strong bleach (20). Despite some of these mutations in the *RDH5* gene resulting in inactive enzyme, patients harboring null mutations ultimately recover their visual pigments. Therefore, 11-*cis*-retinal must be produced by alternative dehydrogenase-catalyzed oxidation of 11-*cis*-retinol that compensates for the loss of RDH5 (18,21,22). In accord with these results, transgenic mice missing the *RDH5* gene also display delayed dark adaptation, but only at very high bleach levels (23). The molecular characterization of this other dehydrogenase(s), however, has not yet been carried out. A remarkable phenotype of *11-cis-rdh*−/− mice is the accumulation of 11-*cis*-retinol/13-*cis*-retinol and 11-*cis*-retinyl/13-*cis*-retinyl esters. This has led to the suggestion that 11-*cis*-RDH could also be important in eliminating 13-*cis*-retinoids from the RPE (23). Furthermore, the *RDH5* gene has recently been found widely expressed in non-ocular tissues (24–27). Due to this wide expression, it has been hypothesized to catalyze the oxidation of 9-*cis*-retinol in tissues in which 11-*cis*-retinoids are not present. It is also worth noting that the expression levels are much lower in these tissues compared with the RPE, yet 11-*cis*-RDH has not been detected by immunocytochemistry in cells of the retina other than RPE using both polyclonal (22) and monoclonal[2] antibodies. This observation, in conjunction with the fundus albipunctatus phenotype, suggests that the 11-*cis*-RDH present in the RPE is involved in the production of 11-*cis*-retinal for both rod and cone cells. 11-*cis*-RDH could be also important for the photochemical production of 11-*cis*-retinal from all-*trans*-retinal by the retinal G protein-coupled receptor (RGR) of RPE and Müller cells (28,29). Recently, Fong and co-workers (30) have shown that 11-*cis*-RDH co-purifies with RGR. The possible role 11-*cis*-RDH plays in this complex could be the reduction of 11-*cis*-retinal to 11-*cis*-retinol, which is stored in a form of 11-*cis*-retinyl esters.

[1]The abbreviations used are: RDH, retinol dehydrogenase; CRALBP, cellular retinaldehyde-binding protein; CRBP, cellular retinol-binding protein; HPLC, high pressure liquid chromatography; MES, 4-morpholineethanesulfonic acid; MOPS, 3-(*N*-morpholino) propanesulfonic acid; RGR, retinal G protein-coupled receptor; ROS, rod outer segments; RPE, retinal pigment epithelium.

In this study, we investigated the effects of age and light conditions on 11-*cis*- and 13-*cis*-retinoid accumulations in *11-cis-rdh*−/− mice. We then evaluated the flow of retinoids in the eyes of these mice after an intense bleach. Isolated RPE membranes from *11-cis-rdh*−/− mice were used to investigate the presence of an alternative oxidation enzyme(s), stereospecificity and isomeric retinoid preference, and the effect of the lack of 11-*cis*-RDH on the isomerization reaction. These studies revealed potential mechanisms by which 13-*cis*-retinol is produced and provide an extensive characterization of an alternative dehydrogenase enzyme responsible for 11-*cis*-retinal production.

MATERIALS AND METHODS

11-cis-rdh Mice

All animal experiments were approved by the University of Washington Animal Care Committee and conformed to the recommendations made by the American Veterinary Medical Association Panel on Euthanasia. The animals were maintained under a 24-h dark and/or 12-h light-dark cycle. All manipulations were performed under dim red light employing a Kodak No. 1 safelight filter (transmittance, >560 nm). *11-cis-rdh*−/− mice were derived from a 129/Sv embryonic stem cell line generated by homologous recombination with a targeting vector that eliminated exons I, II, and III (23). Chimeric male mice were mated with C57BL/6 females and maintained as a recombinant inbred line. Progeny of matings were genotyped by RDH5-specific primers and polymerase chain reaction (23). Typically, 1–2-month-old mice were used in all experimental procedures. All procedures were performed as described previously (31).

Retinoid Extraction and Analysis

All procedures were performed under dim red light as described previously (11,23,31,32). In addition to previously described methods, retinoid analysis was performed on an HP1100 liquid chromatograph equipped with a diode array detector and HP Chemstation (A.07.01) software, allowing identification of retinoid isomers according to their specific retention times and absorption maxima. A normal-phase column (Beckman Ultrasphere Si 5 μ, 4.6 × 250 mm) and an isocratic solvent system of 0.5% ethyl acetate in hexane (v/v) for 15 min followed by 4% ethyl acetate in hexane for 60 min at a flow rate of 1.4 ml/min (total, 75 min) with detection at 325 nm allowed the separation of 11-*cis*-retinyl esters, 13-*cis*-retinyl esters, and all-*trans*-retinyl esters at 20 °C.

Preparation of Retinyl Palmitates

Retinols (1 μmol) were dissolved in dry dichloromethane (dried and distilled over CaH_2) (5 ml), and 3 μmol of diisopropylethylamine and 2 μmol of palmitoyl chloride (Aldrich/Sigma) were added to the reaction mixture, which was kept on ice for 24 h with occasional shaking. The reaction mixture was washed with 3 × 5 ml of water, 5 ml of saturated Na_2CO_3, and was dried with anhydrous $MgSO_4$. Organic solvent was dried under a stream of argon, and esters were dissolved in hexane and purified employing HPLC.

Preparation of Mouse RPE Microsomes

Fresh mouse eyes were enucleated immediately after either cervical dislocation or CO_2 asphyxiation. Typically 30–40 eyes were dissected for each preparation. The anterior segment, vitreous, and retina were carefully removed by microdissection. RPE cells were separated by placing 12 dissected eyecups in 400 μl of 10 mM MOPS, pH 7.0, 250 mM sucrose, 1 mM dithiothreitol, and 1 μM leupeptin with vigorous shaking for 20 min. The eyecups were then gently brushed with a fine brush to further dislodge RPE cells. The cell suspension was removed, another aliquot of 400 μl of MOPS buffer was added, and the eyecups were shaken again for 20 min at room temperature. The cell suspensions were combined and subjected to

glass-glass homogenization. The homogenate was centrifuged at 10,000 × *g* for 10 min, and then the supernatant was centrifuged at 275,000 × *g* for 1 h. The pellet was reconstituted in 200 μl of 10 mM MOPS, pH 7.0, 1 mM dithiothreitol, and 1 μM leupeptin and homogenized (glass-glass) again. The protein concentration (typically 0.5–1 mg/ml) was determined using the Bradford method (33).

Preparation of Pro-S-[4-^{3}H]NADH, Pro-R-[4-^{3}H]NADPH, and Pro-S-[4-^{3}H]NADPH

Syntheses of *pro-S*-[4-^{3}H]NADH and *pro-S*-[4-^{3}H]NADPH were carried out with L-glutamic dehydrogenase (Sigma), NAD, and NADP (Sigma), respectively, and L-[2,3-^{3}H]glutamic acid (PerkinElmer Life Sciences) as described previously (22). Synthesis of *pro-R*-[4-^{3}H]NADPH was prepared from [4-^{3}H]NADP and L-glutamic acid, employing L-glutamic dehydrogenase (18).

Preparation of Pro-R-[4-^{3}H]NADH

The preparation of *pro-R*-[4-^{3}H]NADH was accomplished by utilizing the *pro-R*-specific yeast alcohol dehydrogenase (Sigma) to reduce NAD with [1-^{3}H]EtOH (ARC). Yeast alcohol dehydrogenase (1.5 mg in 1.2 ml of 25 mM bis-tris-propane, pH 8.8, 0.1% bovine serum albumin) was mixed with NAD (14 μmol in 70 μl of H_2O) and [1-^{3}H]EtOH (100 mCi/mmol, 0.25 mCi in 250 3l of H_2O), and the mixture was incubated at room temperature for 45 in. The reaction was stopped by addition of 1.5 ml of MeOH, and the resulting mixture was dried in a Speedvac. The residue was dissolved in 700 μl of H_2O, flash-frozen with liquid nitrogen, and again dried in the peedvac. This was repeated once more to ensure the complete removal of residual [1-^{3}H]EtOH. The product was purified on a Mono Q HR 5/5 column (Amersham Pharmacia Biotech) equilibrated with 10 mM bis-tris-propane, pH 7.3, using a linear gradient of 0–500 mM NaCl over 60 min at a flow rate of 1 ml/min. Concentrations of NADH and NADPH (pH 7.4) were determined using ε = 6220 at 340 nm, and concentrations of NAD and NADP (pH 7.4) were determined using ε = 18,000 at 260 nm (34). [4-^{3}H]NADH and [4-^{3}H] NADPH (100–250 μM) were stored at −20 or −80 °C in their original elution buffers.

