Selective Upregulation of the A3 Adenosine Receptor in Eyes with Pseudoexfoliation Syndrome and Glaucoma

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PURPOSE. Adenosine is increasingly released in metabolic stress conditions, like hypoxia or ischemia, and regulates many physiologic processes, such as aqueous humor secretion and intracellular pressure, via activation of four adenosine receptors. In the current study, the role of the adenosine system in the pathophysiology of pseudoexfoliation (PEX) syndrome, which is typically associated with anterior chamber hypoxia and elevated intracocular pressure, was examined.

METHODS. RT-PCR, Northern hybridization, and immunohistochemistry were applied to analyze the mRNA and protein expression of the adenosine receptor subtypes A1, A2A, A2B, and A3 in anterior segment tissues of PEX eyes, without and with glaucoma, in comparison to eyes with primary open-angle or angle-closure glaucoma and normal control eyes. Real-time PCR was used to study the effect of hypoxia and oxidative stress on adenosine receptor expression by nonpigmented ciliary epithelial cells in vitro. Levels of adenosine and its metabolites inosine, hypoxanthine, and xanthine were measured in cell culture supernatants and aqueous humor samples by HPLC.

RESULTS. All four adenosine receptor subtypes (A2A > A1 > A2B > A3) were coexpressed but differently distributed in the ciliary epithelium of control eyes, with the A3 receptor being localized to the basolateral membrane infoldings of the nonpigmented epithelial cells. A selective, approximately 10-fold upregulation of A3 receptor mRNA and protein was consistently found in the nonpigmented ciliary epithelium of all PEX eyes, without and with glaucoma, compared with the normal and glaucomatous control eyes. Significant upregulation of A3 receptor message in nonpigmented epithelial cells was induced by both hypoxia and oxidative stress in vitro, together with increased levels of inosine, hypoxanthine, and xanthine in the supernatants. Levels of adenosine and its metabolites, however, were not significantly elevated in the aqueous humor of patients with PEX.

CONCLUSIONS. Considering the known role of the A3 adenosine receptor in modulating aqueous humor secretion, its selective, probably hypoxia-induced upregulation in the ciliary epithelium may not only confer cytoprotection but also influence aqueous humor dynamics and may be accessible to therapeutic intervention in patients with PEX. (Invest Ophthalmol Vis Sci. 2005;46:2023–2034) DOI:10.1167/iovs.04-0915

The purine nucleoside adenosine is present in all tissues and body fluids and is known to function as a modulator of a variety of physiologic processes, such as the regulation of cellular growth and differentiation, vasodilation and blood flow, inflammatory responses, central and peripheral neural function, neuroprotection, and apoptosis. Adenosine levels in tissues change with activity and energy demand. In metabolic stress conditions, such as hypoxia or ischemia, the concentrations of adenosine and its metabolites inosine, hypoxanthine, and xanthine in the extracellular fluid increase dramatically, mainly through breakdown of adenosine triphosphate (ATP). One of the primary roles of adenosine is cytoprotection against ischemia-induced cell damage, mainly in tissues such as the heart, brain, and kidney, which are especially prone to ischemic injury. The cytoprotective role of adenosine is thought to be mediated by vasodilation, reduction of oxygen demand, suppression of formation of reactive oxygen species, increase in glucose uptake, decrease in the release of excitatory neurotransmitters, and inhibition of calcium influx. These protective effects are mediated by activation of four pharmacologically and biochemically distinct adenosine receptors—A1, A2A, A2B, and A3—which belong to the family of G-protein-coupled receptors with different intracellular signaling pathways. The A1 and A3 receptor subtypes couple to G-proteins, mediating the inhibition of adenyl cyclase and a decrease in cAMP levels, whereas the A2A and A2B receptors activate adenyl cyclase and increase cAMP levels via the stimulatory G-proteins.

