Localization of Organic Anion Transport Protein 2 in the Apical Region of Rat Retinal Pigment Epithelium

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PURPOSE. The organic anion transporting protein (Oatp)-2 has been cloned from brain and retina. It mediates transport of many endogenous and exogenous amphiphilic compounds across the plasma membrane in a sodium-independent manner. In the brain it resides at the luminal and abluminal membrane of the capillary endothelium and at the basolateral membrane of the choroid plexus epithelium. In the liver, it is expressed at the basolateral membrane of hepatocytes. Its exact localization and function in the retina are unknown. Therefore, the purposes of the present study were to determine the cellular and subcellular localization and the potential functional aspects of Oatp2 in the retina.

METHODS. Oatp2 was detected in rat retinal tissue by immunofluorescence confocal microscopy and by Western blot analysis, with a specific antibody. A Xenopus laevis oocyte expression system was used for functional transport studies.

RESULTS. Oatp2 immunoreactivity was abundantly present at the apical microvilli of the rat retinal pigment epithelium and to a lesser degree in small retinal vessels. In the oocyte expression system, N-retinyl-N-retinylidene ethanolamine (A2E), an unusual cationic, amphiphilic retinoid, exhibited competitive inhibition of Oatp2-mediated digoxin transport with an estimated IC50 of ~37 μM.

CONCLUSIONS. In rat retina, Oatp2 is localized at the interface between the pigment epithelium and the photoreceptor outer segments. A2E is a competitive inhibitor of Oatp2-mediated substrate transport, suggesting that A2E or A2E-like compounds and some retinoids may be substrates for Oatp2 transport. (Invest Ophthalmol Vis Sci. 2002;43:510–514)

The retinal pigment epithelium (RPE) is located between the photoreceptor cell layer and the choroid and plays a vital role in the maintenance and function of photoreceptors. The apical surface consists of microvilli that interact with the outer segment (OS) of rod and cone photoreceptors and constitute a key element for the shuttling of retinoids between the RPE and OS during regeneration of the retinoid chromophore of the visual pigment rhodopsin. So far, the molecular mechanism underlying the shuttling process has not been established. The unusual retinoid N-retinyl-N-retinylidene ethanolamine (A2E) is a cationic amphiphilic molecule that accumulates in the lipofuscin of the RPE.1,2 Lipofuscin and its major fluorophore A2E are believed to contribute to the pathogenesis of age-related macular degeneration (AMD) due to A2E’s lysosomotropic properties3–5 and its capacity to inhibit mitochondrial function.6 A2E may form and accumulate in lipofuscin as a consequence of ingestion and degradation of shed OS tips by the RPE phagolysosomal system.1,6 The precursors of A2E, namely N-retinylidene phosphatidylethanolamine (NRPE) and dihydro-N-retinylidene-retinyl phosphatidylethanolamine (A2PE-H2) are formed on light exposure in the rod OS.7–9

Organic anion-transporting proteins (rat, Oatps; human, OATPs) are a gene family of transmembrane transporters with a rapidly growing number of members.10 These sodium-independent transporters represent a polyfunctional organic solute carrying amphiphilic compounds with mostly overlapping and partially distinct substrate specificity.11 So far, identified substrates include endogenous and exogenous compounds, such as bile salts, steroid conjugates, leukotriene C4, thyroid hormones, cardiac glycosides, peptic drugs, and certain bulky type II organic cations.10 Members of the Oatp/OATP family (gene classification: SLC21A) are widely expressed in many organs, including the liver, kidney, intestine, and brain, where they are localized at distinct membrane domains of several polarized cells. Although the definite physiological significance(s) of Oatps/OATPs remains to be established, their broad substrate spectrum and their heterogeneous distribution indicate a functional diversity of these transporters.

Oatp2 (Slc21a5), a polypeptide consisting of 661 amino acids with 12 predicted transmembrane domains, has been cloned by Noc et al.12 and by Abe et al.13 from the rat brain and retina, respectively. Besides sharing substrates with other Oatps (as just described), Oatp2 specifically mediates the high-affinity transport of the cardiovascular drug digoxin.11 It is localized at the luminal and abluminal membranes of the brain capillary endothelium, the basolateral membrane of the choroid plexus epithelium,14 and the sinusoidal membrane of hepatocytes.15 In the retina, however, its exact localization is unknown. In the present study, using immunofluorescence confocal microscopy, Oatp2 was shown to be predominantly located at the apical microvilli of the RPE. Evidence was provided that the amphiphilic molecule A2E may be an Oatp2 substrate, thus opening the possibility that A2E can be transported actively across pigment epithelial cell membranes. Because A2E may be produced within the cells of the RPE itself and therefore may not normally be a substrate for RPE import, it can be hypothesized that A2E-like molecules, especially precursors of A2E may be able to enter the pigment epithelium through Oatp2-mediated transport.

