Endogenous Production of Extracellular Adenosine by Trabecular Meshwork Cells: Potential Role in Outflow Regulation

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PURPOSE. To investigate the mechanisms for endogenous production of extracellular adenosine in trabecular meshwork (TM) cells and evaluate its physiological relevance to the regulation of aqueous humor outflow facility.

METHODS. Extra-cellular levels of adenosine monophosphate (AMP) and adenosine in porcine trabecular meshwork (PTM) cells treated with adenosine triphosphate (ATP), AMP, cAMP or forskolin with or without specific inhibitors of phosphodiesterases (IBMX) and CD73 (AMPCP) were determined by high-pressure liquid chromatography fluorometry. Extracellular adenosine was also evaluated in cell cultures subjected to cyclic mechanical stress (CMS) (20% stretching; 1 Hz) and after disruption of lipid rafts with methyl- β -cyclodextrin. Expression of CD39 and CD73 in porcine TM cells and tissue were examined by Q-PCR and Western blot. The effect of inhibition of CD73 on outflow facility was evaluated in perfused living mouse eyes.

RESULTS. PTM cells generated extracellular adenosine from extracellular ATP and AMP but not from extracellular cAMP. Increased intracellular cAMP mediated by forskolin led to a significant increase in extracellular adenosine production that was not prevented by IBMX. Inhibition of CD73 resulted, in all cases, in a significant decrease in extracellular adenosine. CMS induced a significant activation of extracellular adenosine production. Inhibition of CD73 activity with AMPCP in living mouse eyes resulted in a significant decrease in outflow facility.

CONCLUSIONS. These results support the concept that the extracellular adenosine pathway might play an important role in the homeostatic regulation of outflow resistance in the TM, and suggest a novel mechanism by which pathologic alteration of the TM, such as increased tissue rigidity, could lead to abnormal elevation of IOP in glaucoma. (*Invest Ophthalmol Vis Sci.* 2012;53:7142–7148) DOI:10.1167/iovs.12-9968

The conventional outflow pathway is believed to be the main site of homeostatic regulation of IOP.^{1–5} It has been proposed that such homeostatic regulation of IOP could involve the release by trabecular meshwork (TM) cells of factors capable of increasing outflow facility in response to

mechanical strain induced by elevated IOP.^{6–15} One of the extracellular signaling mechanisms that could potentially contribute to a feedback regulation of outflow facility is the extracellular adenosine pathway.

There is substantial evidence that functional A₁, A_{2a}, and A₃ adenosine receptors are expressed in the cells of the outflow pathway^{10,16–19} and their activity has been shown to exert significant effects in aqueous outflow facility and IOP.^{20–29} Several adenosine receptor agonists and antagonists are currently being evaluated as potential therapeutic agents for the treatment of glaucoma.³⁰ Because of the observed effects of adenosine receptor agonists and antagonists in the physiology of the outflow pathway, it has been proposed that endogenous production of extracellular adenosine by TM cells in response to different stimuli, such as hypotonic stress or cyclic mechanical stress (CMS), could potentially contribute to the physiologic regulation of aqueous humor outflow and IOP.³¹ However, the specific mechanisms that might be involved in the endogenous production of extracellular adenosine in TM cells have not been investigated.

Extracellular adenosine is generated in multiple cell types, including proximal tubular cells, cardiac fibroblasts, and glomerular mesangial cells, by efflux of cyclic adenosine monophosphate (cAMP) mediated by members of the adenosine triphosphate (ATP)-binding cassette transporter family. cAMP is then converted into adenosine monophosphate (AMP) by ecto-phosphodiesterase (ecto-PDE), which is dephosphorylated into adenosine via CD73 ecto-nucleotidase.^{32–35} In contrast, the main route for generation of extracellular adenosine in human urinary tract epithelial cells, lens cells, and retinal pigment epithelial cells, appears to involve the extracellular release of ATP followed by dephosphorylation to AMP by ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1), also known as CD39, and metabolization of AMP into adenosine by CD73.^{36,37} Although it has been hypothesized that the release of ATP observed in TM cells in response to several stimuli might contribute to the production of extracellular adenosine in the outflow pathway,³¹ the potential contribution of the different extracellular adenosine pathways to the production of extracellular adenosine in the cells of the outflow pathway has not been investigated. Similarly, the potential physiologic relevance of endogenous production of extracellular adenosine in the regulation of outflow facility has yet to be investigated.

