Retinol Secretion by the Lacrimal Gland

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In order to determine the source of the retinol which has been identified in the tear fluid, the lacrimal gland ducts of rabbits and rats were cannulated and the collected lacrimal gland fluid was analyzed by high performance liquid chromatography. Retinol was identified in the lacrimal gland fluid of rabbits and rats, and it is concluded that the lacrimal gland is the source of retinol in the tears. Dose-response studies show that intravenously administered pilocarpine and intra-arterial acetylcholine stimulate secretion of retinol by the lacrimal gland. Intravenous administration of vasoactive intestinal peptide (VIP) also stimulates retinol secretion in a dose-response manner. These observations are similar to the effects of cholinergic drugs and VIP on protein secretion by the lacrimal gland. Invest Ophthalmol Vis Sci 27: 1261-1268, 1986

Analysis of tear fluid of rabbits, cynomologous monkeys, and humans has shown that vitamin A is present in the tears as all-trans retinol.1,2 This finding suggests that the tear fluid may serve as a nutrient source supplying retinol to the avascular cornea which has an absolute requirement for this vitamin. In testing this hypothesis, it is essential that the source of retinol in the tear fluid be identified since the tears consist of a mixture of mucin from goblet cells, lipids from meibomian and Harderian glands (rabbit), and the aqueous secretion containing electrolytes and proteins from the lacrimal glands. It has also been reported that some tear components, such as transferin and IgG, may be present as the result of extravasation of material from conjunctival capillaries or breakdown of exfoliated epithelial cells.3 Since lacrimal gland fluid (LGF) uncontaminated by other secretions can be collected from the lacrimal gland ducts of rabbits4-6 and rats7 and analyzed by high pressure liquid chromatography (HPLC),1 these methods were chosen as a first step in identifying the source of retinol in tears. It will be shown in this paper that the lacrimal gland is a source of retinol in tear fluid.

The lacrimal gland is known to be under parasympathetic control with cholinergic stimulation causing secretion of fluid, electrolytes, and protein.5,6,8 It has also been shown that vasoactive intestinal peptide (VIP) stimulates secretion of enzymes and fluid by the lacrimal glands of rats and rabbits.9,10 Therefore, the effects of several cholinergic agonists and VIP on retinol secretion by the lacrimal gland were also investigated.

Materials and Methods

Experimental Animals

The animals used in these experiments were New Zealand White rabbits of either sex weighing 2.5-3.2 kg and either Sprague-Dawley or WKY rats weighing 200-400 g. Rabbits were anesthetized with an initial dose of ketamine HCl (30 mg/kg) and xylazine (5 mg/kg) given intramuscularly, unless otherwise noted, and anesthesia was maintained by additional doses every 30 min throughout the experiment. Rats were anesthetized with sodium pentobarbital (50 mg/kg) given intraperitoneally. At the end of each experiment, the animals were euthanized with a sodium pentobarbital overdose.

Vitamin A-deficient rabbits were prepared as previously described. Briefly, weanling rabbits weighing 0.5 kg were placed on a casein base diet deficient in vitamin A (Teklad Test Diets, Madison, WI) fed ad libitum for 14-20 weeks.1,1 The animals were used for experiments when their weight had plateaued (1.5-1.8 kg) and the corneas had reached stage 3 to 4 of keratinization. Animal use procedures conformed to the ARVO Resolution on the Use of Animals in Research.

Experimental Protocols

The opening of the excretory duct of the lacrimal gland of the rabbit is located in the palpebral conjunctiva near the lateral canthus. The duct opening was cannulated with a tapered glass cannula as described by Botelho and co-workers.4-6 The cannula measured 0.3-0.5 mm in outside diameter at the tapered end, with an inner diameter of 1 mm at the open end, and

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Supported by grants R23 EY04069, R01 EY05640, and P30 EY01931 from the National Institutes of Health and by a grant from Alcon Laboratories, Fort Worth, Texas.

Submitted for publication: October 21, 1985.