Molecular and pharmacological studies have provided evidence that all adenosine receptor subtypes are expressed in ocular tissues. Activation of these receptors has been shown to regulate retinal neurotransmission and neuroprotection, retinal and choroidal blood flow, photoreceptor phagocytosis, and integrity of the blood–retinal barrier. The adenosine system has also been shown to regulate ion transport in the cornea and the ciliary epithelia and to modulate aqueous humor in- and outflow. Recent animal studies in animal models have demonstrated that adenosine receptor agonists and antagonists can alter intraocular pressure (IOP) in vivo. However, in eyes of healthy subjects, parenteral infusion of adenosine induced a small but significant decrease in IOP. Apart from an involvement in IOP modulation, adenosine and its receptors have been implicated in many ocular and
systemic ischemic diseases (e.g., retinal ischemia) and in conditions associated with oxidative stress.\(^7\) Ischemia and anterior chamber hypoxia are well-recognized features of eyes with pseudoexfoliation (PEX) syndrome, a common age-related extracellular matrix disorder that often leads to the development of ocular hypertension and secondary open-angle glaucoma.\(^5\) Marked alterations of the iris vasculature leading to a progressive oblitative vasculopathy have been well documented clinically, angiographically, and morphologically and account for significantly reduced oxygen partial pressure in the anterior chamber of PEX eyes.\(^6\) In addition, increased concentrations of oxidative stress markers, such as 8-isoprostaglandin-F\(_2\alpha\), have been measured in the aqueous humor of PEX eyes.\(^7\)

An involvement of the adenosine system in ischemia/hypoxia and IOP elevation in PEX eyes may therefore be hypothesized. This hypothesis has been substantiated by previous findings, in differential gene expression analyses, of a more than 30-fold overexpression of A3 adenosine receptor mRNA in the ciliary processes of PEX eyes compared with control eyes (Schlötzер-Schrehardt U, et al. IOVS 2004;45:ARVO E-Abstract 3535). We therefore further investigated the role of the adenosine system in eyes with PEX syndrome, without and with glaucoma, in comparison to eyes with primary open-angle (POAG) or angle-closure (ACG) glaucoma and normal donor eyes. In particular, we analyzed the expression of adenosine receptors in anterior segment tissues on the mRNA and protein level and measured the concentration of adenosine and its catabolites inosine, hypoxanthine, and xanthine in aqueous humor samples. In addition, we studied the effects of hypoxia and oxidative stress on adenosine synthesis and adenosine receptor expression by ciliary epithelial cells in vitro. The findings provided evidence of a selective upregulation of the A3 adenosine receptor in the nonpigmented ciliary epithelium of all PEX eyes, independent of the presence of glaucoma, that was also induced by hypoxia or oxidative stress in vitro.

**METHODS**

**Tissues and Samples**

Anterior segment tissues from 10 donor eyes with PEX syndrome (75.1 ± 7.9 years), 8 donor eyes with a history of POAG (78.8 ± 13.7 years), and 10 normal-appearing donor eyes (76.5 ± 6.2 years) were obtained at autopsy and fixed or processed within 10 hours after death. In addition, two pairs of donor eyes (79 and 81 years of age) with clinically and macroscopically unilateral PEX syndrome were used. The diagnosis of PEX syndrome was established by macroscopic observation of the presence of characteristic PEX deposits on ciliary processes, zonules, and lens and iris surfaces and confirmed by electron microscopy. Optic nerve cross sections were cut to exclude the presence of glaucomatous optic nerve atrophy. The diagnosis of PEX was taken from the patients’ files, and glaucomatous optic atrophy was confirmed by optic nerve cross sections. The normal donor eyes had no history or morphologic evidence of any known ocular disease.

We further included 5 eyes with PEX-associated open-angle glaucoma (80.2 ± 7.6 years), 5 eyes with PEX-associated closed-angle glaucoma (79.7 ± 5.1 years), 3 eyes with POAG (81.3 ± 1.5 years), and 10 eyes with secondary ACG due to rubecious iridis (77.3 ± 9.8 years), all of which had to be surgically enucleated because of painful absolute glaucoma or associated malignant melanoma of the posterior choroid. These eyes were fixed or processed immediately after enucleation for optimal preservation of RNA.

Aqueous humor was aspirated intraoperatively from eyes of 10 patients with PEX syndrome without glaucoma (mean age, 79.8 ± 7.9 years), 10 patients with PEX glaucoma (mean age, 76.2 ± 8.4 years), 10 patients with POAG (mean age 75.4 ± 7.5 years), and 10 patients with cataract (mean age, 78.8 ± 8.0 years) during cataract or filtration surgery. One hundred microliters of aqueous humor were withdrawn through an abexterno limbal paracentesis site with a 27-gauge needle on a tuberculin syringe. The samples were immediately frozen in liquid nitrogen and stored at −80°C for up to 3 months. Patients with ophthalmic diseases other than glaucoma or cataract or with previous surgery were excluded from the study.

Informed consent to tissue and aqueous humor donation was obtained from the patients or, in case of donor eyes obtained at autopsy, from the donors’ relatives. The protocol of the study was approved by the local Ethics Committee and adhered to the tenets of the Declaration of Helsinki for experiments involving human tissue and samples.