Given the observed effects of the adenosine receptors in the modulation of outflow facility, elucidating the specific pathways involved in the endogenous production of extracellular adenosine by TM cells might be particularly relevant to the understanding of the physiologic mechanisms by which the TM modulates outflow facility and maintains normal levels of IOP. Therefore, we investigated the specific molecular pathways that might contribute to the production of extracel-

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lular adenosine in primary porcine TM (PTM) cells and evaluated whether endogenous production of extracellular adenosine exerts a significant effect on aqueous humor outflow facility in vivo.

METHODS

Cell Culture and Treatments

PTM cells were obtained from fresh pig eyes.¹⁹ PTM was dissected from surrounding tissue, digested in 10 mg collagenase/20 mg bovine serum albumin (BSA)/5 mL phosphate buffer saline (PBS) solution. The cells were seeded on gelatin-coated 10-cm Petri dishes and maintained at 37°C in 5% CO₂ in medium (low glucose Dulbecco's Modified Eagle Medium with L-glutamine, 110 mg/mL sodium pyruvate, 10% fetal bovine serum, 100 µM nonessential amino acids, 100 units/mL penicillin, 100 µg/mL streptomycin sulfate and sulfate (Invitrogen, Grand Island, NY). For evaluation of potential precursors of extracellular adenosine, cells were incubated with ATP (15 µM), AMP (15 µM), cAMP (15 µM), or forskolin (15 µM) in the absence and presence of specific inhibitors of phosphodiesterase (IBMX, 10 µM) or ecto-5'-nucleotidase (AMPCP, 0.1 mM) for 30 and 90 minutes. Lipid rafts were disrupted with methyl-β-cyclodextrin (MβCD) (10 mM) for 30 and 90 minutes (Sigma, St. Louis, MO). The extracellular levels of AMP and adenosine production were measured by high-pressure liquid chromatography (HPLC).

Cyclic Mechanical Stress

PTM cells on passage 4 or 5 were plated on type I collagen-coated flexible silicone bottom plates (Flexcell, Hillsborough, NC). One day after confluence, cells were stressed for different periods of time (30 and 90 minutes) (20% stretching, 1 Hz), using the computer-controlled, vacuum-operated FX-3000 Flexercell Strain Unit (Flexcell). A frequency of 1 Hz was selected to mimic cardiac frequency.¹⁹ Control cells were cultured under the same conditions, but no mechanical force was applied. The extracellular levels of AMP and adenosine production were measured by HPLC.

Analysis of Extracellular AMP and ADO by HPLC

HPLC-fluorometry analysis was performed according to a published method³⁸ modified to accommodate available instrumentation and the study sample matrix. In brief, 50 µL of each sample was combined with 50 µL of 1 M acetic acid (pH 4.5 adjusted by NaOH) and 5 µL of chloroacetaldehyde, and incubated for 30 minutes at 80°C. A 10 µL aliquot of this mixture was directly injected into a Waters 2695 Alliance separations module HPLC system (Waters, Milford, MA) equipped with a Phenomenex-Luna C18 HPLC fully porous silica column (150 × 3 mm, 5 µm particle size) (Phenomenex, Torrance, CA) and Waters 2475 fluorescence detector (Waters). Reverse-phase separation was conducted using a mixture of 0.01 M sodium dihydrogen phosphate (pH 3.0)-methanol as the mobile phase at a flow rate of 0.5 mL/min. Samples were separated using a tray temperature of 4°C and a column temperature of 45°C. Fluorescence detection of AMP and adenosine (ADO) was conducted using the following settings: excitation at 280 nm, emission at 420 nm, gain 1. Identification of AMP and ADO was based on retention time when co-injected with external standards (Sigma-Aldrich, St. Louis, MO). Quantification was based on the external standard method. Standard curves for AMP and ADO were generated with a concentration sequence of 0.162, 0.054, 0.18, 0.6, and 2 µM.

Quantitative Real-Time PCR

Total RNA was extracted from PTM cells and PTM tissue using RNeasy micro kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. One µg RNA was reversely transcribed into cDNA using

the superscript first-strand synthesis system (Invitrogen, Carlsbad, CA). Quantitative RT-PCR was performed with SYBR Green Supermix (Biorad, Hercules, CA). Each PCR reaction mixture (20 µL) contained 1 µL of template, 10 µL SYBR Green supermix, 1 µL forward primer (10 µM) and 1 µL reverse primer (10 µM). The mixture was initially incubated at 94°C for 10 minutes, followed by 40 cycles of 94°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. PCR reactions were performed in triplicate. Primers for quantitative PCR were as follows: PTM (CD39): 5'-ACTGAAATCTGCTGGCTGGAGTGA (forward), 5'-TTGGTGAGCAAGGAACGATGACCT (reverse). PTM (CD73): 5'-TGCAACCCGAAGTCGACAAGCTAA (forward), 5'-TGCAACCCGAAGTCGACAAGCTAA (reverse). Relative expression levels of each gene were evaluated as $\Delta\Delta C_t$ using actin as the internal control.