RDH Assays for Mouse RPE Microsomes

Activities of RDH in RPE microsomes of *11-cis-rdh*+/+ and *11-cis-rdh*−/− mice were assayed by monitoring the production of [15-^{3}H]retinol (reduction of retinal) (18). The reduction reaction mixture (100 μl) contained MES (final concentrations, 62–66 mM, pH 5.5), dithiothreitol (1 mM), *pro-R* (or *pro-S*)-[4-^{3}H]NADH (21 μM) or *pro-R* (or *pro-S*)-[4-^{3}H] NADPH (21 μM), *11-cis-rdh*+/+ (2.2–3.8 μg) or *11-cis-rdh*−/− (3.2–5.4 μg) mice RPE microsomes in the presence or absence of NAD(P)H (420 μM), and 2 μl of retinal (132 μM) substrate stock was added last to initiate the reaction. The reaction was incubated at 34–37 °C for 50 min and then terminated with 400 μl of methanol, 100 μl of 1 M NaCl and extracted with 500 μl of hexane. Radioactivity was measured in 400 μl of the organic phase by scintillation counting. In some cases, 400 μl of the upper phase was removed and dried down by a stream of argon or by Speedvac. The residue was dissolved in 120 μl of hexane, and 100 μl of the hexane solution was analyzed by HPLC using 10% ethyl acetate in hexane to confirm the formation of the corresponding retinol. The retinol fractions were collected and subjected to scintillation counting.

RDH Assays for Bovine RPE Cytosol

Bovine RPE cytosol was prepared as described previously (35). The resulting cytosol was adjusted with $CaCl_2$ and $MgCl_2$ (final concentration of both, 1 mM) and centrifuged at 146,000 × *g* for 50 min. The supernatant was then filtered through a 0.22 μm Millex-GV filter (Millipore), and 30 μl (1.32 μg/μl) of the filtrate was used for RDH assays as described above.

Isomerization of All-trans-retinol to 11-cis-retinol Using Mouse RPE Microsomes

The assay used for determining isomerization to 11-cis-retinol was reported previously (35–37). Briefly, the reaction mixture, containing 1% bovine serum albumin, 30 mM bis-tris-propane, pH 7.5, 1 mM ATP, 15 μM apo-rCRALBP, 40 μl of mouse RPE (typically ~20–40 μg of total protein), and 10 μM all-*trans*-retinol (in dimethylformamide), was incubated for 2 h at 37 °C. The reaction was quenched using 300 μl of MeOH, and retinoids were extracted with 200 μl of hexane. The mixture was shaken vigorously for 2 min and then centrifuged at 14,000 rpm for 4 min for phase separation. The upper organic layer was removed, and an aliquot of 100 μl was analyzed by HPLC using 10% ethyl acetate in hexane.

RESULTS

Separation of Retinoids

To analyze the retinoid flow in *11-cis-rdh* mice, and for the biochemical assays used in this study, retinoids were separated using two chromatographic conditions. In the first system, the concentration of the modifier solvent, ethyl acetate, was initially set at 0.5% in hexane to separate 13-*cis*-, 11-*cis*-, and all-*trans*-retinyl esters and then increased to 4% in hexane (Fig. 1). The separation of 13-*cis*-from 11-*cis*-retinyl esters was particularly challenging, and only partial separation of these esters was accomplished (identified by characteristic UV/visible spectra). 11-*cis*-Retinol and 13-*cis*-retinol could not be separated by employing this system given that both retinols always co-elute, whereas all other retinoids were separated to baseline. Partial separation of 11-*cis*-retinol and 13-*cis*-retinol was accomplished using an isocratic elution system of 10% ethyl acetate in hexane, as discussed below. In addition, all retinoids were identified by co-elution with authentic retinoids and in many cases by mass spectrometry. With this separation technique, we analyzed changes in retinoid concentration *in vivo* using *11-cis-rdh*−/− mice and *11-cis-rdh*+/+ mice as controls.

Retinoids in 11-cis-rdh Mice

Previous analysis of retinoids in *11-cis-rdh*−/− mice revealed a high accumulation of *cis*-retinyl esters as compared with *11-cis-rdh*+/+ mice, whereas *11-cis-rdh*+/− mice showed intermediate characteristics (23). The ester hydrolysis of *cis*-retinyl esters, however, introduced uncontrollable spontaneous isomerization of retinoids. In this study, we used 1–2-month-old animals reared under 24-h dark and/or 12-h light-dark cycle. HPLC separation profiles of *11-cis-rdh*−/− and *11-cis-rdh*+/+ mice are presented in Fig. 2. In the eyes of *11-cis-rdh*−/− mice, retinyl ester concentration increased 4.2-fold compared with *11-cis-rdh*+/+ mice. One-month-old *11-cis-rdh*−/− mice contained prominently higher concentrations of 13-*cis*-retinyl esters and 11-*cis*-retinyl esters (Fig. 2). This result was in agreement with previous studies (23). For *11-cis-rdh*+/+ mice, only a minimally elevated absorption was observed in the region that corresponds to *cis*-retinyl esters. Such minimal peaks and the breakthrough of the column raised uncertainty about the origin of this absorption. However, if these peaks are indeed *cis*-retinyl esters, they are in trace amounts compared with all-*trans*-retinyl esters. For further identification, the *cis*-retinyl ester peaks from *11-cis-rdh*−/− mice were collected and chemically isomerized using I_2. This resulted in the decrease in *cis*-retinyl esters and production of all-*trans*-retinyl and 13-*cis*-retinyl esters in our experimental conditions in a ratio of 3:1, respectively (Fig. 2, *inset*). Hence, 15% of the *cis*-retinyl esters in *11-cis-rdh*−/− mice are present as 11-*cis*-retinyl esters. No significant amounts of free retinols were observed in fresh samples, as compared with the previous studies. This could be a result of longer time periods between eye dissection and analysis in the original studies.

The Effect of Light and Age on the Accumulation of cis-Retinyl Esters

The question arose whether the formation of *cis*-retinyl-esters is dependent on age and exposure of eyes to light. Therefore, three groups of *11-cis-rdh*−/− mice were reared under different light regimens. The first group was reared under a 12-h light-dark cycle. HPLC retinoid analysis confirmed that under these conditions, *11-cis-rdh*−/− mice stored their esters predominantly in the *cis*-retinyl conformation (Fig. 3*A*). Retinoid analysis of the second group, reared under 24-h dark, also showed the presence of retinyl esters predominantly in the *cis*-retinyl conformation. The third group, reared under 24-h dark, but exposed to seven light flashes (each bleaching ~40% of rhodopsin) and subsequently dark-adapted for 12 h also had a high concentration of *cis*-retinyl esters present in the posterior pole of the eye. Representative chromatograms are presented in Fig. 3*A*. No differences were observed compared with control *11-cis-rdh*+/+ mice reared under 24-h dark and/or a 12-h light-dark cycle (results not shown). Hence, exposure to light does not influence the retinyl ester conformation in *11-cis-rdh*−/− mice.

To investigate the effect of age on the accumulation of *cis*-retinyl esters, 2-week-old, 3-month-old (Fig. 3*B*), and 6-month-old mice were tested. For 2-week-old *11-cis-rdh*−/− mice, ~300 pmol of rhodopsin was produced, compared with 500–600 pmol for adult mice. No 11-*cis*-retinyl ester and very low amounts of all-*trans*-retinyl ester were observed, whereas 13-*cis*-retinyl ester was very prominent. For the 2-month-old and older mice, an increase in total amount of esters was apparent; however, a similar ratio between 13-*cis*-, 11-*cis*-, and all-*trans*-retinyl esters was observed (discussed below). Because these measurements were taken at one time point, it was important to look at the flow of retinoids immediately after intense bleaching.

