Rhodopsin, Vitamin A, and Interstitial Retinol-Binding Protein in the rd Chicken

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In order to determine whether blindness in the rd strain of Rhode Island Red chickens is due to a defect in the vitamin A (visual) cycle, spectroscopy, high performance liquid chromatography, and immunochcmical techniques were used to compare the amounts of rhodopsin, interstitial retinol-binding protein, and vitamin A compounds in the dark-adapted eyes of homozygous rd and heterozygous carriers. In both groups of chickens, (up to 6 weeks post-hatching) the distribution of stored vitamin A differed from other vertebrates (mammals, amphibians, fish) in that more than half of the retinyl palmitate/stearate occurred in the neurosensory retina. The 11-cis isomer accounted for nearly 100% of the retinyl palmitate/stearate in the neurosensory retinas of both groups. In the pigmented layers (pigment epithelium and choroid) the 11-cis isomer amounted to 70.1 ± 4.2% in the carrier, and 65.1 ± 2.9% in the rd birds. With respect to their content of rhodopsin, IRBP, retinyl palmitate/stearate and unesterified retinol, (both 11-cis and all-trans isomers) no significant difference could be demonstrated between the eyes of rd and carrier chickens (3 days and 28 days post-hatching). These results therefore demonstrate that the ocular tissues of rd chickens do not lack IRBP, the putative extracellular transport protein for vitamin A, that these tissues synthesize and store the 11-cis isomer of vitamin A, and that the 11-cis isomer is used to form rhodopsin. It is suggested that the absence of vision in rd chickens is similar to the human condition of dominantly-inherited night blindness, and may be due to a defect in the transduction process. Invest Ophthalmol Vis Sci 28:613-617, 1987

The newly-hatched rd strain of Rhode Island Red chicken1-4 is behaviorally blind and has no measurable ERG, although their neurosensory retinas are morphologically identical to those of age-matched normal chicks. The chicken mutation is unusual. Blindness precedes the onset of photoreceptor degeneration, which is not evident until 1-2 weeks post-hatching. The current study was designed to address the question of whether there is a defect in the systems transporting vitamin A to the outer segments, or in the ability of the eye to isomerize all-trans retinoid to 11-cis, and to use this isomer for rhodopsin synthesis. This study demonstrates that 3 days after hatching, the quantities of rhodopsin, 11-cis retinyl esters, 11-cis retinol and interstitial retinol-binding protein (IRBP) appear to be comparable in rd and normal chicks. We conclude that the cause of blindness is not due to a defect in the vitamin A cycle, but may be connected with a defect further on in the transduction process, perhaps involving G-protein, PDE, cGMP or the sodium channel. Retinal degeneration is probably secondary to one or more of these biochemical defects in this animal model. A preliminary report of this work has been published.5

Materials and Methods

Animals

Colonies of homozygous rd and heterozygous carrier breeder chickens are maintained at the University of Florida Animal Care Facility, under NIH guidelines. Controlled matings occurred, which produced either all rd or half rd and half carrier offspring. Eggs were incubated in an automatic rotating, forced draft incubator until 1-2 days before hatching, when they were...
transferred to a brooder. Vision or blindness of offspring was verified by behavioral tests on the day of hatching. Hatchlings were maintained on a 12L:12D cycle until the age of study.

**Tissue**

Chickens were dark-adapted for 2–4 hr, and subsequent operations were carried out in dim red light. In early experiments, the neurosensory retinas were detached cleanly from the pigment epithelium-choroid. Later, however, both layers were collected and processed together. In either event, the tissues were frozen on dry ice for shipment to Houston.

**Procedure**

The tissue samples (usually from 6–12 eyes) were thawed and homogenized by hand in 2 ml of phosphate-buffered saline (150 mM NaCl, 5 mM Na phosphate, pH 7.5) containing 0.1 mM phenylmethylsulfonyl fluoride as a protease inhibitor. The homogenate was divided into two 1-ml fractions, designated A and B, respectively (Fig. 1). Fraction A was used to determine rhodopsin and IRBP, fraction B for retinol and its esters.

**Rhodopsin**

The homogenate was centrifuged at 100,000 g for 60 min (Sorvall AH 650 rotor, Dupont Instruments, Newton, CT). The supernatant was collected and retained for IRBP analysis. To obtain optically stable visual pigment solutions, the pellet was not extracted directly, but was suspended in 5 ml 65% (w/v) sucrose in 67 mM phosphate buffer (pH 7.0). This suspension was overlaid with successive 1-ml fractions, designated A and B, respectively (Fig. 1). Fraction A was used to determine rhodopsin and IRBP, fraction B for retinol and its esters.