METHODS

Chemicals

Radiolabeled [3H] digoxin (19 Ci/mmol) and [3H] all-trans-retinol (52 Ci/mmol) were from DuPont-NEN (Boston, MA). Unlabeled all-trans-retinol was from Sigma (St. Louis, MO). A2E was from Christoph

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Richter (Institute of Biochemistry, Swiss Federal Institute of Technology, Zürich, Switzerland) and was synthesized as previously described. All other chemicals were obtained from Merck (Dietikon, Switzerland), Sigma, and Fluka (Buchs, Switzerland).

**Animals**

Procedures concerning animals were in accordance with the regulations of the veterinary authority of Zürich and with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. Male (200–250 g) Sprague-Dawley rats (SUT/SDT9) were obtained from the Institut für Laboratoriumsmedizin, University of Zürich (Zürich, Switzerland). Mature *Xenopus laevis* females were purchased from the African *Xenopus* facility at Noord-dijk, Republic of South Africa.

**Antibodies**

The rabbit polyclonal Oatp2 antibody used in this study was raised against a synthetic peptide consisting of 15 amino acids at the C-terminal of Oatp2 and was extensively characterized by Western blot analysis and immunofluorescence microscopy previously. Affinity purification of this antiserum was performed with N-hydroxy succinimide agarose gel (Affigel 10; Bio-Rad Laboratories, Hercules, CA) coupled to synthetic peptides according to the manufacturer’s instructions. The Cy3-conjugated goat anti-rabbit and horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies were purchased from Jackson Immunoresearch (West Grove, PA) and Santa Cruz (Santa Cruz, CA), respectively.

**Immunoblot Analysis**

For eyecup preparations, lens, vascular, and retina were removed through a slit in the cornea. The remaining eyecup was enucleated and blood and optics, conjunctiva, and muscle tissue were carefully removed. After homogenization with an ultrasound tip in 0.1 M Tris (pH 7.5), the homogenate was centrifuged at 1000×g for 5 minutes. Protein content of the supernatant was assessed using the Bradford assay (Bio-Rad), with BSA serving as the standard. The supernatant was mixed with SDS sample buffer, heated to 70 °C for 10 minutes, and stored at −20 °C until use. Basolateral rat liver plasma membrane was isolated as described before. Proteins were separated on 10% SDS-polyacrylamide minigels and transferred to nitrocellulose membranes.

Blots were blocked for 1 hour at room temperature in 10 mM Tris (pH 7.5), the homogenate was centrifuged at 1000×g for 5 minutes. Protein content of the supernatant was assessed using the Bradford assay (Bio-Rad), with BSA serving as the standard. The supernatant was mixed with SDS sample buffer, heated to 70 °C for 10 minutes, and stored at −20 °C until use. Basolateral rat liver plasma membrane was isolated as described before. Proteins were separated on 10% SDS-polyacrylamide minigels and transferred to nitrocellulose membranes.

**Immunofluorescence Staining**

Rats were decapitated and the eyes were immediately enucleated, immersed in phosphate-buffered saline (PBS) containing 3% paraformaldehyde and 0.2% glutaraldehyde (pH 7.4) at 4 °C for 2 hours and cryoprotected in 30% sucrose overnight at 4 °C. After carefully removing the lens, 10-μm cryostat sections were cut and mounted on glass slides coated with 3-aminopropyltriethoxysilane (Sigma). Sections were incubated overnight at 4 °C with the Oatp2 antibody diluted to 1:5000 for 1 hour at room temperature. Immunoreactivity was visualized with a Western blot detection kit (Renaissance; DuPont NEN).