Kinetics of the Retinoid Flow in 11-cis-rdh Mice Post-bleach

To test the flow of retinoids after an intense bleach, ~1-month-old *11-cis-rdh*−/− and *11-cis-rdh*+/+ mice were used. Mice were dark-adapted for 12 h and exposed to a flash that bleached ~40% of their visual pigment. Immediately after the flash, mice were transferred to the dark and sacrificed at different times; their eyes were then enucleated. The amount of 11-*cis*-retinal decreased from ~400 pmol/eye to ~250 pmol/eye immediately after the flash, whereas the amount of all-*trans*-retinal increased from 5 to 150 pmol/eye (Fig. 4, *e* and *a*, respectively). The rate of reduction of all-*trans*-retinal in ROS of *11-cis-rdh*−/− mice was identical to that in ROS of *11-cis-rdh*+/+ mice, and statistically, no differences were observed in free all-*trans*-retinol (Fig. 4). Retinyl esters were significantly elevated (2–3-fold) in *11-cis-rdh*−/− mice, with the most noticeable difference being a higher concentration of *cis*-retinyl esters (~10% in 11-*cis*-configuration, ~60% in 13-*cis*-configuration, and the rest as all-*trans*-retinyl esters) (Fig. 5). It appears that the higher concentration of esters could be accounted for by extra *cis*-esters, compared with *11-cis-rdh*+/+ mice, the ester concentrations of which did not significantly fluctuate during dark adaptation after intense flash. Only slightly higher concentrations of *cis*-retinols were observed in *11-cis-rdh*−/− mice as compared with *11-cis-rdh*+/+ mice after the bleaching and subsequent dark recovery, indicating that an alternative 11-*cis*-RDH is less efficient in oxidizing 11-*cis*-retinol to 11-*cis*-retinal. Most importantly, the recovery of 11-*cis*-retinal was indistinguishable between *11-cis-rdh*−/− mice and *11-cis-rdh*+/+ mice (Fig. 4). These results suggest that other enzymes, in addition to 11-*cis*-RDH, are responsible for the oxidation of 11-*cis*-retinol. Further characterization of this activity was necessary.

Stereospecificity and Isomeric Preference of an Alternative RDH Activity Present in RPE Microsomes of 11-cis-rdh−/− Mice

Mouse RPE microsomes from *11-cis-rdh*−/− mice were used to establish isomeric preference and specificity of an alternative enzyme activity responsible for the production of 11-*cis*-

retinal. Because the redox reactions are readily reversible, we used the reduction of retinals to retinols with radioactive dinucleotides in our assays, employing phase partition to separate the radioactive product (*e.g.* reduction of 11-*cis*-retinal to [15-^{3}H]11-*cis*-retinol) from the excess of radioactive dinucleotide substrate (38). The assay was independently verified by HPLC separation of retinoid substrates and products. Studies have shown that 11-*cis*-RDH has a higher affinity for 11-*cis*-retinal and 13-*cis*-retinal than for 9-*cis*-retinal and all-*trans*-retinal (18). In stereospecific assays, 11-*cis*-RDH uses *pro-S*-[4-^{3}H]NADH to yield *pro-S*-[15-^{3}H]11-*cis*-retinol; hence, 11-*cis*-RDH displays dual *pro-S* stereospecificity. We tested *pro-S*-[4-^{3}H] NADPH nucleotide substrate. For both *11-cis-rdh*−/− and *11-cis-rdh*+/+ mice, no differences were found when *pro-S*-[4-^{3}H]NADPH and all-*trans*-retinal in the presence or absence of excess NADH was used. Incubation with *pro-S*-[4-^{3}H]NADPH and all-*trans*-retinal in the absence of excess NADH resulted in 2.1 and 4.6 nmol/min/mg [^{3}H]retinol, whereas in the presence of excess NADH, no inhibition of [^{3}H]retinol production was found (Fig. 6). This could result from undefined amounts of ROS membrane contamination containing *pro-S*-NADPH-specific all-*trans*-RDH (18,39). Highly purified ROS membranes do not have *cis*-RDH activity, so all measured *cis*-RDH activity in our microsomal RPE fractions was derived from the RPE. Incubation of RPE protein fractions of 11-*cis-rdh*−/− mice using *pro-S*-[4-^{3}H] NADPH with 9-*cis*-retinal or 11-*cis*-retinal produced ~0.3 nmol/min/mg [15-^{3}H]9-*cis*-retinol or 0.2 nmol/min/mg [15-^{3}H]11-*cis*-retinol (confirmed by HPLC), respectively. In contrast, incubation of RPE protein fractions of 11-*cis-rdh*−/− mice with *pro-S*-[4-^{3}H]NADPH and 13-*cis*-retinal produced very little (<0.01 nmol/min/mg), if any, [15-^{3}H]13-*cis*-retinol. NADH was a poor substrate for *11-cis-rdh*−/− RPE microsomal proteins, as was determined by direct assay with radioactive [^{3}H]NADH and by lack of inhibition of the [^{3}H]NADPH-dependent reaction with cold excess of NADH (Fig. 6). RPE protein fractions of *11-cis-rdh*−/− mice assayed with *pro-S*-[4-^{3}H]NADH and 9-*cis*-retinal, 11-*cis*-retinal, or 13-*cis*-retinal in the presence or absence of excess NADPH resulted in the formation of <0.1 nmol/min/mg [^{3}H]retinol. Incubating RPE protein fractions of wild-type mice with *pro-S*-[4-^{3}H]NADH and 9-*cis*-retinal, 11-*cis*-retinal, or 13-*cis*-retinal in the absence of excess NADPH, on the contrary, resulted in the formation of 1.2, 1.1, and 1.5 nmol/min/mg [^{3}H]retinol, respectively, whereas with *pro-S*-[4-^{3}H]NADPH, [^{3}H]retinol production was much lower. Therefore, NADH-dependent reduction of 11-*cis*-retinal and 9-*cis*-retinal was significantly decreased in the RPE membranes from *11-cis-rdh*−/− mice.

Incubation of RPE protein fractions of *11-cis-rdh*−/− mice with *pro-R*-[4-^{3}H]NADPH and 9-*cis*-retinal, 11-*cis*-retinal, or 13-*cis*-retinal in the absence of excess NADH resulted in ~0.01 nmol/min/mg [^{3}H]retinol, whereas in the presence of excess NADH, [^{3}H]retinol production seemed to be less. When RPE protein fractions of *11-cis-rdh*+/+ mice were assayed with *pro-R*-[4-^{3}H]NADH and 9-*cis*-retinal, 11-*cis*-retinal, or 13-*cis*-retinal in the presence or absence of excess NADPH, ~0.1 nmol/min/mg [^{3}H]retinol was formed, whereas in *11-cis-rdh*−/− mice only 0.05, 0.013, and 0.03 nmol/min/mg [^{3}H]retinol was obtained. Hence, *pro-R*-NADPH and *pro-R*-NADH activities were >10 times lower (Fig. 7) in both *11-cis-rdh*−/− and *11-cis-rdh*+/+ mice, suggesting that no *pro-R*-specific enzyme is responsible for the alternative 11-*cis*-RDH activity. In conclusion, when using RPE protein fractions from *11-cis-rdh*−/− mice, 11-*cis*-retinal reduction was most efficient using *pro-S*-[4-^{3}H]NADPH, resulting in ~0.2 nmol/min/mg [^{3}H]retinol production, which is less efficient compared with RPE protein fractions of wild-type mice with *pro-S*-[4-^{3}H]NADH and 11-*cis*-retinal, resulting in ~1.1 nmol/min/mg [^{3}H] retinol.

In addition, we tested bovine RPE for possible soluble RDH activity. A bovine soluble RPE protein preparation was used because larger and more concentrated preparations could be obtained. Only very residual activity was found (Fig. 8), which cannot account for the alternative oxidation of retinols.

Isomerization of All-trans-retinol to cis-Retinols in RPE Membranes from 11-cis-rdh −/− Mice

As mentioned in the Introduction, 11-*cis*-RDH was first identified by immunoprecipitation with RPE65, a protein thought to be involved in the isomerization of all-*trans* retinol to 11-*cis*-retinol. This suggested that 11-*cis*-RDH might closely interact with the protein(s) that catalyzes isomerization. It was therefore important to determine whether isomerization is affected in the RPE microsomes of *11-cis-rdh*−/− mice. We adapted an isomerase assay, described previously, (35–37) for bovine RPE, to measure the isomerase activity in mouse RPE microsomes. After isolating RPE microsomes from mice, isomerization was performed in the presence of CRALBP according to previously published procedures (35–37). The results showed that, indeed, isomerization to 11-*cis*-retinol still takes place in the RPE microsomes of *11-cis-rdh*−/− mice; however, the data obtained furthermore suggest that isomerization occurs at a reduced rate in *11-cis-rdh*−/− mice compared with *11-cis-rdh*+/+ mice (Fig. 9).