Western Blot

Porcine TM cells were washed twice in cold PBS and harvested using lysis buffer (150 mM NaCl, 20 mM Tris [pH 8.0], 1% NP-40, 0.1% SDS, and 1× protease inhibitor cocktail; Thermo Scientific, Rockford, IL) with a cell scraper. Tissue was homogenized in 20 mM Tris buffer, pH 7.4 containing 1 mM sodium orthovanadate, 0.2 mM EDTA, 0.2 mM PMSE, 0.1 M NaCl, 50 mM NaF, 1× protease inhibitor cocktail (Thermo Scientific). Protein concentration was determined using Micro BCA Protein Assay Kit (Pierce, Rockford, IL). Equal amounts of protein were separated by 10% SDS-PAGE gels and transferred to polyvinylidene fluoride membrane (Bio-Rad). Membranes were blocked with 5% nonfat dry milk and incubated overnight at 4°C with the primary antibodies anti-CD39, -CD73 (Santa Cruz Biotechnology, Santa Cruz, CA). They were incubated with secondary antibodies conjugated to horseradish peroxidase for 1 hour at room temperature. Immunoreactive bands were visualized by chemiluminescence using ECL Plus Western Blotting System (GE Healthcare, Piscataway, NJ). Membranes were reprobed with antitubulin β antibody (Sigma) for protein loading control.

Analysis of Outflow Facility in Living Mice Eyes

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and with all protocols and regulations established by the Institutional Animal Care University Committee of Duke University. CD1 mice were anesthetized with ketamine (60 mg/kg) and xylazine (5–10 mg/kg). Outflow facility was evaluated using a previously published method.³⁹ Briefly, a glass micro-needle was inserted in the anterior chamber of each eye through the cornea with the help of micromanipulators. To evaluate outflow facility simultaneously in both eyes of each mouse, each micro-needle was connected with pressure tubing and a three-way stopcock to a 10-mL syringe filled either with PBS or with 0.2 mM AMPCP in PBS, a vertical fluid column, and a pressure transducer (Honeywell model 140 PC; Honeywell Sensing and Control, Freeport, IL) linked to a data acquisition system (ML870/P PowerLab 8/30; AD Instruments, Colorado Springs, CO). Flow was calculated by monitoring the slight decline in pressure over time resulting from fluid exiting the system from the vertical fluid column. Although the pressure declined during the perfusion, this method was still considered a constant pressure perfusion because the pressure did not decrease more than 1.0 mm Hg for each experiment. Outflow was analyzed simultaneously in the control and experimental eye of each mouse. For each eye, outflow was calculated at an initial pressure of 35 mm Hg for 20 minutes. Then pressure was adjusted to 25 mm Hg for a second set of measurements.

Statistical Analysis

The data were presented as the mean ± SD. The significance of the data was analyzed using non-paired Student's *t*-test for experiments conducted with cultured cells, and paired Student's *t*-test for analysis of

FIGURE 1. PTM cells were incubated with different substances for 30 and 90 minutes, with and without ATP (15 μM) and AMPCP (0.1 mM) (A, B); with and without AMP (15 μM) and AMPCP (0.1 mM) (C, D); with and without AMPCP (0.1 mM) (E, F). The amounts of extracellular AMP (A, C, E) and adenosine (B, D, F) were determined by HPLC. Values are mean $\pm$ SD of three preparations. (a, b, and c) indicate $P < 0.05$ compared with control, ATP and AMP, respectively.

outflow facility. A probability of less than 5% was considered statistically significant.

RESULTS

Production of Extracellular Adenosine from ATP in PTM Cells

The ability of PTM cells to generate adenosine from extracellular ATP was evaluated in cultures incubated in the presence or absence of 15 μM ATP in the culture media. The presence of extracellular ATP resulted in a noticeable increase in the production of extracellular AMP that was increased more by CD73 inhibition with AMPCP and by a similar increase in extracellular adenosine that was almost completely inhibited by AMPCP (Figs. 1A, 1B). To further confirm the functionality of CD73, PTM cell cultures were incubated with 15 μM AMP in the presence and absence of the CD73 inhibitor AMPCP. As shown in Figures 1C and 1D, the addition of AMP to the media resulted in a sharp increase in adenosine concentration after 30 minutes of incubation that was abolished in the presence of AMPCP. In the absence of extracellular ATP or AMP, AMPCP led to significant accumulation of extracellular AMP and inhibited adenosine production (Figs. 1E, 1F).