**Semiquantitative and Real-Time RT-PCR**

Total RNA was isolated from ciliary tissues, iris tissue, lens epithelium, and trabecular meshwork specimens obtained from normal donor eyes or donor eyes with PEX syndrome and from surgically enucleated eyes with PEX-associated OAG or ACG or with POAG or ACG. RNA extraction was then performed (RNeasy kit; Qiagen, Hilden, Germany) and included an on-column DNase I digestion step, according to the manufacturer’s instructions.

First-strand cDNA synthesis was performed with 1 μg of total RNA, 200 U reverse transcriptase (Superscript II; Invitrogen, Karlsruhe, Germany), and 500 ng oligo dT primers (Roche Diagnostics, Mannheim, Germany) in a 20-μL reaction volume. Gene-specific primers (MWG Biotech, Anzing, Germany) were designed to anneal with sequences located in different exons by means of Primer 3 software.\(^6\) The identity of PCR fragments was subsequently confirmed by sequence analysis with a sequence analyzer (Prism 3100; Applied Biosystems [ABI], Foster City, CA).

For semiquantitative RT-PCR, normalization of cDNA samples from different specimens was performed in 25-μL PCR reaction volumes with primers for glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) and dilutions of the first-strand products. Dilutions resulting in the same band intensities were used for analytic amplifications. Amplification of each gene was performed at the exponential phase in 25-μL reaction volumes containing 0.5 μL of the normalized first-strand reaction product, 0.2 μM of 5′ and 3′ primers, 200 μM of each dNTP, 0.65 U Taq DNA polymerase (HotStar; Qiagen), in a program with an initial denaturation step of 95°C for 15 minutes, and 35 to 45 cycles of 95°C for 15 seconds, 56°C (58°C and 60°C, respectively) for 30 seconds, and 72°C for 1 minute. PCR products were analyzed in 1.2% agarose gels containing 250 ng/mL ethidium bromide. Images were captured, and band intensities were quantitated by densitometry (EagleEye II system; Stratagene, La Jolla, CA).

Quantitative real-time PCR was performed with a thermal cycler (Cycler IQ Thermal Cycler; Bio-Rad, Munich, Germany). A typical PCR reaction (25 μL) contained 0.5 μL of first-strand product (corresponding to 25 ng of total RNA), 0.4 μM of 5′ and 3′ primers, 3.5 mM MgCl\(_2\), (GAPDH, A3 adenosine receptor) or 4 mM MgCl\(_2\) (A1, A2A, and A2B receptors), respectively, and PCR mix (IQ SYBR Green Supermix; Bio-Rad) according to the manufacturer’s instructions. All samples were analyzed in triplicate, in a program with an initial denaturation step of 95°C for 3 minutes and 40 cycles of 95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds. For quantification, standard curves using serial dilutions (10\(^1\) to 10\(^3\) copies) of plasmid-cloned specific amplification products were run in parallel, and amplification specificity was checked by using melting-curve analysis. For standardization of levels of gene expression, mRNA ratios relative to the housekeeping gene GAPDH were calculated.

**Northern Hybridization**

Five micrograms of total RNA was separated by denaturing agarose/formaldehyde gel electrophoresis, transferred to nylon membranes (ZetaProbeGT; Bio-Rad), and fixed by UV cross-linking. The membranes were prehybridized for 6 hours at 42°C in hybridization buffer containing 53% formamide, 5× SSC, 5% Denhardt’s solution, 0.5% SDS, 100 μg/mL of denatured salmon sperm DNA, and 100 mM potassium phosphate (pH 6.5). The hybridization probe against the human A3...
adenosine receptor was labeled with [α-32P]dCTP (Prime-It II Random-Primer labeling kit; Stratagene), according to the manufacturer’s instructions, and column-purified (Mini Quick Spin columns; Roche Diagnostics). The specificity of the probe was confirmed by sequence analysis. The membranes were hybridized with the cDNA probe at a concentration of 2×10^6 cpm/ml overnight at 42°C. After hybridization, the membranes were washed in 2×SSC and 0.5% SDS and in 0.5× SSC and 0.1% SDS. Blots were exposed on imaging plates (Fuji; Düsseldorf, Germany) and analyzed with a phosphorescence imager (BAS-2000 Phosphor-Imager; Fujifilm). The band intensities were quantitated on computer (Tina 2.0 software; Raytest, Straubenhardt, Germany). For normalization, blots were hybridized with a cDNA probe for the housekeeping gene GAPDH.