DISCUSSION

Accumulation of 11-cis-Retinyl Esters in 11-cis-rdh−/− mice

Accumulation of 13-*cis*-retinoids in RPE of *11-cis-rdh*−/− mice is, to our knowledge, the first example of a significant presence of this isomer in the vertebrate system. A low difference in free energy between all-*trans*-retinol and 13-*cis*-retinol (ΔG = ~1 kcal) leads to spontaneous formation of 13-*cis*-retinol, and therefore, trace amounts of 13-*cis*-retinoids are frequently found in all biological samples in which all-*trans*-retinoids are present. In *11-cis-rdh*−/− mice, the phenomenon is different because there is a highly significant accumulation of 13-*cis*-retinyl esters (100 pmol/eye) in the RPE, a much higher level than the trace amounts observed in *11-cis-rdh*+/+ mice (5 pmol) (Fig. 2). *11-cis-rdh*−/− mice also accumulate significant amounts of 11-*cis*-retinyl esters (~30–50 pmol/eye), which are present only in trace amounts in *11-cis-rdh* +/+ mice. This accumulation does not consequently results from light conditions to which these *11-cis-rdh*−/−animals were exposed to (Fig. 3), suggesting that some thermodynamic equilibrium governs this process and that 11-*cis*-retinol is not derived from a default pathway during the regeneration of photoisomerized chromophore but rather from a constitutive activity of RPE in *11-cis-rdh*−/− mice. We could not exclude the possibility that *in vivo* spontaneous isomerization occurs. This could be due to an accumulation of all-*trans*-retinol. However, in another set of experiments, we looked at the accumulation of *cis*-retinoids as a function of age. 13-*cis*-Retinyl esters were present in all tested ages from 14 days to 6 months. Only in very young mice (14 days old) were 11-*cis*-retinyl esters absent (Fig. 3*B, top*). Spontaneous *in vivo* formation of 13-*cis*-retinoids is very unlikely in these animals because only trace amounts of all-*trans*-retinoids are present. Another argument against the idea of spontaneous isomerization is the fact that in mice with a targeted disruption of the RPE65 gene, a huge accumulation of all-*trans*-retinyl esters does not lead to formation of 13-*cis*-retinyl esters *in vivo* and *in vitro* (11). Around 14 days after birth, rhodopsin is produced at a very high rate, as ROSs elongate and 50–75% of the adult rhodopsin content is present (data not shown). It is possible that 11-*cis*-retinyl esters are quickly hydrolyzed and used to regenerate newly synthesized rhodopsin. With aging, when the synthesis of rhodopsin is balanced by phagocytosis, a large fraction of the chromophore bound to opsin is recycled; thus, the demand for *de novo* synthesis of 11-*cis*-retinal is lowered. In adult *11-cis-rdh* mice, the production of 11-*cis*-isomer keeps up with the demand for this isomer, because the rate of rhodopsin regeneration is unaffected in these animals (23) (Fig. 4*e*).

In previous studies, formation of 13-*cis*-retinol was observed in dissected eyes (23). In dark-adapted animals, using freshly dissected eyes, we were not able to observe this retinoid in significant amounts. Therefore, it is possible that 13-*cis*-retinol observed in the previous studies was a result of hydrolysis of the esters during the processing of these samples. Lack of accumulation of *cis*-retinols could be explained by low affinity of retinoid-binding proteins to

retinols as compared with aldehydes (40) and powerful lecithin:retinol acyl transferase activity in RPE (35,37,41). After bleaching, there is a ~30% elevation of *cis*-retinols (Fig. 4*d*), most likely resulting from lowered oxidation capacity of *11-cis-rdh*−/− RPE membranes, before rhodopsin is fully regenerated, retinols are re-esterified, and normal *cis*-retinol concentrations are re-established again.

Utilization of cis-Retinyl Esters

Isomerization of all-*trans*-retinol to 11-*cis*-retinol requires ΔG = ~4 kcal (2). One model concerning the isomerization step in the retinoid cycle assumes that the hydrolysis of retinyl esters provide the excess energy necessary for the isomerization process (42,43). It has been hypothesized that potent inhibition by *cis*-retinols prevents isomerization of all-*trans*-retinyl esters (44). This model is, however, inadequate to explain the formation of *cis*-retinoids in RPE of *11-cis-rdh*−/− mice (Fig. 2); thus, other models that would explain this perturbed isomerization system by lack of 11-*cis*-RDH should be considered.

McBee *et al*. have proposed that light provides the input of energy for both formation of MII and rhodopsin regeneration. The action of light is a two-step process: first, energy (~35 kcal/mol) (reviewed in Ref. 2) is absorbed by the chromophoreopsin complex, allowing consequent changes in the rhodopsin conformation and the hydrolysis of the photoisomerized chromophore all-*trans*-retinal; second, exothermic formation of rhodopsin from opsin and 11-*cis*-retinal is regenerated through the retinoid cycle (~10 kcal/mol). Re-formation of rhodopsin would allow the unfavorable isomerization reaction to overcome the needed ΔG = +4 kcal/mol by moving retinoids from one step of the reaction to another by mass action. Binding of 11-*cis*-retinal to CRALBP would drive the isomerization/oxidation process, providing 8–10 kcal/mol based on the relation between ΔG and K_d. A lack of acceptor for the isomerization process would halt isomerization observed in RPE microsomes (35). This explanation is also consistent with the observation that when CRALBP is replaced with cellular retinol-binding protein, a retinoid-binding protein with different specificities, enzymatic formation of 13-*cis*-retinol was observed (4). In *11-cis-rdh*−/− mice, inefficient oxidation could perturb the isomerization system, leading to accumulation of 13-*cis*-retinoids, as dictated *in vivo* by equilibrium constants between binding proteins, slower oxidation, and lecithin:retinol acyl transferase and hydrolase activities toward different isomers. It is unclear from our studies whether 13-*cis*-retinyl esters could be utilized for the conversion to 11-*cis*-retinol/retinal. Most likely, double isomerization around C_{13} to form the *trans*-configuration around this bond and C_{11} to form the *cis*-configuration around this bond would not be energetically feasible. Therefore, we favor the idea that all-*trans*-retinyl-esters, through an as yet unconfirmed intermediate (a carbocation intermediate has been postulated in the most recent studies (4)), is the substrate for further formation of 11-*cis*-retinol.

Other transgenic animals in which genes encoding proteins thought to play a role in the retinoid cycle are disrupted include CRALBP (45), cellular retinol-binding protein type 1 (CRBPI) (46), ABCR (47), interphotoreceptor retinoid-binding protein (IRBP) (31,48,49), and RPE65 (10) knockout mice (reviewed in Ref. 50). In addition to *11-cis-rdh*−/− mice, ABCR and CRALBP knockout mice were reported to have delayed dark adaptation. Recently, more compelling evidence has emerged indicating that mutations in some of the genes encoding proteins with a function in the retinoid cycle result in a more severe retinal pathology in humans compared with mice. For example, in *abcr*−/− mice that are lacking ABCR (which is believed to function as an outwardly directed flippase for *N*-retinylidene-PE (51,52)), no RPE cell enlargement or degeneration, as is evident in advanced Stargardt's disease, was present. Furthermore, photoreceptor degeneration developed slowly in *abcr*−/− mice. Another example is *cralbp*−/− mice, which show little or no reduction in the number of photoreceptor cells, whereas mutations in the CRALBP gene in humans are found to be associated with autosomal

recessive retinitis pigmentosa and retinitis punctata albescens (53–55). Finally, *11-cis-rdh*−/− mice display normal dark adaptation kinetics at bleaching levels under which patients suffering from fundus albipunctatus display slower dark adaptation. Additionally, in the fundus of *11-cis-rdh*−/− mice, no punctata typical for fundus albipunctatus are present. A question arises as to what causes the more severe outcome for humans. One theory suggests that complete absence of proteins, which is the case in most mouse knockout models, is less deleterious than the continual production of mutant proteins, which can be toxic, as is the case in most human cases. Another explanation might be that, contrary to humans, mice are night animals with eyes adapted to dim light vision in which the compensatory mechanisms may be much more evolved than in humans, resulting in less pronounced retinal pathology. Further research is needed to explore this possibility.

