Effect of adenosine on GLAST expression in the retina of a chronic ocular hypertension rat model

ZI-JIAN YANG and YI-SHENG ZHONG

Department of Ophthalmology, Ruijin Hospital Affiliated Medical School,
Shanghai Jiaotong University, Shanghai 200025, P.R. China

Abstract. This study was performed to evaluate the effect of adenosine and an adenosine receptor antagonist on the expression of the L-glutamate/L-aspartate transporter (GLAST) in the retina of a chronic ocular hypertension (COH) rat model. COH models were established via the cauterization of three episcleral veins. Measurements of the intraocular pressure of the right eye (COH eye) were taken weekly by a handheld digital tonometer. A total of 10 μM adenosine or 10 μM adenosine + 100 nM SCH442416 solution (2 μl) was injected into the rat vitreous space. The reverse transcription-quantitative polymerase chain reaction, western blotting and immunohistochemistry were used to detect GLAST expression. Compared with the COH group, GLAST mRNA expression was decreased by 33.6% in the group treated with adenosine (n=6, P=0.020) and was increased by 159.6% in the group treated with SCH442416 (n=6, P=0.001). Administration of adenosine decreased GLAST protein expression by 34.7% (n=6, P<0.001), while treatment with the adenosine A_{2A} receptor antagonist SCH442416 increased GLAST protein expression by 48.3% compared with the control COH group (n=6, P<0.001). Immunohistochemical experiments showed that administration of adenosine decreased GLAST protein expression, as compared with expression in the control COH rat retina. Administration of SCH442416 markedly increased GLAST protein expression. The results of the present study may provide a novel method for retinal neuron protection.

Introduction

Adenosine is an endogenous bioactive substance widely present in the intracellular and extracellular fluid in mammals. Almost all cells, including Müller cells, can release adenosine. Müller cells release adenosine through a calcium-independent facilitated transport method (1,2). Adenosine is a local hormone that is present at a low level in physiological conditions, normally at a concentration of 20-200 nM. Tissue hypoxia or ischemia can lead to adenosine triphosphate breakdown and the increased generation of adenosine (3). Adenosine, which exerts anti-inflammatory effects, is released from the retina and has an important role in pathological conditions, such as ischemia-hypoxia and glaucoma (4). To a certain extent, adenosine protects neurons from glutamate toxicity by suppressing excitatory neurotransmission (5).

Glutamate is a neurotransmitter that can be found in brain tissue and the retina of the eye (6). In conditions of depolarization, Müller cells release glutamate and the increased non-vesicular release of glutamate causes excitotoxic damage to neurons (7). The L-glutamate/L-aspartate transporter (GLAST) is a primary glial enzyme in the clearance of extracellular glutamate in physiological conditions (8). In pathological conditions, the functional downregulation of GLAST induces an increased glutamate concentration in the retina; however, the association between the high concentration of adenosine and GLAST downregulation in the retina is not clear. The aim of the present study, therefore, was to explore the effects of adenosine and an adenosine receptor antagonist, SCH442416, on rat retina GLAST expression.

Materials and methods

Rat chronic ocular hypertension (COH) models. All experimental procedures described were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The present study was approved by the laboratory animal ethics committee of Ruijin Hospital (Shanghai, China). Male Sprague Dawley rats (weight, 200-250 g) were purchased from Shanghai SIPPR-BK Laboratory Animal Co., Ltd. (Shanghai, China), and housed in an air-conditioned animal room at ~23°C under a 12-h light/dark cycle. All efforts were made to minimize the suffering of the rats in this study. Prior to all surgeries and procedures, the animals were anesthetized with an intraperitoneal injection of xylazine and ketamine hydrochloride (7.4 mg/ml and 5 mg/kg, respectively) (Jiangsu Hengrui Medicine Co., Ltd., Lianyungang, China). Two drops of 0.3% Ocuflox® solution (Santen Pharmaceutical Co., Ltd.,

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Correspondence to: Professor Yi-Sheng Zhong, Department of Ophthalmology, Ruijin Hospital Affiliated Medical School, Shanghai Jiaotong University, 197 Ruijin No. 2 Road, Shanghai 200025, P.R. China
E-mail: yszhong68@126.com

Osaka, Japan) were topically applied prior to and following the eye surgeries to prevent infection.

The rat COH model was reproduced following a procedure described in previous studies (9,10). An increase in the intraocular pressure (IOP) was induced in 60 rats by cauterizing three episcleral veins in the right eye of each rat. Briefly, three veins (including two episcleral veins beside the superior rectus muscle and one episcleral vein beside the lateral rectus muscle) were separated and cauterized precisely to avoid nearby tissue damage. The right eyes of a further 6 rats were subjected to a sham procedure. IOP measurements were taken weekly following surgery at the same time in the morning. Proxymetacaine hydrochloride solution (Alcon Laboratories, Inc., Fort Worth, TX, USA) was used topically prior to the IOP measurements, which were conducted with a handheld digital tonometer (TonoPen®; Mentor Ophthalmics, Inc., Norwell, MA, USA). The probe of the TonoPen was applied vertically to the cornea surface, and touched the cornea surface with the same force in all measurements. If the confidence interval was ≥95%, numerical values were accepted. Ten numerical values were recorded in each eye.