### In Situ Hybridization

The cDNA probe specific for the human A3 adenosine receptor was cloned into the pCR II vector (Invitrogen). In vitro transcription of the linearized constructs was performed with T7 and Sp6 RNA polymerase (Promega, Mannheim, Germany) in the presence of digoxigenin-11-uridine triphosphate (DIG-UTP) to produce DIG-labeled single-strand antisense or sense RNA probes with the DIG-RNA labeling kit (Roche Diagnostics) used according to the instructions of the manufacturer. The quality of the transcripts was controlled by using denaturing formaldehyde-agarose gels before and after DNase I digestion. Whole eyes, with and without PEX syndrome, were fixed in freshly prepared 4% buffered paraformaldehyde overnight and embedded in paraffin. For in situ hybridization of 6-μm-thick paraffin sections, deparaffinized sections were rehydrated, pretreated with proteinase K (20 μg/ml), postfixed, acetylated in 0.25% acetic anhydride, and hybridized for 16 hours at 44°C in chemiluminescent gold hybridization buffer (ECL; Amersham Biosciences, Freiburg, Germany) containing 0.3 M NaCl, 5% blocking reagent (Roche Diagnostics) and 1 ng/ml DIG-labeled RNA probe. After hybridization, the sections were washed at 40°C in 1× SSC for 10 minutes and in 0.5× SSC for 1 hour; treated with RNase A (5 μg/ml) and RNase T1 (25 U/ml) in 2.5 M NaCl, 50 mM Tris-HCl, and 25 mM EDTA (pH 7.5) for 30 minutes at 40°C; and washed again in 1× SSC for 10 minutes at room temperature for 2 hours at 50°C in 0.1× SSC and for 30 minutes at room temperature in 0.5× SSC. For detection of hybridized probes, slides were incubated with a sheep anti-DIG antibody conjugated to alkaline phosphatase (Roche Diagnostics) diluted 1:2000 in 0.1 M maleate buffer (pH 7.5) for 30 minutes at room temperature, and the color reaction was processed with 0.5 mM nitroblue tetrazolium chloride (NBT) and 0.5 mM 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) for 3 days at room temperature.

Hybridization with sense strand riboprobes served as the negative control and with 18S rRNA probes served as the positive control.

### Immunohistochemistry

Light microscopic indirect immunofluorescence and electron microscopic immunogold labeling were performed on anterior segment tissues of PEX and control eyes, as previously described. 37 Goat polyclonal antibodies against adenosine receptors A1, A2A, A2B, and A3 (Santa Cruz Biotechnology, Santa Cruz, CA) were used. Antibody binding was detected by Cy3-conjugated secondary antibodies (Molecular Probes, Eugene, OR) or by 10 nm gold-conjugated secondary antibodies (BioCell, Cardiff, UK). In negative control experiments, the primary antibodies were replaced by PBS or equimolar concentrations of non-immune goat IgG, or an irrelevant primary antibody.

### Cell Culture and Cell Viability Assay

To study the effect of hypoxia and oxidative stress on adenosine receptor expression, the immortalized human pigmented ciliary epithelial cell line ODM-235 was used at passage 16. Cells were maintained in DMEM/Ham’s F12 (Invitrogen-Gibco, Grand Island, NY) containing 10% fetal calf serum (FCS) and 50 μg/ml gentamicin in a 95% air and 5% CO2 humidified atmosphere at 37°C. Cells were grown to subconfluence and then exposed to hypoxia (2% oxygen) in an incubator equipped with a polarographic oxygen electrode for 1 to 3 days or treated with different concentrations (0.025–10 mM) of hydrogen peroxide (H2O2) for 1 hour. Cultures maintained under normoxia (21% oxygen) or without addition of H2O2 served as the control. Cells were harvested, the total RNA was extracted as described earlier, and cell culture supernatants were analyzed by HPLC.

A quantitative assessment of cell viability was made with a commercially available fluorescent system (Live/Dead Viability/Cytotoxicity Kit; Molecular Probes), according to the instructions of the manufacturer. Cells were grown in 12-well plates and underwent the same exposure protocols as the experimental cells. All experiments were performed in six replicates. Incubating the cells in culture medium under normoxic conditions without addition of hydrogen peroxide for 24 hours served as the control. Fluorescence was measured with a microtiter plate reader (Fluoroscan Ascent 2.4; Tecan, Germany) using
The observed differences in A3 adenosine receptor mRNA expression in ciliary process tissue of PEX and control eyes were further analyzed by quantitative real-time RT-PCR, by Northern hybridization, and by in situ hybridization. Real-time PCR confirmed a moderate basal expression of A3 receptor mRNA in the ciliary body of normal and glaucomatous control eyes without PEX (n = 3), and a significant upregulation (9- to 12.5-fold; mean, 10.5 ± 1.3; P < 0.0001) in all PEX eyes with and without open- and closed-angle glaucomas (n = 3; Fig. 2). PCR analysis also confirmed an ~10-fold increase in A3 receptor mRNA levels in PEX-positive eyes (n = 2) compared with their noninvolved contralateral eyes (n = 2), in clinically unilateral cases (Fig. 2).