11-cis-rdh−/− Mice: A Model for Human Fundus Albipunctatus

Mutations in the *RDH5* gene in humans have been associated with a night blindness disease called fundus albipunctatus (56). Several mutations have been identified (20–22,57–61), and longer follow-ups of the progress of the disease and more careful electroretinogram testing have showed that the disease is not stationary as originally thought; instead, these studies show a slow deterioration of vision and photoreceptor degeneration (60).

Recently, we extensively studied the consequences of a lack of 11-*cis*-RDH in a family with fundus albipunctatus. An Arg-157 to Trp mutation in the *RDH5* gene that replaces an amino acid residue conserved among short-chain alcohol dehydrogenases causes destabilization of the enzyme and its inactivation. Physiological tests revealed delayed resensitization of rod and, even more profoundly, cone photoreceptor cells following full bleaches. Cones showed a biphasic recovery with an initial rapid component and an elevated final threshold. It is unclear whether this observation is related to a recently reported complex between the photoisomerization protein RGR and 11-*cis*-RDH (30). Under these conditions, RGR could be involved in photoregeneration that support cones, whereas rods are saturated. At lower bleaching levels, normal rod recovery is observed following 0.5% bleaching and abnormal slow recovery following bleaches in the 2–12% range. These intermediate bleaches showed a rapid partial recovery of rods with a transitory plateau. Pathways in addition to 11-*cis*-RDH likely provide 11-*cis*-retinal for both rods and cones and can maintain normal kinetics of visual recovery, but only under certain constraints they are less efficient for cone than rod function (22,62). Additional biochemical assays using specifically [^{3}H]labeled dinucleotides pointed out the existence of an alternative oxidizing system for the production of 11-*cis*-retinal. The *11-cis-rdh*−/− mice are an interesting model of fundus albipunctatus; however, they show some striking differences. The electroretinograms show only a delay in dark adaptation after >50% bleach (23). Unfortunately, no biochemical data are available yet for human donor eyes with this disease to analyze whether there is any abnormal accumulation of retinoids in these patients, as we observed in mice. The most important observation from both animal and human studies on 11-*cis*-RDH defects is that there are alternative mechanisms (enzymes) present that allow production of 11-*cis*-retinal. However, these enzymes have not been yet characterized at the molecular level.

At high bleaching levels, RGR may be one of the alternative pathways playing an important role in production of 11-*cis*-retinal. Because in this reaction all-*trans*-retinal is directly converted to 11-*cis*-retinal, no oxidation step is needed. This route could support an alternative method of 11-*cis*-retinal generation, but only if light is continuously present. In the case of a pulse of illumination, this pathway is not likely to be involved; however, *11-cis-rdh*−/− mice submitted to flash were still able to regenerate rhodopsin. Therefore, another alternative oxidoreductase must be present.

Characterization of a Novel Dehydrogenase Involved in Oxidation of 11-cis-Retinol

cis-RDH activity in *11-cis-rdh*+/+ RPE preparations is *pro-S*-[4-^{3}H]NADH-dependent. This *proS*-[4-^{3}H]NADH dependence and retinoid substrate specificity are also similar to those reported in bovine RPE microsomes, in which 13-*cis*-retinal is best utilized (18). In *11-cis-rdh* −/− RPE microsomes, activities for 9-*cis*-retinal, 11-*cis*-retinal, and 13-*cis*-retinal can also be observed with *pro-S*-[4-^{3}H]NADH, although they are very minor compared with those in 11-*cis-rdh*+/+ animals. In contrast, *11-cis-rdh*−/− mice are clearly able to reduce 9-*cis*-retinal and 11-*cis*-retinal with *pro-S*-[4-^{3}H]NADPH at an increased rate compared with *pro-S*-[4-^{3}H] NADH. However, this is not observed for 13-*cis*-retinal with *pro-S*-[4-^{3}H]NADPH. In addition, there are trace but detectable activities with *pro-R*-[4-^{3}H]NAD(P)H and all four retinal isomers, and no differences in activity can be distinguished between the *11-cis-rdh*−/− and *11-cis-rdh*+/+ animals. These lower activities toward 9-*cis*-retinol and 11-*cis*-retinol activities in 11-*cis-rdh*−/− animals are probably sufficient to maintain the retinal retinoid flow but with less efficiency (22,23). Although bovine RPE cytosol has been carefully prepared by ultracentrifugation in the presence of Mg^{2+} and Ca^{2+} and filtration through a 0.22 μm filter, there is only very residual activity (Fig. 8) detected with *pro-S*-[4-^{3}H]NADH, showing the same retinoid substrate specificity as in RPE microsomes (18). These results suggest that the soluble enzymes are not likely to be responsible for a significant contribution to 11-*cis*-retinol oxidation in the RPE cytosol. All-*trans*-RDH activity is detected in both 11-*cis-rdh*+/+ and 11-*cis-rdh*−/− RPE membranes in a *pro-S*-[4-^{3}H]NADPH-dependent manner, and are most likely a result of ROS contamination during the RPE microsomal preparations (18,63).

As the chromophore regeneration capacity in *11-cis-rdh*−/− mice is only mildly affected, it was rather surprising to find that 11-*cis*-retinol oxidation was reduced at least 5-fold in the RPE of these animals. This could be easily explained if other steps in the retinoid cycle are rate-limiting. Interestingly, reduction of all-*trans*-retinal in ROS has been proposed to be the rate-limiting step in the retinoid cycle (31,32). This is also supported by the fact that disruption of 11-*cis*-RDH produced only a modest accumulation of *cis*-retinols after an intense bleach (Fig. 4); in *11-cis-rdh*−/− mice, the rate of 11-*cis*-retinol oxidation is comparable to other rate-limiting steps. An alternative explanation might be that the procedure employed to isolate RPE membranes results in reduced activity of the alternative enzyme.

Napoli and co-workers (64) have reported on the cloning of a member of the short-chain alcohol dehydrogenase family termed CRAD2. CRAD2 has steroid 3- and 17-dehydrogenase and *cis*/*trans*-retinol catalytic activities. Although the enzyme is expressed predominantly in liver, CRAD2 transcripts were also found in the RPE (64). Our intensive characterization of the alternative enzyme involved in the oxidation reaction of 11-*cis*-retinol is very useful in eliminating CRAD2 as a major contributor to the 11-*cis*-RDH activity in the RPE because in contrast to the NAD-preferring CRAD2 (~10-fold preference), the alternative 11-*cis*-RDH prefers NADP.

In summary, these results provide a detailed characterization of a mouse model of the human disease fundus albipunctatus. They point out the importance of 11-*cis*-RDH in keeping the balance between different components of the retinoid cycle. Lack of 11-*cis*-RDH perturbs these relationships and leads to accumulation of *cis*-retiniods, and in particular, it is likely the first vertebrate model system in which a 13-*cis*-retinol isomer is present in high concentrations. This finding implies indirectly that the presence of 11-*cis*-RDH somehow impacts the removal of this isomer in wild-type mice. The analysis of *11-cis-rdh*−/− mice is also convenient for the characterization of the RDH that is responsible for an alternative pathway of 11-*cis*-retinal production. Cloning and further characterization of this enzyme and double knockout mice for both dehydrogenases should be very helpful in understanding the individual contributions of these dehydrogenases in the retinoid cycle.