**Transport Assays in *X. laevis* Oocytes**

Oatp2 cRNA was transcribed in vitro from NotI-linearized cDNA using a kit (mMESSAGE mMachine T3; Ambion, Austin, TX). Oocytes were prepared and incubated overnight at 18 °C. Healthy oocytes were microinjected with either 50 nL of water (control) or 50 nL of water containing 5 ng Oatp2 cRNA. After injection, oocytes were cultured for 5 days to allow the expression of the transporter protein in the plasma membrane. Uptake of radiolabeled substrates (all-trans-retinol, A2E, and digoxin) was performed at 25 °C for 30 to 40 minutes in 100 μL Na1⁻-free medium containing 100 mM choline chloride, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM HEPES-Tris, and 1% dimethyl sulfoxide (DMSO, pH 7.5). Subsequently, oocytes were washed with 3 × 6 mL ice-cold incubation buffer, and each oocyte was dissolved in 10% SDS. After addition of 5 mL scintillation fluid (Ultima Gold; Canberra Packard, Zürich, Switzerland), the oocyte-associated radioactivity was measured in a liquid scintillation analyzer (CA Tri-Carb 2200; Canberra Packard).

**Results**

On sections incubated with the Oatp2 antibody, immunoreactivity was predominantly present in the RPE layer and was only weakly scattered within the inner nuclear layer (INL) and ganglion cell layer (GCL; Figs. 1A, 1B). The immunostaining was absent on sections incubated with the antibody preabsorbed with the peptide used for immunization (Fig. 1C), indicating the specificity of the staining for Oatp2. High magnification revealed that the intense immunostaining appeared predominantly at apical microvilli of RPE cells with only minor reactions of the cytoplasm and no positive staining of the basolateral plasma membrane (Figs. 2B, 2C, 2E). The stained microvilli appeared to extend along the distal part of the OS (Figs. 2B, 2C, 2E). On sections prepared for double-labeling experiments, Oatp2 immunoreactivity colocalized with phalloidin, a marker for filamentous actin that is abundant in apical microvilli of the RPE (Fig. 2F). Within the INL and GCL, weak immunoreactivity was mainly associated with small retinal vessels (Fig. 1B).

We have shown that Oatp2 at the basolateral membrane of hepatocytes has an apparent molecular mass of ~92 kDa, indicating that it is considerably more glycosylated than its analogue in the brain capillary endothelium (~76 kDa). In eyecup homogenates, the antiserum recognized a protein band with the same molecular mass (~92 kDa, Fig. 3) as in basolateral liver plasma membranes, thus indicating that in RPE cells, Oatp2 undergoes an extent of glycosylation similar to that in liver. This band disappeared when the antiserum was preabsorbed with the peptide used for immunization (data not shown).

The strikingly abundant expression of Oatp2 at apical microvilli of RPE cells raises the possibility that this polyspecific membrane transporter is involved in the shuttling of retinoids and its derivatives between photoreceptors and RPE cells during the visual cycle and in the course of photoreceptor membrane turnover. To test this hypothesis, we performed transport experiments with Oatp2-expressing oocytes. Incubation of Oatp2-expressing oocytes in a medium containing 10 μM of radioactive labeled all-trans-retinol did not result in increased uptake compared with water-injected oocytes, either in the
presence or absence of 30 μM serum albumin (data not shown). In search of other potential substrates, we considered A2E because of its amphiphilic organic cationic properties, as a possible candidate. Because radiolabeled A2E with high specific activity was unavailable, we performed Cis inhibition experiments to determine A2E’s ability to compete for Oatp2-mediated transport of digoxin. At a concentration of 200 μM, A2E exhibited a 100% inhibition of Oatp2-mediated transport of digoxin (Fig. 4), whereas all-trans-retinal had no effect (not shown). The absence of an inhibitory effect of all-trans-retinal in the competition assay was in accordance with the failure of Oatp2 to recognize all-trans-retinol as a substrate for direct transport (discussed earlier). The inhibition kinetics, as analyzed by Dixon and Cornish Bowden plot analysis, were consistent with a competitive type of inhibition of Oatp2 mediated digoxin transport by A2E (Fig. 5). The $K_i$ value was estimated at approximately 37 μM.

**DISCUSSION**

In the present study, Oatp2 was found to be abundantly present in apical microvilli of the rat RPE. Our data suggest that

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A2E, and possibly some of its precursors, may be novel Oatp2 substrates.