Evaluation of the Ability to Convert Extracellular cAMP into AMP and Adenosine by PTM Cells

In order to test whether PTM cells could generate AMP and adenosine from extracellular cAMP, fresh culture media (i.e., PBS) containing 15 μM cAMP was added to the PTM cell cultures, and the concentrations of AMP and adenosine at 30 and 90 minutes of incubation were compared with those of control cultures without cAMP. As shown in Figures 2A and 2B, the presence of extracellular cAMP did not result in an increase of either AMP or adenosine by PTM cells. Consistent with this observation, Figures 2C and 2D indicate that the inhibition of phosphodiesterase activity with IBMX had no effects on AMP and adenosine production. To further confirm that cAMP was not a significant source of extracellular adenosine in PTM cells, we analyzed the effects of forskolin-induced cAMP on the generation of extracellular AMP and adenosine. As shown in Figures 2E and 2F, treatment with forskolin resulted in a statistically significant increase in extracellular AMP and adenosine; such an increase was not prevented by the inhibition of phosphodiesterase activity with IBMX. The results suggested that the increase in extracellular adenosine induced by forskolin did not result from metabolism of cAMP into adenosine.

FIGURE 2. PTM cells were incubated with different substances for 30 and 90 minutes, with and without cAMP (15 μM) for 30 and 90 minutes (A, B); with cAMP (15 μM) in absence or presence of IBMX (10 μM) and AMPCP (0.1 mM) (C, D) and with and without forskolin (15 μM) and IBMX (10 μM) (E, F). The amounts of extra-cellular AMP (A, C, E) and adenosine (B, D, F) were determined by HPLC. Values are mean $\pm$ SD of three preparations. a and b indicate $P < 0.05$ compared with control and cAMP, respectively.

Effects of Lipid Raft Disruption on Extracellular Adenosine

Disruption of lipid rafts in cultured PTM cells with M β CD resulted in a significant increase in the concentration of both AMP (fold = 5.2 for 30 minutes, fold = 39.06 for 90 minutes, both $P < 0.001$) and adenosine (fold = 3.47 for 30 minutes, fold = 4.35 for 90 minutes, both $P < 0.001$) in the media, which suggested that an important fraction of the extracellular production of adenosine might take place in compartments of the cell membrane (Figs. 3A, 3B).

Activation of the Extracellular Adenosine Pathway by CMS

Since CMS has been reported to induce an efflux of ATP in TM cells,^{15,19,40,41} we evaluated whether this increase in extracellular ATP could result in increased production of extracellular adenosine. As shown in Figures 4A and 4B, CMS (20% stretching, 1 Hz) caused a significant increase in the extracellular levels of AMP ($P < 0.001$) and adenosine ($P < 0.001$) at both 30 and 90 minutes compared with parallel control cultures not subjected to CMS.

Expression of CD39 and CD73 in PTM Cells and Tissue

The two enzymes involved in the production of extracellular adenosine from ATP, CD39 and CD73, were confirmed in PTM

cells and PTM tissue by quantitative real time polymerase chain reaction (qPCR) and Western blot. The ΔCt value obtained by qPCR for CD39 and CD73 in cells were 9.320 ± 0.092 and 8.770 ± 0.020 , and for tissue were 7.173 ± 0.242 and 9.330 ± 0.149 , respectively ($n = 3$). Figure 5 shows the expression of CD39 and CD73 proteins in cells and tissue.

Effect of Inhibition of the Extracellular Adenosine Pathway on Outflow Facility In Vivo

To evaluate the physiologic relevance of the endogenous production of extracellular adenosine by the cells of the outflow pathway, we inhibited CD73 in living mice eyes by perfusion of AMPCP (0.2 mM). Outflow facility was calculated for two levels of initial perfusion pressure (35 and 25 mm Hg). At both pressures, inhibition of extracellular adenosine production resulted in a significant decrease of outflow facility of approximately 50% ($n = 10$, $P < 0.001$ for both 35 and 25 mm Hg) (Fig. 6). These results suggested that the levels of activity of the extracellular adenosine pathway exerted a significant effect on outflow facility, and that constant production of extracellular adenosine might be needed to maintain normal levels of IOP.

DISCUSSION

Our results demonstrate the presence of a functional extracellular adenosine pathway in TM cells that involves the

FIGURE 3. Confluent cultures of PTM cells were incubated in the presence of MβCD (10 mM) or vehicle control, AMP and adenosine concentrations in the media were evaluated by HPLC at 30 and 90 minutes ($n = 3$, a indicates $P < 0.001$).