Northern hybridization confirmed overexpression of mRNA coding for the A3 adenosine receptor in ciliary process tissues from two PEX eyes with glaucoma compared with two glaucomatous control eyes without PEX (Fig. 3). Densitometric analysis of the normalized band intensities demonstrated an eight- to ninefold (mean, 8.6 ± 1.3) overexpression in the PEX samples.

By in situ hybridization, signals for A3 adenosine receptor mRNA were localized mainly to the nonpigmented epithelium of the ciliary processes and were confirmed to be increased in eyes with PEX syndrome and PEX glaucoma compared with normal and glaucomatous control eyes (Fig. 4). Increased signals were also observed in the walls of iris blood vessels of PEX eyes. In addition, weak signals for A3 receptor mRNA were detected in the basilar layer of the corneal epithelium, walls of conjunctival blood vessels, trabecular meshwork, dilator and sphincter muscles of the iris, and ciliary muscle cells of PEX and control eyes without showing clear differences between the groups. Tissue sections hybridized with the sense probe revealed no signals. Hybridization with the 18S rRNA probe revealed equally distributed intense signals throughout all anterior segment tissues of PEX and control eyes indicating proper RNA preservation.
Antibody binding was abolished completely when nonimmune serum or PBS was used instead of the primary antibodies (Figs. 5G, 5H).

Levels of Adenosine, Inosine, Hypoxanthine, and Xanthine in Aqueous Humor

To determine the concentration of adenosine and its catabolic reaction products inosine, hypoxanthine, and xanthine in aqueous humor, 10 aqueous samples from each of four groups of patients (PEX syndrome, PEX glaucoma, POAG, and cataract) were analyzed. Whereas the concentration of adenosine was below the limits of detection (<200 nM) in all aqueous humor samples, inosine, hypoxanthine, and xanthine were reliably measured in all samples. The aqueous concentrations of inosine were 1.13 ± 0.45 μM; of hypoxanthine, 0.88 ± 0.83 μM; and of xanthine, 9.43 ± 2.64 μM, in all groups of patients.
Although the hypoxanthine and xanthine levels were generally higher in both PEX groups (1.13 ± 0.70 and 10.07 ± 3.14 μM, respectively; n = 20) compared with the cataract group (0.66 ± 0.26 and 8.49 ± 2.46 μM, respectively; n = 10), the differences were not statistically significant (P = 0.07).

Effects of Hypoxia and Oxidative Stress on A3 Adenosine Receptor Expression and Adenosine Secretion

In search of pathogenetic factors that could be responsible for A3 receptor upregulation, we studied the effect of hypoxia (2% oxygen) and oxidative stress (0.025–10 mM hydrogen peroxide) on A3 receptor mRNA expression and on secretion of adenosine and its catabolites by human nonpigmented ciliary epithelial cells in vitro. Viability assays, conducted in parallel, revealed an average ratio of living versus dead cells of 39 in control wells (n = 6). Incubation of the cells in experimental wells (n = 6) with various periods of hypoxia or increasing concentrations of hydrogen peroxide up to 4 mM revealed ratios of living versus dead cells that were not significantly different from the control (Fig. 7). Due to significantly lower relative numbers of live cells after exposure to 10 mM H₂O₂ (P = 0.002), this experimental parameter was excluded from further evaluations.

Quantitative real-time PCR demonstrated that A3 receptor mRNA expression is subject to regulation by both hypoxia and oxidative stress, showing peaks of an approximately 5-fold
upregulation after 2 days of hypoxia and an 11-fold upregulation after exposure to 0.25 mM H$_2$O$_2$ for 1 hour (Fig. 8). Statistically significant upregulation of A3 receptor mRNA was observed after 24 (2.5-fold; \( P = 0.005 \)), 48 (4.7-fold; \( P = 0.01 \)), and 72 (2.1-fold; \( P = 0.009 \)) hours of hypoxia (Fig. 8A) as well as after treatment with 0.25 (11.6-fold; \( P = 0.005 \)), 0.5 (7.1-fold; \( P = 0.005 \)), 1.0 (6.7-fold; \( P = 0.001 \)), 2.0 (7.5-fold; \( P < 0.0001 \)), and 4.0 (2.3-fold; \( P = 0.001 \)) mM of H$_2$O$_2$ for 1 hour (Fig. 8B).