The expression of Oatp2 at the apical microvilli of the RPE (Figs. 1, 2) was somewhat unexpected, as in other types of polarized cells, such as hepatocytes and choroid plexus epithelial cells, Oatp2 is present at the basolateral domain of the plasma membrane. The specific membrane localization in different cell types suggests that Oatp2 targeting depends on cell type properties, such as anchoring and/or cytoskeleton proteins, whereas targeting signals within the Oatp2 primary sequence may be of secondary importance.

A2E is a major component of the RPE lipofuscin. Its progressive accumulation in the RPE cells appears to contribute to the pathogenesis of AMD, one of the leading causes of severe visual impairment, which affects 10% and 20% of people of age 65 and older in industrialized nations. A2E impairs lysosomal function and may finally lead to rupture of lysosomal membranes. Furthermore, by specifically targeting cytochrome oxidase, A2E inhibits mitochondrial function and induces apoptosis in RPE and other cells. Precursors of A2E form within the rod OS from the sequential condensation of one phosphatidylethanolamine with two all-trans-retinal molecules followed by proton uptake. A2E is thought to arise within phagolysosomes of the RPE as the degradation of ROS membranes proceeds.

The evidence for the ability of Oatp2 to transport A2E comes from the Cis inhibition experiments, which is an indirect approach to identify new substrates of Oatps/OATPs. That A2E, but not all-trans-retinal, competitively inhibited Oatp2-mediated digoxin transport (Figs. 4, 5) indicates that A2E may also be a substrate of Oatp2. Although A2E is toxic to cells and membranes at high concentrations, it is unlikely that the A2E inhibitory effect was due to oocyte damage for the following reasons: First, oocytes were exposed to A2E for only 30 minutes at the same concentration range as that used in a previous study, in which cultured rat RPE and neuronal cells showed signs of significant mitochondrial and cellular toxicity only after exposure to A2E for more than 18 hours. Second, we did not observe any signs of morphologic damage after oocytes had been exposed to A2E concentrations of up to 200 μM for 1 hour (data not shown). And third, A2E inhibition of Oatp2-mediated digoxin uptake could be overcome by increasing the concentration of digoxin (Fig. 5). Thus, the data are compatible with A2E’s potentially being a specific substrate of Oatp2 at the apical microvilli of the RPE.

The direction of Oatp2-mediated transport varies in different systems. For example, in the oocyte expression system, it was demonstrated that Oatp2 can mediate bidirectional substrate transport. In hepatocytes, Oatp2 mediates uptake of bile salts and other amphiphilic compounds from blood plasma under physiological conditions, whereas in the choroid plexus Oatp2 may also mediate efflux of organic solutes. The directionality of Oatp2-mediated substrate transport in RPE cells remains to be determined. The potential substrate A2E matures from precursors within the cells of the RPE. Thus, Oatp2 may not be normally involved in the import of A2E into the pigment epithelium. However, Oatp2 may instead have the capacity to transport A2E across the RPE cell membrane into the extracellular matrix under some conditions. Release of A2E from lysosomal compartments may lead to mitochondrial damage and therefore be highly toxic to the cell. Potential export of A2E by Oatp2 may thus lower intracellular levels of free A2E and may therefore constitute a measure to counteract the toxicity of A2E in the RPE. Alternatively, A2E may be formed under some (unknown) pathologic conditions outside the RPE.
and, under these conditions, Oatp2-mediated transport may contribute to the accumulation of A2E in the pigment epithelium.

Furthermore, considering the multispecificity of Oatp2, the A2E precursors NRPE and A2-PEH, and fatty acids, particularly docosahexaenoic acid (DHA), which is known to be shuttled between photoreceptors and RPE, are also potential candidates for Oatp2. Finally, Oatp2 may exert synergistic effects with other transporter(s) identified recently in the RPE, in transporting various drugs and other xenobiotics into and/or out of the RPE.

In conclusion, this study provides evidence that A2E can be actively transported by Oatp2 across cell membranes. This may suggest a role for the Oatp2 transporter in the (patho-)physiology of A2E in the RPE. Further studies are needed to elucidate the role of a potential A2E transport into or out of the pigment epithelium. Also, whether Oatp2 is involved in the transport of other retinoids in the RPE cells in vivo should be investigated. Given the clinical relevance of the retinoid metabolism, the identification of a human orthologue of Oatp2 in human RPE is essential.

**ADDENDUM**

During preparation of this manuscript, localization of Oatp2 at the apical microvilli of rat RPE was reported also by Ito et al., in abstract form.

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