FIGURE 4. PTM cells were incubated with and without cyclic mechanical stress (20% stretching, 1 Hz) for 30 and 90 minutes, and the amounts of extra-cellular AMP (A) and adenosine (B) were determined by HPLC. Values are mean $\pm$ SD of three preparations, and a indicates $P < 0.05$ compared with control.

FIGURE 5. The expression levels of CD39 and CD73 were evaluated by Western blot analysis in PTM cell and PTM tissue.

dephosphorylation of extracellular ATP into AMP by ecto-ATPases and the further conversion of AMP into adenosine by CD73. The role of extracellular ATP, as the main source for extracellular adenosine in TM cells, was also supported by the observation that inhibition of cAMP conversion to AMP by IBMX (which inhibits both intracellular and extracellular phosphodiesterases) had no effect on the generation of extracellular adenosine, which suggested that neither extracellular nor intracellular generation of adenosine from cAMP had a significant contribution to extracellular adenosine.

Interestingly, increased production of intracellular cAMP induced by forskolin resulted in a significant increase in extracellular adenosine. This increase in extracellular adenosine was independent of conversion of cAMP into adenosine because it could not be prevented by inhibition of both endo- and ecto-phosphodiesterases with IBMX. Therefore, the effects of forskolin on extracellular adenosine production are not likely to result from conversion of cAMP into adenosine, but rather suggested that intracellular cAMP signaling might activate the ATP-dependent extracellular adenosine pathway.

We have previously reported that a significant fraction of the ATP release by TM cells in response to mechanical stress was localized in lipid rafts.¹⁹ There is considerable evidence that CD73 and adenosine receptors are concentrated in discrete membrane microdomains, such as lipid rafts or caveolae, where newly synthesized adenosine can efficiently stimulate adenosine receptors in an autocrine manner.^{42–48} The observed effects of lipid raft disruption on the concentration of extracellular adenosine detected in the media suggested a similar compartmentalization of the extracellular adenosine pathway in TM cells.

Also consistent with previous reports showing the release of ATP in response to mechanical stress in TM cells,¹⁹ our results demonstrated a robust increase in the production of

FIGURE 6. CD73 were inhibited in living mouse eyes by perfusion of AMPCP (0.2 mM), outflow facility was calculated for two levels initial perfusion pressure (35 and 25 mmHg). Significance was analyzed using paired Student's *t*-test, $n = 10$, a indicates $P = 0.00047$, b indicates $P = 0.00059$.

extracellular adenosine in PTM cells subjected to CMS. It has long been hypothesized that the cells of the TM might contribute to homeostatic regulation of IOP by releasing factors that increase aqueous humor outflow facility in response to the mechanical stress associated with increased IOP. Although, because of the current technical limitations, we were able to demonstrate a similar increased production of extracellular adenosine production in vivo, the observed activation of the extracellular adenosine pathway by mechanical stress in cultured primary PTM cells together with the known effects of adenosine on outflow facility, suggested a potential mechanism for such homeostatic regulation of IOP at the level of the outflow pathway. Importantly, our results showed that decreased production of extracellular adenosine by inhibition of CD73 in living mouse eyes led to a significant decrease in outflow facility. This observation supports the physiologic relevance of the extracellular adenosine pathway in the maintenance of normal levels of IOP in vivo. Although additional studies in human eyes will be needed to evaluate the functional relevance of this pathway in relationship to human glaucoma, the current studies in mouse eyes are particularly valuable because of the important similarities to human eyes, including no detectable washout rate and a linear pressure-flow relationship over a broad range of intraocular pressures and presence of a true Schlemm's canal.^{49,50} In addition, these results suggested that loss of functionality of the extracellular adenosine pathway could potentially contribute to a pathologic increase in IOP. Since the TM is believed to be subjected to constant mechanical stimulation because of IOP fluctuations associated with systole and diastole,⁵¹ it is expected that the extracellular adenosine pathway will exhibit some constant basal level of activity in the TM providing constant activation of adenosine receptors. Any factors capable of limiting the ability of the TM cells to sense and respond to mechanical stimuli, such as the increase in tissue rigidity recently reported in the TM of glaucomatous donors,^{52,53} could potentially contribute to decreased activity of the extracellular adenosine pathway and thus lead to increased outflow resistance and elevated IOP.

In conclusion, our results showed that the main route of extracellular adenosine production in PTM cells is the metabolization of extracellular ATP by ecto-ATPases and CD73, and that functionality of this pathway appears to be necessary for the maintenance of normal levels of aqueous humor outflow in living mouse eyes. These results support the concept that the extracellular adenosine pathway might play an important role in the homeostatic regulation of outflow resistance in the TM.

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