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was 6–10 mm long. As LGF filled the cannula, it was collected using a tapered, L-shaped glass pipette. Initial experiments were designed to allow collection of large amounts of LGF for HPLC analysis to determine the presence of retinol. LGF flow was therefore maximally stimulated by injection of 5 μg of carbachol (carbachol) into the marginal ear vein of normal and vitamin A-deficient rabbits. The drug was injected every 15 min for up to 3 hr, and LGF samples were pooled to allow analysis of as much as 100 μl of LGF by HPLC.

In a second series of experiments, tears were sampled from three vitamin A-deficient rabbits as previously described and analyzed by HPLC to confirm the absence of retinol. The animals were then given a 100,000 IU dose of retinyl palmitate orally and placed on a normal diet (Purina Rabbit Chow). Additional 25,000 IU doses of retinyl palmitate were given every other day for 4–6 days. When the corneal pathology had cleared, tears were again sampled and analyzed to confirm the presence of retinol. The animals were then anesthetized, the lacrimal gland duct was cannulated, and LGF was collected following carbachol stimulation.

Because of the long-acting, widespread systemic effects of carbachol, further experiments on the effect of cholinergic stimulation on retinol secretion by the rabbit lacrimal gland were conducted using pilocarpine and acetylcholine. Pilocarpine was administered intravenously via the marginal ear vein or the femoral vein. The dose-response characteristics of the effect of pilocarpine on retinol secretion were studied in five animals using the following protocol. The flow rate of LGF was minimized by application of two drops of proparacaine HCl to the cornea. After 5 min, a 100–400 μg/kg dose of pilocarpine was administered and 10–25 μl of LGF was collected in a pre-weighed pipette for a precisely timed period of no more than 5 min. The volume of LGF collected was immediately determined gravimetrically and the flow rate was calculated. The sample was transferred to a 25 μl Hamilton syringe and injected onto the HPLC column. When the LGF flow rate had returned to basal levels (usually after about 10–15 min), proparacaine was again applied to the cornea and the procedure was repeated until the animal had received two injections at each dosage of 100, 200, 300, and 400 μg/kg of pilocarpine. Doses were administered in randomized order.

The effect of acetylcholine (Ach) on retinol secretion by the lacrimal gland was studied on five animals by injecting Ach directly into the arterial supply of the gland. The animals were anesthetized with an initial dose of 35 mg/kg sodium pentobarbital. Catheters were placed in the femoral artery and vein for monitoring arterial pressure and administration of drugs. The animal was ventilated through a tracheostomy tube using a Harvard 607 respirator. The internal maxillary artery which supplies the lacrimal gland was isolated by ligation of the posterior occipital, superficial temporal, and external maxillary arteries. The lingual artery was catheterized in the retrograde direction and infused with lactated Ringer’s solution at 0.6 ml/min. Acetylcholine introduced into the infusate was carried to the lacrimal gland by blood flowing from the common carotid artery into the internal maxillary artery. The effect of Ach on retinol secretion by the lacrimal gland was studied by infusion of bolus doses of 20–60 μg Ach following the same protocol used with pilocarpine. Prior to each infusion of Ach, the rabbit received a 0.35 mg dose of d-tubocurarine chloride intravenously to prevent movement due to the effect of Ach on skeletal muscle.

LGF secretion was also stimulated by intravenous infusion of vasoactive intestinal peptide (VIP) at doses ranging from 2–8 μg/kg. Dose-response effects were studied in six animals using the same protocol used in the pilocarpine study except that LGF was collected for 10 min following each VIP injection. Because of the cardiovascular effect of VIP, which causes vasodilation, after each dose the arterial pressure was allowed to return to control levels and remain there for 15 min before a subsequent dose was administered.

The duct of the exorbital lacrimal gland of the rat was cannulated in similar fashion to the rabbit, as described by Thorig et al. A tracheostomy tube was placed in the trachea so that salivary secretions in response to cholinergic stimulation would not interfere with respiration. Pilocarpine (50 μg/kg) was infused into the femoral vein and the LGF secreted was collected. The LGF secreted in response to repeated doses of pilocarpine was pooled until a 20 μl volume sufficient for HPLC analysis was obtained.