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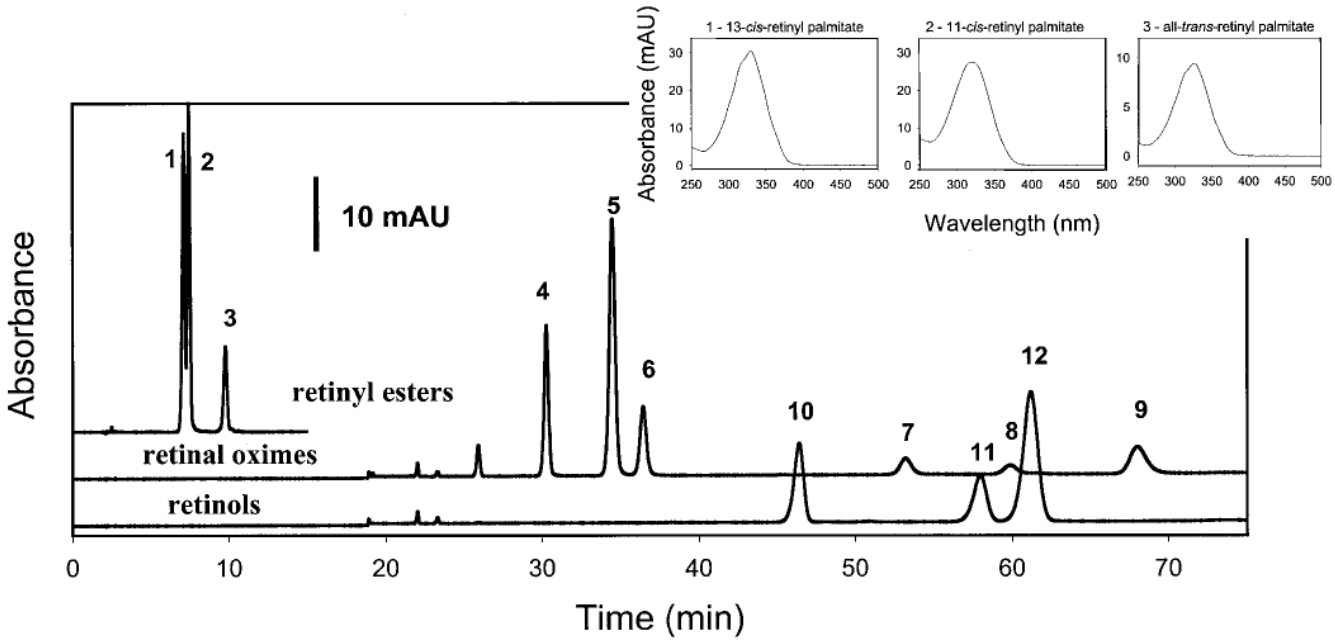


Fig. 1. Chromatograms for separation of retinyl palmitate, retinal oxime, and retinol standards

Peak 1, 13-*cis*-retinyl palmitate; *peak 2*, 11-*cis*-retinyl palmitate; *peak 3*, all-*trans*-retinyl palmitate; *peak 4*, *syn*-11-*cis*-retinal oxime; *peak 5*, *syn*-all-*trans*-retinal oxime; *peak 6*, *syn*-9-*cis*-retinal oxime; *peak 7*, *anti*-11-*cis*-retinal oxime; *peak 8*, *anti*-9-*cis*-retinal oxime; *peak 9*, *anti*-all-*trans*-retinal oxime; *peak 10*, 11-*cis*-retinol; *peak 11*, 9-*cis*-retinol; *peak 12*, all-*trans*-retinol. *Insets*, UV/visible spectra of *peaks 1*, *2*, and *3*.

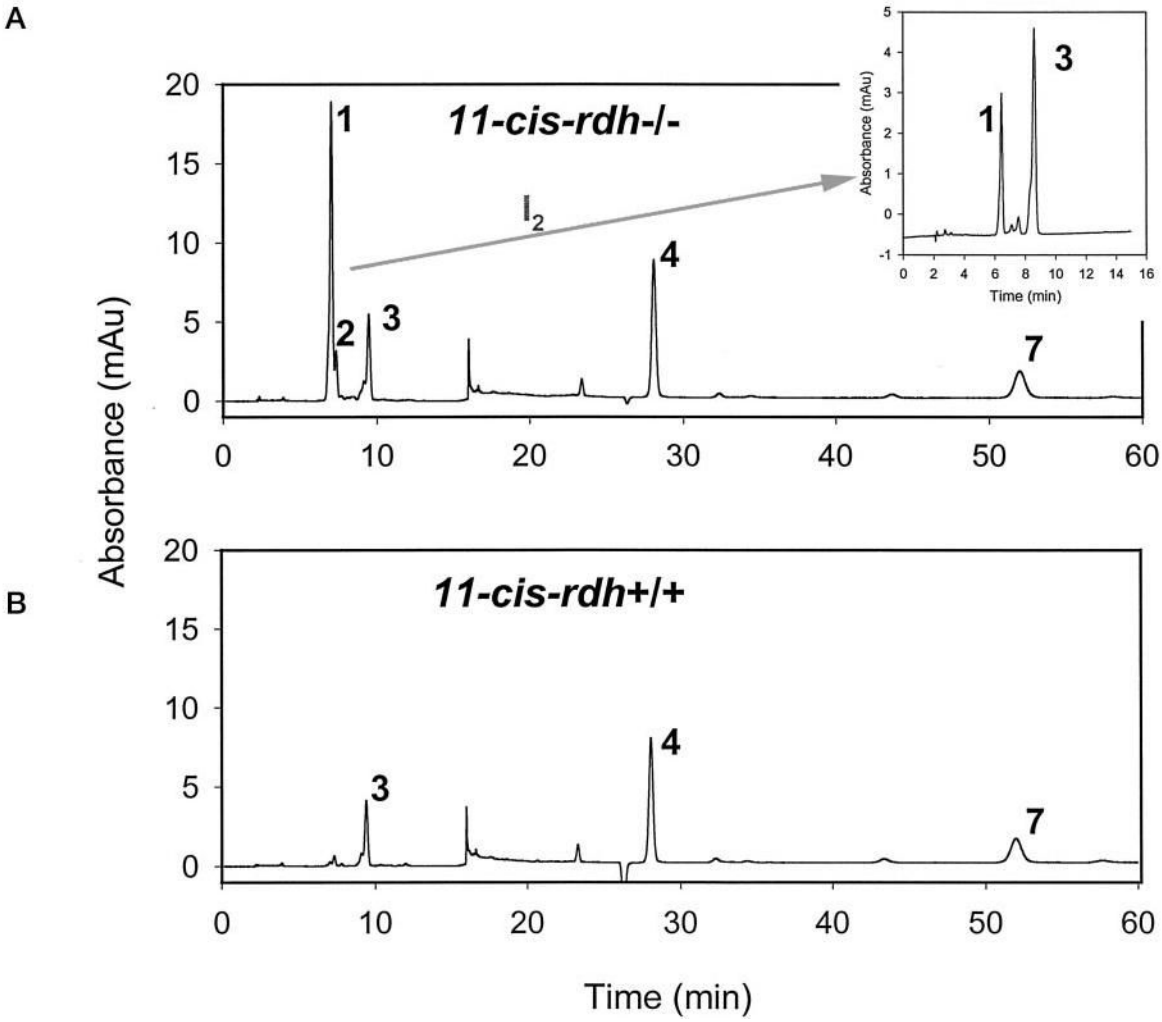


Fig. 2. HPLC separation of retinoids for 1-month-old *11-cis-rdh* mice

A, *11-cis-rdh*−/− reared under 12-h light-dark cycle. *Inset*, chemical isomerization of collected 11-*cis*- and 13-*cis*-retinyl esters employing I_2 (see under "Materials and Methods"). *Peak 1*, 13-*cis*-retinyl ester; *peak 2*, 11-*cis*-retinyl ester; *peak 3*, all-*trans*-retinyl ester; *peak 4*, *syn*-11-*cis*-retinal oxime; *syn*-all-*trans*-retinal oxime; *peak 7*, *anti*-11-*cis*-retinal oxime. *B*, *11-cis-rdh* +/+ reared under 12-h light-dark cycle.