HPLC analysis of cell culture supernatants showed that hypoxia for 1 to 3 days and oxidative stress for 1 hour had a dose-dependent effect on the extracellular levels of inosine, hypoxanthine, and xanthine, whereas concentrations of adenosine (<200 nM) again could not be reliably measured (Figs. 9A, 9B). Hypoxia induced a significant increase in hypoxanthine (approximately fivefold) and xanthine (approximately fourfold) levels after 3 days of exposure to 2% oxygen (\( P = 0.01 \)). The increase in inosine levels (approximately twofold) was not statistically significant (Fig. 9A). Oxidative stress induced significant increases in inosine levels (approximately threefold) at a concentration of 4 mM H$_2$O$_2$ and in hypoxanthine levels (approximately twofold) at concentrations of \( \geq 2 \) mM H$_2$O$_2$ (\( P = 0.04 \)). The increase in xanthine levels (approximately 1.5-fold) was not statistically significant (Fig. 9B). Although the ratio of living versus dead cells treated with hypoxia did not correlate with metabolite levels, there was a positive correlation between the decreased ratio of living cells and the increased concentrations of inosine (\( P = 0.02 \)), hypoxanthine (\( P = 0.008 \)), and xanthine (\( P = 0.02 \)) after H$_2$O$_2$ treatment.

### Discussion

**Expression and Functional Significance of Adenosine Receptors in Intra- and Extraocular Tissues**

In the eye, molecular and pharmacological studies have provided evidence that all adenosine receptor subtypes are expressed in ocular tissues.\(^8\)–\(^8\) Using in situ hybridization, Kvanta et al.\(^8\) localized weak adenosine receptor mRNA expression to the ciliary body and retina of the rat eye. They showed that A1, A2A, and A2B mRNA were weakly expressed in the ciliary processes, but found no significant A3 receptor mRNA expression. Mitchell et al.,\(^4\) however, clearly demonstrated expression of A3 receptor message in cultured human ciliary epithelial cells and rabbit ciliary processes by RT-PCR.

Activation of these receptors has been shown to modulate retinal function and blood flow.\(^8\)–\(^11\)–\(^21\) Neuroprotection,\(^1\) corneal deturgescence,\(^2\)–\(^7\) outflow facility,\(^8\)–\(^27\) and ion transport in human trabecular meshwork cells\(^1\) and ciliary epithelial cells.\(^2\)–\(^4\)\(^,\)\(^10\) A1 and A2 receptors have been associated with reduction and increase of IOP, respectively, in rabbits\(^2\)–\(^4\) and monkeys.\(^2\) The hypotensive effect resulted primarily from increased outflow facility in the trabecular meshwork, whereas aqueous humor inflow was not influenced by A1 and A2 receptor agonists.\(^2\) In contrast, A3 agonists have been shown to activate chloride channels in nonpigmented ciliary epithelial cells in vitro, leading to the hypothesis that A3 receptor agonists would increase aqueous humor secretion and thereby IOP in vivo.\(^4\) These predicted effects could be subsequently confirmed by Avila et al.,\(^2\) who provided pharmacological evidence that A3-selective agonists and antagonists modulate IOP in the mouse eye, with A3 receptor agonists increasing aqueous humor secretion and IOP. Moreover, in A3 receptor knockout mice, baseline IOP was significantly lower than in wild-type animals and A3-selective agonists and antagonists did not affect IOP.\(^2\) The physiologic and nonselective agonist adenosine produced only a moderate elevation of IOP in the knockout animals that was 7 to 10 times lower than in control mice. Further along this line, topical administration of selective A3 receptor antagonists was recently reported to reduce IOP in monkey eyes.\(^3\) These observations supported the conclusion that adenosine acts mainly through A3 adenosine receptors to regulate IOP.