**High Pressure Liquid Chromatography (HPLC)**

Samples of LGF were analyzed for retinol on a Beckman (Fullerton, CA) Model 334 Gradient Liquid Chromatograph equipped with a Beckman Model 165 Variable Wavelength UV Detector, a Dupont (Wilmington, DE) Zorbax ODS reversed-phase column, and a Rhodyne 7302 column inlet filter (Rainen Instrument Co., Woburn, MA). The mobile phase for most experiments was 90% methanol:10% water containing 10 mM ammonium acetate (NH₄Ac) at a flow rate of 1 ml/min. This is a standard method for separation of retinoids and has previously been used in our laboratory for separation of retinoid metabolites extracted from cornea and identification of retinol in...
tears. This system is capable of resolving isomers of retinol and separating retinol from retinaldehyde as was confirmed during the course of the present study using a retinaldehyde standard and a retinol isomer produced by UV irradiation of all-trans retinol. A second solvent system consisting of 90% acetonitrile:10% water containing 10 mM ammonium acetate was also used to confirm the results obtained using methanol:water. The detector was set at a wavelength of 330 nm and a sensitivity of 0.005 absorbance units full scale. The scanning channel of the detector was set at a sensitivity of 0.005 absorbance units full scale, and absorbance spectra were obtained by scanning peaks from 300–400 nm as they passed through the flow cell of the detector.

All-trans retinol and retinaldehyde (Sigma Chemical, St. Louis, MO) standards were dissolved in HPLC grade methanol and stored under nitrogen at −80°C. Standard curves were generated and retinol in LGF was quantified based on peak height as previously described.1 The solvents used were HPLC grade and were filtered through a 0.2 μm filter and degassed before use. All experiments were conducted in a room equipped with amber lights to protect the retinoids from exposure to light with a wavelength of less than 380 nm.

Results

Identification of Retinol in Lacrimal Gland Fluid

Chromatographic analysis of samples of LGF collected during cholinergic stimulation resulted in elution of a peak with the same retention time as a retinol standard (Fig. 1). Scanning of this peak from 300–400 nm revealed an absorption spectrum corresponding to that for retinol with an absorbance maximum of 330 nm, as shown in Figure 2 for a 100 μl LGF sample. The retinol peak was absent from the LGF of vitamin A-deficient rabbits (Fig. 3). In agreement with our previous study, retinol was absent from the tear fluid of vitamin A-deficient rabbits but returned after repletion of the animals with vitamin A. Subsequent collection and analysis of LGF from the same animals showed that this retinol was secreted by the lacrimal gland (Fig. 4). Mean retinol concentrations in LGF collected during maximal cholinergic stimulation from normal rabbits and three vitamin A-repleted rabbits were 62.3 ± 5.9 ng/ml (n = 10) and 130 ± 17.5 ng/ml (n = 7), respectively.

These results were confirmed using the acetonitrile:water mobile phase. In order to detect retinol in LGF using this system, it was found to be necessary to first extract the LGF with methanol in a 1:1 ratio. This resulted in precipitation of protein which was sedimented by centrifugation. The supernatant was then analyzed for retinol. The retinol peak detected using the methanol:water system was also detected by this method (Fig. 5).

Retinol was also detected in the LGF of three rats (Fig. 6); however, since it took 3–4 hr to collect the 20 μl of LGF needed for analysis, this model has not been pursued.

Dose-Response Experiments

Administration of increasing doses of pilocarpine had no effect on retinol concentration in LGF. However, the LGF flow rate increased from 1.8 ± 0.27 μl/
min in response to a 100 μg/kg dose of pilocarpine to 11.2 ± 1.3 μl/min in response to a 400 μg/kg dose of pilocarpine. Retinol concentration remained constant at 40.3 ± 2.1 ng/ml (n = 30) over this range of flow rates (Fig. 7A). In order for retinol concentration to remain constant with increasing LGF flow rate, the rate of retinol secretion by the gland must also increase with increasing levels of stimulation. Therefore, the retinol secretion rate (pg/min) for each dose of pilocarpine (μg/kg) was calculated from the LGF flow rate and corresponding retinol concentration for each sample. A range from 67.1 ± 11 pg/min at 200 μg/kg to 458.8 ± 72.8 pg/min at 400 μg/kg (Fig. 7B) was measured.