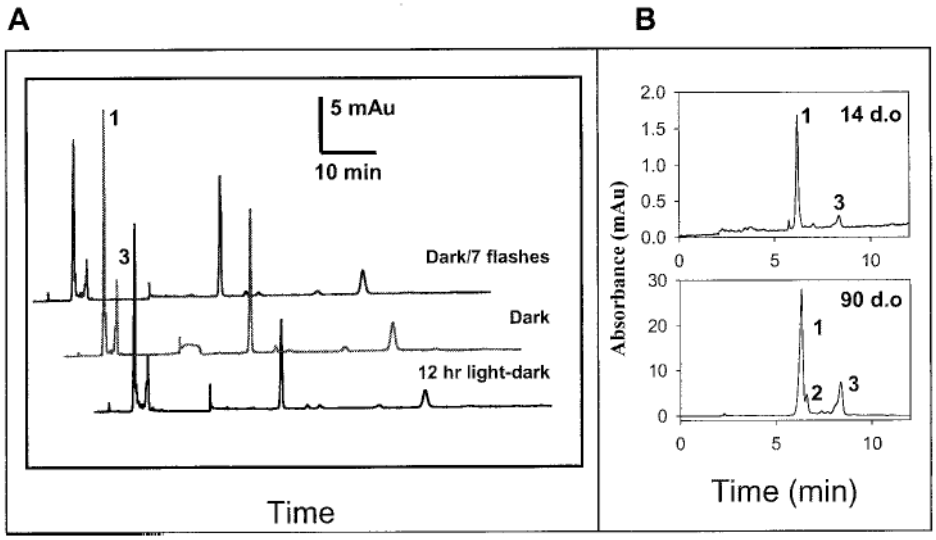


Fig. 3. HPLC separation of retinoids for 2-month-old *11-cis-rdh* mice

A, *11-cis-rdh*−/− reared under 24-h dark and exposed to seven flashes, each of which bleached ~40% of rhodopsin, at 1-h intervals and subsequently dark-adapted for 12 h (*top trace*); *11-cis-rdh*−/− reared under 24-h dark (*middle trace*); *11-cis-rdh*−/− reared under a 12-h light-dark cycle and dark-adapted for 12 h (*bottom trace*). *Peak 1*, 13-*cis*-retinyl ester; *peak 2*, 11-*cis*-retinyl ester; *peak 3*, all-*trans*-retinyl ester; *B*, 14-day-old *11-cis-rdh*−/− (*top panel*) and 3-month-old *11-cis-rdh*−/− reared under 24-h dark (*bottom panel*).

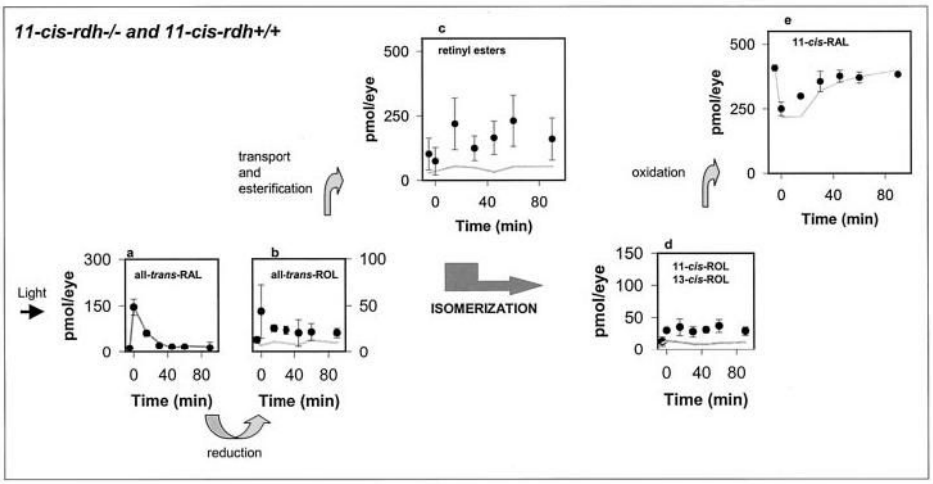


Fig. 4. Kinetics of retinoid recovery in *11-cis-rdh*+/+ and *11-cis-rdh*−/− mice

Mice were reared under 12-h light-dark cycle. HPLC retinoid analysis was either before or after a flash that bleached ~40% of the visual pigment. Photolyzed rhodopsin released all-*trans*-retinal (*a*), which was reduced to all-*trans*-retinol (*b*) and then transported to the RPE and esterified to retinyl esters (*c*). All-*trans*-retinol, or its derivative, was isomerized to 11-*cis*-retinol (*d*), which in turn, was oxidized to 11-*cis*-retinal (*e*). *Closed circles* represent *11-cis-rdh*−/− mice. The *gray line* represents data obtained from *11-cis-rdh*+/+ mice. *Error bars* indicate the standard error of the mean (n = 3).

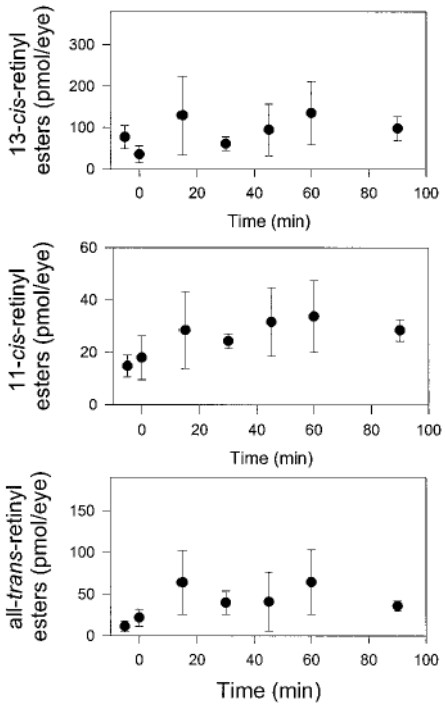


Fig. 5. Kinetics of retinyl esters in 11-*cis*-*rdh*−/− mice

Mice were reared under 12-h light-dark cycle. HPLC retinoid analysis was either before or after a flash that bleached ~40% of the visual pigment. Note the scaling on the *y* axis. *Error bars* indicate the standard error of the mean (n = 3).

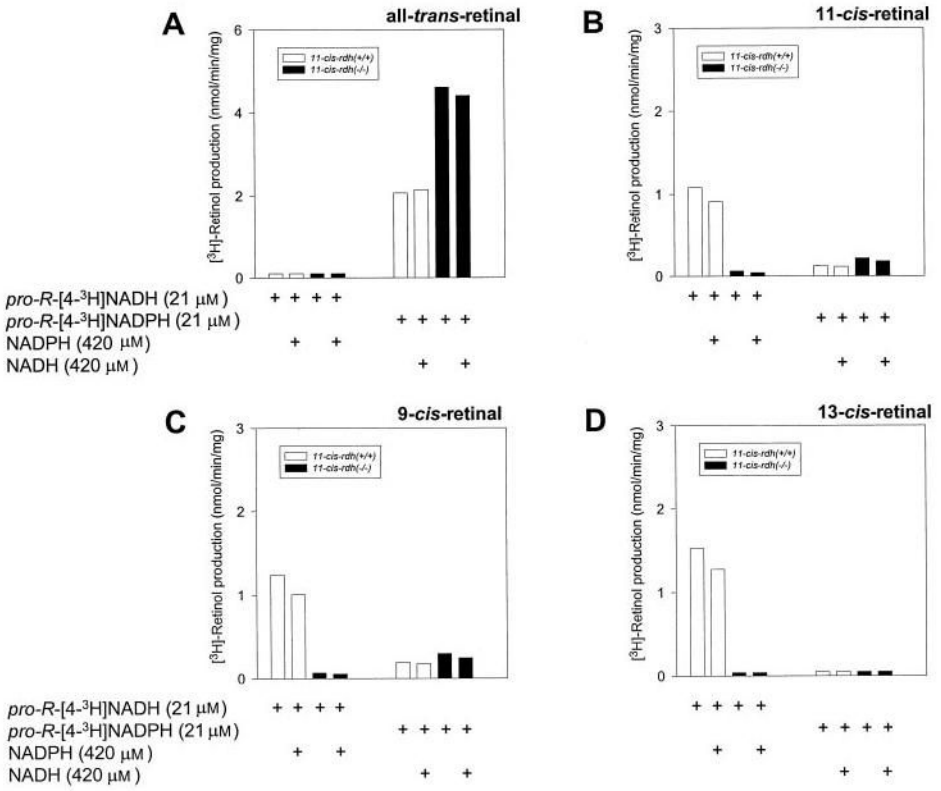


Fig. 6. Relative RDH activity in the RPE of *11-cis-rdh*+/+ and *11-cis-rdh*−/− mice with *pro-S*-[4-^{3}H] NAD(P)H and geometric isomers of retinals

The assay was carried out for 50 min at 34–37 °C using the phase partition assay as described under "Materials and Methods." All-*trans*-retinal (*A*), 11-*cis*-retinal (*B*), 9-*cis*-retinal (*C*), and 13-*cis*-retinal (*D*) were used as substrates. Indicated is the amount of the corresponding [^{3}H] retinol isomer formed (confirmed by HPLC; see under "Materials and Methods") in nmol/min/mg of protein.