**IRBP**

The protein concentration of the supernatant was determined. Aliquots containing 100 or 300 μg of protein were precipitated by addition of 5 vol of acetone, and subjected to electrophoresis in 7.5% polyacrylamide slab gels containing sodium dodecyl sulfate. Standard quantities of purified bovine IRBP were electrophoresed in the same gel. The separated proteins were then transferred to nitrocellulose paper, which was probed with rabbit antibovine IRBP IgG as described by Gonzalez-Fernandez et al. The second antibody was horseradish peroxidase-labeled goat antirabbit IgG.

**Retinol and Retinyl Esters**

One milliliter of methanol was added to the remaining 1 ml of homogenate, and the mixture was extracted twice under argon with 4 ml volumes of petroleum ether (37–58°C, Allied Fisher Scientific, Pittsburgh, PA). This extract was dried under N₂, dissolved in 1 ml 10% dioxane in hexane, and polar lipids were removed by chromatography on a 1 cm x 1 cm alumina column (Woelm 200 basic, deactivated with 5% water) eluted with 10 ml of the same solvent. The eluted retinol and retinyl esters were transferred to the appropriate mobile phase (see below) and analyzed by high-performance liquid chromatography (hplc) on 4.6 x 150 mm columns (Supelcosil LC-Si 3 μm or Ultrasphere Si 5 μm). The eluant was hexane containing 0.2 or 0.5% methyl-t-butyl ether (retinyl esters and their isomers) or 5% dioxane (retinol isomers): flow rate was 0.7 ml/min, detection was by absorbance at 325 nm (Kratos Spectroflow 773 absorbance detector, Kratos Analytical Instruments, Ramsey, NJ). Quantitation was carried out by injecting the authentic compounds and constructing calibration curves of peak area versus amount.
Results

Retinyl Esters

In the first part of this investigation, the neurosensory retina and pigment epithelium-choroid were separated and extracted separately. Typical hplc profiles of retinyl esters extracted from the neurosensory retina and corresponding pigment epithelium-choroid layer of an 11-day heterozygous carrier (sighted) chicken are shown in Figure 2. The major peak in each case was attributed to 11-cis retinyl palmitate (peak 3), with a minor peak due to the stearate (peak 2) on its rising phase. The all-trans palmitate was represented by peak 5. In some chromatograms, a minor peak attributable to the corresponding stearate was distinguishable. The eluted fraction corresponding to peak 4 had $\lambda_{\text{max}}$ at 322 nm (n-hexane), suggesting that it contained a retinyl ester. Although it did not appear to be an 11-cis isomer (the 11-cis retinyl palmitate had $\lambda_{\text{max}}$ at 318 nm in n-hexane), this material was not further characterized. Its omission from all the quantitative measurements presented here, which are based only on the palmitate and small amounts of stearate, does not materially affect our conclusions.

More than half of the retinyl palmitate/stearate isomers was found in the neurosensory retinas. In four groups of eyes from sighted carriers aged 1 day, 10 days, 11 days, and 6 weeks (32 eyes were analyzed), 61.7 ± 13.1% ($\bar{x} \pm \text{SD, } n = 4$) of the retinyl palmitate/stearate occurred in the neurosensory retina. The fraction of retinyl palmitate/stearate in the 11-cis configuration varied according to location. In the neurosensory retinas of sighted carriers, 11-cis accounted for nearly 100% of the palmitate/stearate, while in the pigment epithelium-choroid it was 70.1 ± 4.2% ($\bar{x} \pm \text{SD, } n = 4$).

Essentially similar results were found for three groups of rd chickens aged 1 day, 10 days and 6 weeks (26 eyes were analyzed). The neurosensory retinas accounted for 65.5 ± 15.4% ($\bar{x} \pm \text{SD, } n = 3$) of the total retinyl palmitate/stearate. Of particular importance was the finding that large proportions of 11-cis isomer were present in these blind chicks, the levels being comparable to the sighted controls. In the neurosensory retinas, almost 100% of the retinyl palmitate/stearate was 11-cis, compared with 65.1 ± 2.9% in the pigment epithelium-choroid ($\bar{x} \pm \text{SD, } n = 3$).

Following these observations, a more comprehensive examination of tissue from the eyes of two age groups (3 days and 28 days) was initiated. In these studies, the neurosensory retina and pigment epithelium-choroid were not separated, permitting analysis of the IRBP in the interphotoreceptor matrix (IPM). Typical hplc profiles for these combined tissues from 3-day-old chicks (Fig. 3) show that both sighted (panel A) and rd (panel B) were similar in the isomeric composition of the retinyl esters stored in their dark-adapted eyes. The summarized results for 3-day-old and 28-day-old
sighted and rd birds in Figure 4 (panel B) show that there do not appear to be significant differences in the quantity of retinyl palmitate/stearate stored in the eyes of these four groups.