A similar response was observed with stimulation of LGF secretion by injection of bolus doses of Ach into the arterial supply of the lacrimal gland. Flow increased from 3.5 ± 0.4 μl/min in response to a 20 μg dose to 8.4 ± 0.9 μl/min in response to a 60 μg dose. Retinol concentration remained constant at 45.7 ± 5.5 ng/ml (n = 35) over this range of flow rates (Fig. 8A). Calculation of the retinol secretion rate from these data resulted in a rate of 156.9 pg/min at 20 μg Ach to 437.0 pg/min at 60 μg Ach (Fig. 8B).

Intravenous administration of VIP also results in stimulation of LGF secretion. Flow rates ranged from 1.22 ± 0.11 μl/min for a 2 μg/kg dose to 2.06 ± 0.28 μl/min for an 8 μg/kg dose. Again, the retinol concentration remained constant over this range of flow rates although at a higher level than that observed with cholinergic stimulation, 85.9 ± 5.5 ng/ml (n = 33) (Fig. 9A). Calculation of the retinol secretion rate over this range of VIP doses resulted in values ranging from 109.8 ± 28.0 pg/min at 2 μg/kg VIP to 181.8 ± 41.7 pg/min at 8 μg/kg (Fig. 9B).

**Fig. 3.** A, Chromatogram of lacrimal gland fluid from a vitamin A-deficient rabbit. Note lack of retinol peak at 14-15 ml. B, Absorbance spectrum from 200-400 nm of the peak which elutes from lacrimal gland fluid at 7 ml. The absorbance maxima at 210 nm and 280 nm suggest that the peak which elutes at 7 ml in this and other rabbit lacrimal gland fluid samples is a peptide. Mobile phase, methanol:water, 10 mM NH₄Ac.

**Fig. 4.** Effect of repletion of a vitamin A-deficient rabbit on retinol levels in tears and lacrimal gland fluid. Mobile phase, methanol:water, 10 mM NH₄Ac.
Discussion

Analysis of rabbit lacrimal gland fluid by HPLC shows that vitamin A is present in this secretion as all-trans retinol. This conclusion is based on the following evidence: (1) the presence of a chromatographic peak with the same retention time as a retinol standard, (2) an absorbance spectrum and absorbance maximum (330 nm) which corresponds to that for retinol, (3) the absence of this peak from the LGF of vitamin A-deficient rabbits, and (4) the reappearance of the peak in the LGF of vitamin A-repleted rabbits.

The alternative explanation of the data would be that the chromatographic peak is a retinol metabolite such as retinoic acid, retinyl phosphate, a retinyl ester, retinaldehyde, 3,4 didehydroretinol (vitamin A₂), or a retinol isomer. Retinoic acid and retinyl phosphate are more polar than retinol and elute from the column at or near the solvent front in our HPLC system. Using a mobile phase of 80% methanol:20% water, we have been unable to detect retinoic acid in LGF or tears. A retinyl ester such as retinyl palmitate would have a retention volume of at least 100 ml in our system. Metabolism of retinol to retinaldehyde is known to occur in liver, retina, and intestine; however, retinaldehyde has not been identified in other tissues. Our HPLC system gives baseline separation of retinol (elution volume 14 ml) and retinaldehyde (elution volume 16 ml). Vitamin A₂ and retinol isomers have been identified in fish; however, they do not appear to be important in mammals. Our HPLC system is able to separate a retinol isomer produced by UV irradiation which elutes at 13 ml from all-trans retinol (14 ml). In chromatograms of LGF, there is always a single peak corresponding to all-trans retinol. It is, therefore, concluded that the lacrimal gland is the source of the retinol.
which has previously been reported to be present in tears. In preliminary studies, we have also cannulated the duct of the Harderian gland of rabbits and extracted the collected Harderian gland fluid with chloroform/methanol. Analysis of these extracts gave no evidence of the presence of retinol, indicating that this lipid secreting gland is not a source of retinol in tears.