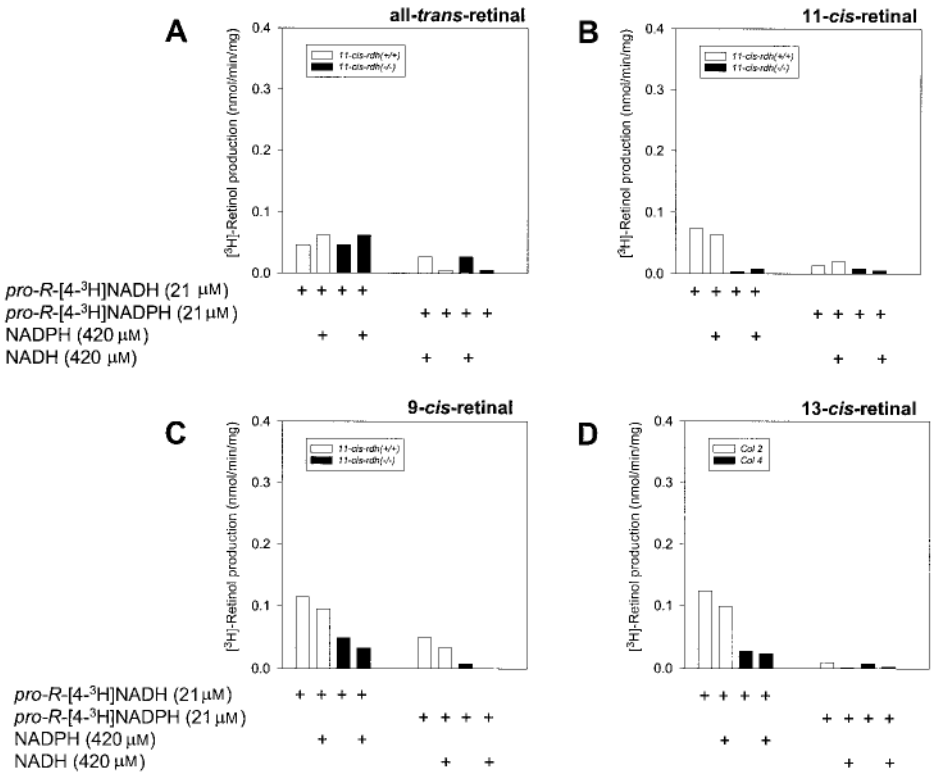


Fig. 7. Relative RDH activity in the RPE of *11-cis-rdh*+/+ and *11-cis-rdh*−/− mice with *pro-R*-[4-^{3}H]NAD(P)H and geometric isomers of retinals

The assay was carried out for 50 min at 34–37 °C using the phase partition assay as described under "Materials and Methods." All-*trans*-retinal (*A*), 11-*cis*-retinal (*B*), 9-*cis*-retinal (*C*), and 13-*cis*-retinal (*D*) were used as substrates. Indicated is the amount of the corresponding [^{3}H]] retinol isomer formed in nmol/min/mg of protein.

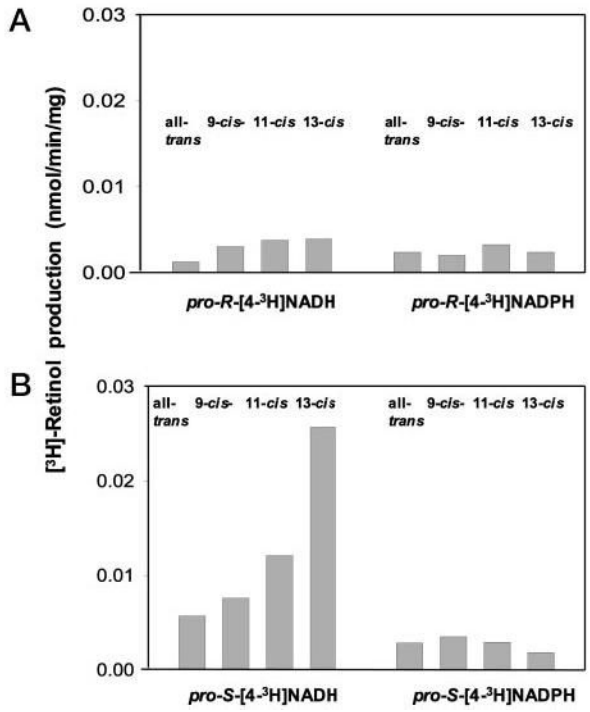


Fig. 8. Relative RDH activity in the cytosol of bovine RPE with *pro-R*-[4-^{3}H]NAD(P)H or *pro-S*-[4-^{3}H]NAD(P)H and geometric isomers of retinals

The assay was carried out for 40 min at 33–37 °C using the phase partition assay as described under "Materials and Methods." *A*, *pro-R*-[4-^{3}H]NAD(P)H. *B*, *pro-S*-[4-^{3}H]NAD(P)H. Indicated is the amount of the corresponding [^{3}H]]retinol isomer formed in nmol/min/mg of protein.

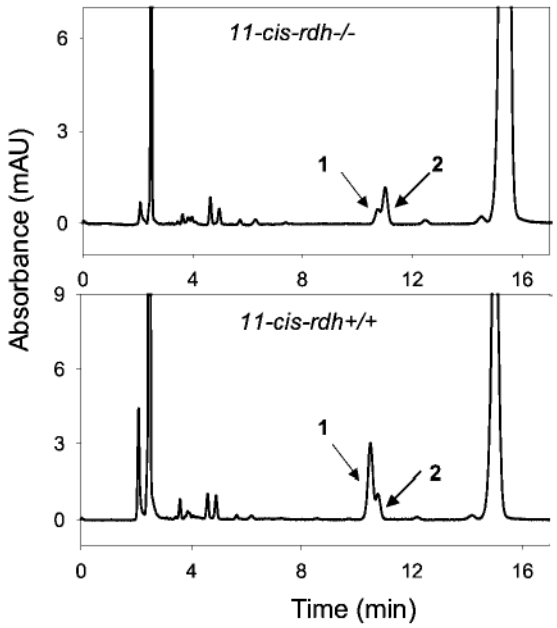
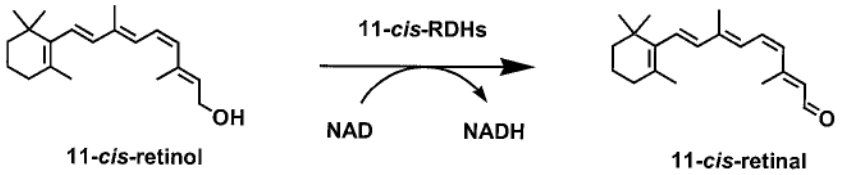


Fig. 9. Isomerization to 11-*cis*-retinol remains active in *11-cis-rdh*−/− mice

Mouse RPE microsomes were prepared as described under "Materials and Methods." Approximately 20–40 μg of protein (40 μl) was added to a solution of 1% bovine serum albumin, 1 mM ATP, 15 μM apo-rCRALBP, and 50 mM bis-tris-propane, pH 7.5, to a final volume of 220 μl. The reaction was incubated by addition of 0.5 μl of all-*trans*-retinol in dimethylformamide (4 mM stock) for 2 h at 37 °C. Retinoids were extracted through addition of 300 μl of MeOH and 200 μl of hexane and analyzed by HPLC, as described under "Materials and Methods." *Arrows 1* and *2* indicate elution of 11-*cis*-retinol and 13-*cis*-retinol, respectively. The *top trace* shows reduced production of 11-*cis*-retinol in *11-cis-rdh*−/− mice under identical conditions compared with *11-cis-rdh*+/+ mice RPE (*bottom trace*).



Scheme 1. Redox reactions catalyzed by mouse 11-*cis*-RDH

11-*cis*-RDH is a NAD-specific enzyme, whereas other enzymes in the RPE utilize NADP to produce 11-*cis*-retinal, which is transported to ROS to regenerate rhodopsin.