In extraocular tissues, the A3 receptor has been mainly implicated in ischemic disease, such as ischemic brain damage or cardiac ischemia.\(^4\) Although the physiological implications of A3 receptors are still largely unknown, they have been shown to be involved in chloride transport of epithelial tissues\(^2\) as well as in the cardioprotective effect of adenosine during cardiac ischemia and in ischemic preconditioning, a process by which brief intermittent periods of ischemia provide protection against a more sustained ischemic episode.\(^2\)–\(^6\)\(^,\)\(^10\) Increasing evidence suggests that adenosine mediates its cytoprotective actions mainly by interacting with A3 receptors. Selective A3 receptor upregulation was observed,
for instance, by RBL-2H3 cells in vitro after exposure to oxidative stress and after preconditioning of central nervous tissue, whereas expression of the other receptor subtypes remained unchanged. Activation of the A3 receptor is known to activate the cellular antioxidant defense system by increasing the activities of superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase, along with a reduction in malondialdehyde, a marker of lipid peroxidation. Such a mechanism may provide a means by which adenosine exerts a cytoprotective action in ischemic conditions.

In the present study, all adenosine receptor subtypes were coexpressed but differently distributed in the human ciliary epithelium in a subtype-specific pattern complementing previous studies on animal models and cultured cells. Expression of more than one type of adenosine receptors on the same cells may allow the agonist adenosine to activate multiple signaling pathways, allowing the possibility of reciprocal control and fine tuning of physiological functions, such as ion transport and aqueous humor formation. The A3 receptor subtype was immunolocalized to the basolateral surface infoldings of the nonpigmented ciliary epithelial cells, which is consistent with the receptor’s functional role in aqueous humor secretion.

This study further provided evidence of a selective and significant upregulation of the A3 adenosine receptor on both the mRNA and protein levels, in the nonpigmented ciliary epithelium of all eyes with PEX syndrome, independent of the presence or type of glaucoma. Significant upregulation of A3 receptor message in nonpigmented ciliary epithelial cells was also induced in vitro by hypoxia and oxidative stress in a dose-dependent manner, suggesting that hypoxia/ischemia and/or oxidative stress may promote A3 receptor upregulation in vivo. It has to be kept in mind, however, that the in vitro data were obtained by using an immortalized ciliary epithelial cell line and may not reflect the actual in vivo situation.

**Levels of Adenosine and Its Metabolites in Aqueous Humor**

Adenosine has been reported to be present in aqueous humor. The adenosine concentration in aqueous humor of porcine

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**FIGURE 5.** Immunofluorescence labeling of adenosine receptor subtypes in ciliary processes of normal control (A–D) and PEX (E, F) eyes. (A) Immunolocalization of the A1 receptor to the nonpigmented ciliary epithelium. (B) The A2A receptor is present in both the nonpigmented and pigmented ciliary epithelium. (C) Immunolocalization of the A2B receptor to the apicalateral aspects of the nonpigmented epithelium. (D) The A3 receptor is present along the basal cell membrane of the nonpigmented epithelial layer (arrows). PEX eyes without (E) and with (F) open-angle glaucoma showed an increased immunoreaction for the A3 receptor along the basal aspects of the nonpigmented ciliary epithelium. Negative control sections (PEX syndrome) using PBS (G) or nonimmune serum (H) instead of the primary antibody. PC, posterior chamber; blue fluorescence, DAPI staining of nuclei. Original magnification, ×160.
eyes was determined to be approximately 300 nM in a highly sensitive fluorometric detection protocol. In normotensive patients, mean aqueous adenosine levels were 5.2 ng/100 μL corresponding to approximately 200 nM. In ocular hypertensive individuals, the mean aqueous adenosine and inosine levels were significantly elevated to 500 nM and were significantly correlated with IOP.

Adenosine most likely modulates aqueous humor flow by paracrine and/or autocrine mechanisms. The levels of adenosine are primarily determined from dephosphorylation of ATP and AMP by a series of ectonucleotidases (e.g., ecto-5'-nucleotidase). The ciliary epithelium has been reported to store and release ATP, which can then be converted to adenosine through ectoenzyme activity. To exert any physiological effect, the local concentration of adenosine is likely to be important, because the different adenosine receptor subtypes have different affinities for adenosine. A1 and A2A receptors may be constitutively activated by basal adenosine levels (10–200 nM) under physiological conditions, whereas the A2B and A3 receptors are only activated when adenosine levels are increased (5000–6500 nM) during pathologic conditions. During periods of ischemic stress, extracellular adenosine levels can rise to micromolar levels, but excess adenosine is rapidly metabolized to inosine and hypoxanthine by adenosine deaminase and further to xanthine and uric acid by xanthine oxidase.