The retinol concentration in LGF of normal rabbits (Figs. 7–9) is within the same range previously reported for rabbit tears. The significantly higher levels of retinol in the LGF of vitamin A-repleted rabbits probably reflects the relatively high doses of vitamin A which these animals received. The rapid response of the animals to vitamin A repletion shows that the retinol secretion mechanism of the lacrimal gland is not affected by the deficient state and that the gland will begin to secrete retinol soon after it is again made available. This finding is in agreement with previous observations of the rapid recovery from the effects of vitamin A deficiency upon administration of large doses of retinyl palmitate.

With the detection of retinol in the fluid from the exorbital lacrimal gland of the rat, it has now been shown that retinol is present in the tears or LGF of four species: rat, rabbit, cynomologous monkey, and human. The secretion of retinol into the tear fluid appears to be a general phenomenon in mammals and may serve a nutritive role in delivery of vitamin A to the ocular surface epithelia.

Cholinergic and peptidergic (VIP) stimulation caused an increased rate of LGF secretion in the present study, as has also been previously reported by other investigators. Increasing levels of cholinergic and
peptidergic stimulation also result in an increasing rate of retinol secretion by the lacrimal gland. The increase in retinol secretion rate parallels the increase in LGF flow rate, resulting in a constant retinol concentration over the range of flow rates reported in this study.

Cholinergic drugs and VIP also stimulate protein secretion by the lacrimal gland. Dartt and Botelho have shown that, with injection of Ach, protein concentration remains nearly constant over the same range of flow rates observed in the present study. Protein concentration, however, is lower at basal flow rates than at stimulated flow rates in rabbits and guinea pigs. Retinol concentration at basal LGF flow has not been measured since the minimum volume required for HPLC analysis is approximately 15 μl. Using rat lacrimal gland slices and isolated acini, it has been shown that carbachol stimulates protein and enzyme secretion in a dose-dependent manner and that this protein secretion is an exocytotic process which is calcium dependent.

Protein secretion by the lacrimal gland is also stimulated in a dose-dependent manner by VIP, and peptidergic neurons containing VIP appear to innervate the extrobital lacrimal gland of the rat. VIP is thought to stimulate protein secretion via a cAMP-dependent pathway which converges with the cholinergic pathway.

The similarities between the effects of cholinergic and peptidergic stimulation on protein and retinol secretion by the lacrimal gland suggest that retinol and protein may be secreted by the lacrimal gland via similar mechanisms. Since retinol is a hydrophobic molecule which is unstable in an aqueous medium, it is unlikely that it would be present in free form in the LGF.

Retinol is transported in the blood by serum retinol binding protein, and it would not be expected to reach the LGF via an extracellular route as has been proposed for water and sodium. Retinol binding protein binds to specific target cell surface receptors but does not enter cells, and it is reported to be absent from the tear fluid of monkeys. Our finding that it is necessary to extract LGF with methanol before HPLC analysis using acetonitrile indicates that the retinol in LGF is associated with protein. Acetonitrile is not an extraction medium for protein-bound retinoids, and, therefore, apparently will not separate retinol when LGF is applied directly to the column. The methanol mobile phase, on the other hand, effectively separates retinol when LGF is applied to the column. If retinol is secreted with the protein component of the LGF, it may be bound to a unique retinoid binding protein synthesized by the acinar cells. Alternatively, it may be secreted and transported in LGF in non-specific association with LGF protein as is the case for the relationship between retinoic acid and serum albumin. The relationship between retinol and protein in LGF is currently being investigated.

Key words: retinol, vitamin A, lacrimal gland, tears, cholinergic agonists, vasoactive intestinal peptide

Acknowledgments

The authors thank Dr. Darlene A. Dartt of the Eye Research Institute, Boston, MA, for teaching us the lacrimal gland fluid collection method, Dr. Henry F. Edelhauser for his critical review of the manuscript, and Mrs. Barbara Olson for secretarial assistance.

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