**Retinol**

The amount of unesterified retinol was also determined for some groups of rd and sighted carriers. It was found to be surprisingly high, representing between 16.5 and 26.4% of the palmitate/stearate. As shown in Figure 4, panel C, the proportion of 11-cis isomer was comparable with that present in the palmitate/stearate fraction.

**Rhodopsin**

Not only could rd ocular tissues form 11-cis retinol and 11-cis retinyl esters, but they also had the capacity to use the 11-cis isomer to synthesize rhodopsin. Three-day-old birds averaged 0.16 nmol/eye, compared with 0.14 nmol/eye for sighted carriers (Fig. 4, panel A). At 28 days, the average amount in rd birds was less than that found in a single preparation from age-matched controls, but this difference may not have been significant. It should be noted that because hydroxylamine was always added to the extract, any iodopsin present would have been destroyed (unpublished observations). The molar ratio of rhodopsin to retinyl palmitate/stearate averaged 0.63.

**IRBP**

IRBP was present in the eyes of young rd chickens at levels similar to the controls. This is demonstrated in immunoblots of the soluble proteins from the combined neurosensory retina and pigment epithelium-choroid in Figure 5 (lanes 1–10, 13–15). The blots were probed with rabbit antibovine IRBP antibodies. An immunoreactive band is visible in all the chicken samples: its apparent M, was 138,000, slightly lower than bovine IRBP, and corresponding to the value previously reported for chicken IRBP.13 By comparison with the bovine IRBP standards (lanes a–f), the 2- , 6- and 12-day-old rd chickens had the immunochemical equivalent of 43 ng bovine IRBP per mg soluble protein, compared with 52 ng/mg for the 1- and 11-day-old sighted carriers. Depletion of IRBP was observed in old birds, however. Lane 11 is the immunoblot of soluble proteins from a 1½-year-old rd chicken. The immunoreactive band is fainter than that corresponding to the age-matched control in lane 12. The rd membrane pellet was extracted with L1690 sucrose ester,6 but no rhodopsin was detected in this animal.

**Discussion**

The current studies provide new information on vitamin A in the eyes of normal as well as rd chickens. The amount of retinyl ester present in the dark-adapted chicken neurosensory retina was 1.6 times that in the RPE-choroid. This is an unusually high proportion: at most, only a small percentage of the stored retinyl esters are associated with the neurosensory retina in amphibians, fish, and mammals.13 The site of storage in the chicken neurosensory retina was not identified, although one possibility is the cone oil-droplets. Although the retinyl ester was unusually distributed, its amount relative to the rhodopsin present was similar to that reported for other animals.13 The chicken was unusual in the proportion of 11-cis isomer in the retinyl pal-
mitate/stearate esters, which was higher than observed in other animals, and in the fraction of unesterified retinol, which exceeded the small percentage typically present in frogs and mammals.13 It was not established whether this retinol was bound to soluble binding proteins such as cellular retinol and retinal binding protein.14-16

The results show that absence of visual function in the rd chicken neurosensory retina is not due to loss of its ability to synthesize and secrete IRBP or to form 11-cis isomer, which is abundant in rd chicken eyes and present in amounts comparable to the sighted carriers. Neither do the morphologically normal rod outer segments contain a variety of opsin that cannot form rhodopsin. Since no ERG can be recorded from the rd chicken, even after prolonged dark-adaptation,4 this situation does not appear to resemble the human hereditary disorder of fundus albipunctatus.17 In this condition, rhodopsin regeneration is retarded, and it has been suggested that there may be an abnormality in the vitamin A transport system, perhaps involving IRBP.18

On the other hand, the rd chicken is strikingly similar to the human condition of dominantly inherited night blindness,9,8,19 where the rods have their normal complement of rhodopsin that after bleaching regenerates with normal kinetics. Like the rd chicken, ERG recordings reveal an almost complete absence of electrical activity. The presence of an apparently normal rhodopsin cycle, but no ERG in the human and animal cases, suggests a failure in the mechanism of visual transduction. Further studies are needed to establish whether the target for the genetic defect is rhodopsin, G-protein, PDE, the sodium channel, or another molecular component.

Key words: rhodopsin, interstitial retinol-binding protein, vitamin A, 11-cis isomers, retinyl esters, rd chicken

References