Studies in the retina have demonstrated that brief periods of ischemia produce a rapid, but relatively short-lived elevation in extracellular adenosine, which peaked within 30 minutes after onset of ischemia, and a long-lasting elevation of its metabolites inosine and hypoxanthine, which continued to increase for the entire measurement period of 2 hours. The concentrations of adenosine metabolites exceeded those of adenosine itself, suggesting rapid metabolism of adenosine. These changes in purine levels were reported to be similar to those described for different cerebral ischemia models. Because of such effective clearance and conversion mechanisms, in vivo investigations of adenosine levels have rather relied on the measurement of the more stable intermediate products of adenosine metabolism, such as hypoxanthine and xanthine.

In contrast to previous findings, adenosine could not be reliably detected in aqueous humor samples from nonglaucomatous or glaucomatous PEX and control eyes, possibly because of methodological differences or the failure to add inhibitors of adenosine degradation to the sampling syringe. However, its more stable metabolites inosine, hypoxanthine, and xanthine could be reliably measured, but were not found to be significantly elevated in aqueous samples from patients with PEX. In accordance, adenosine was not detected in cell culture supernatants, whereas levels of inosine, hypoxanthine, and xanthine were increased after exposing NPE cells to hypoxia or oxidative stress. In agreement with our findings, adenosine levels in the vitreous were not consistently detectable.
during retinal ischemia, whereas inosine and hypoxanthine were more easily identified. However, the increased levels of adenosine metabolites in our in vitro experiments may also be related to cell death, since there was a positive correlation of metabolite levels with a decrease in living cells after H₂O₂ treatment.

Despite the lack of direct evidence of increased extracellular adenosine concentrations in the aqueous humor of PEX eyes, A3 receptors are very likely activated in a paracrine or autocrine manner by adenosine concentrations close to the plasma membrane of NPE cells (e.g., by release and metabolism of ATP). Moreover, the magnitude of adenosine receptor stimulation may be determined, not only by the amount of extracellular adenosine, but also by receptor upregulation. It has been reported that overexpression of A3 receptors during the ischemic preconditioning response may cause a hypersensitivity to adenosine, providing a more prolonged period of cytoprotection. Therefore, it may be assumed that A3 receptor upregulation in PEX eyes may also be associated with increased receptor activation.

**Functional Significance of A3 Receptor Upregulation in PEX Syndrome**

A3 receptor upregulation has been well demonstrated to be associated with PEX syndrome and is present in nonglaucomatous eyes as well as in eyes with open- or closed-angle glaucoma. This finding suggests that A3 receptor upregulation is not likely to be involved in glaucoma development. It is suggested that hypoxia and/or oxidative stress, typical of all eyes with PEX syndrome/glaucoma, promote a selective upregulation of A3 adenosine receptors in the nonpigmented ciliary epithelium, which may confer protection against ischemic or oxidative damage to sustain prolonged periods of chronic hypoxia or oxidative stress. Considering, however, the known properties of the A3 receptor in activating chloride transport in epithelial cells, its upregulation in the nonpigmented ciliary epithelium may have an additional influence on aqueous humor secretion and hence on IOP levels in PEX eyes.

It has been previously reported that IOP elevation in PEX patients results from an increased outflow resistance in the trabecular meshwork, whereas the rate of aqueous flow through the anterior chamber was not different or even slightly lower in PEX eyes than in control eyes. Aqueous humor formation may be compromised in PEX eyes due to heavy involvement of the nonpigmented ciliary epithelium in PEX material production. A3 receptor upregulation in the ciliary epithelium may therefore also be a means to counteract this
assumed functional deficit, to maintain a more normal rate of aqueous humor formation.

Although this is the first report to provide evidence of a significant dysregulation of adenosine receptor expression (i.e., the A3 receptor subtype), in ocular disease, the precise role of this receptor in cytoprotection and in the regulation of aqueous humor dynamics in normotensive and hypertensive individuals with PEX syndrome requires additional studies. Nevertheless, the present findings could be of clinical and therapeutic significance and the reduction of choroidal channel activity with A3 receptor antagonists may be an alternative specific approach for treating ocular hypertension in patients with PEX who are refractory to standard medical therapy.

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2. Roth S, Rosenbaum PS, Osinski J, et al. Ischemia induces significant dysregulation of adenosine receptor expression (i.e., the A3 receptor subtype), in ocular disease, the precise role of this receptor in cytoprotection and in the regulation of aqueous humor dynamics in normotensive and hypertensive individuals with PEX syndrome requires additional studies. Nevertheless, the present findings could be of clinical and therapeutic significance and the reduction of choroidal channel activity with A3 receptor antagonists may be an alternative specific approach for treating ocular hypertension in patients with PEX who are refractory to standard medical therapy.

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