Drugs and intravitreal injection. Prior to injection, the rat pupil was dilated with a tropicamide drop (Qianjiang Pharmaceutical Co., Ltd., Qianjiang, China). A total of 10 μM adenosine (Sigma-Aldrich, St. Louis, MO, USA) or 10 μM adenosine + 100 nM SCH442416 (Sigma-Aldrich) solution (2 μl) was injected into the rat vitreous space (n=6 rats/group). Adenosine or adenosine + SCH442416 were first dissolved in dimethyl sulfoxide (DMSO) and then diluted by double-distilled H_2O (final concentration DMSO, 5%). Under a stereoscopic microscope, a microinjector (Hamilton Robotics, Inc., Reno, NV, USA) was inserted 2 mm behind the temporal limbus and directed toward the optic nerve to inject solution into the vitreous space. Eyes that received only an injection of vehicle solution in the same manner served as controls.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. First, total RNA, isolated by TRIzol® (Invitrogen Life Technologies, Carlsbad, CA, USA), was reverse-transcribed into cDNA. The PCR solution contained 2 μl cDNA, the specific primer set (1 μM each) and 11.5 μl QuantiTect SYBR® Green PCR Master Mix from the QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany) in a final volume of 20 μl. The following primer pairs (Invitrogen Life Technologies) were used: GLAST sense, 5'-CCTATGTGGCAGTCGTTT-3' and anti-sense, 5'-CTG TGATGGGCTGGCTAA-3', β-actin sense, 5'-GCGCTCGTC GTCGACAACGG-3' and anti-sense, 5'-GTGTGGTGCCAA ATCTTCTCC-3'. The PCR cycle conditions were as follows: i) Initial denaturation, one cycle at 94˚C for 5 min; ii) amplification and quantification, 40 cycles at 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 30 sec; iii) melting curve, 55˚C with the temperature gradually increased up to 95˚C.

Western blot analysis. For western blotting, the rat retinas were lysed in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Shanghai, China) in the presence of protease inhibitors. The homogenates were centrifuged at 14,000 x g for 30 min at 4˚C. The protein concentrations were determined using the bicinchoninic acid method. Equal quantities of protein (1 μg/μl, 15 μl) were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Subsequent to being blocked with 5% skimmed milk at room temperature for 2 h, the membranes were incubated with rabbit polyclonal antibody against GLAST (1:200 dilution; cat. no. ab416; Abcam, Cambridge, UK) or mouse monoclonal antibody against GAPDH (1:10,000 dilution; cat. no. KC-5G4 KangChen Bio-tech Inc., Shanghai, China) overnight at 4˚C. The membranes were then washed three times with Tris-buffered saline-Tween 20 for 10 min and incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:2,000 dilution) for 1 h at room temperature. Images were captured using the ImageQuant™ Las 4000 mini biomolecular imager (GE Healthcare Life Sciences, Pittsburgh, PA, USA), and the protein bands were quantitatively analyzed with Image-Pro Plus version 6.0.0.260 (Media Cybernetics, Inc., Rockville, MD, USA) image analysis software.

Immunohistochemistry. Rats were perfused with normal saline and 4% paraformaldehyde (PFA) solution. The right eyeballs were fixed in 4% PFA solution for 4 h, and then dehydrated with a graded sucrose solution at 4˚C (4 h in 20% sucrose solution and overnight in 30% sucrose solution). The retinas were vertically sectioned at a 7.5-μm thickness and then mounted on gelatin-coated slides. Subsequent to being rinsed with 0.01 M phosphate-buffered saline (PBS), the retina slices were blocked in 4% goat serum (Gibco Life Technologies, Carlsbad, CA, USA), 0.25% bovine serum albumin (Sigma-Aldrich) and 0.2% Triton X-100 (Solarbio Science & Technology Co., Ltd., Shanghai, China) in PBS at room temperature for 2 h, and incubated with goat polyclonal anti-GLAST primary antibody (1:200 dilution; cat. no. sc-7758; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4˚C for 48 h. Following incubation with the primary antibody, the retina slices were rinsed in 0.01 M PBS and incubated with the fluorescein isothiocyanate-conjugated donkey anti-goat immunoglobulin G secondary antibody (1:100 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 2 h at room temperature. Finally, the retina slices were mounted with anti-fade mounting medium with DAPI (Vector Laboratories, West Grove, PA, USA), and the immunofluorescence images were visualized with a Zeiss Imager M1 laser-scanning microscope (Carl Zeiss AG, Oberkochen, German) using a 20X objective lens.

Statistics. Data were analyzed using SPSS 19.0 software (IBM SPSS, Armonk, NY, USA) and are presented as the mean ± standard deviation. The paired-samples Student's t-test or one-way analysis of variance was used to test the data for statistical significance. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

IOP elevation in the COH models. The mean IOP in the sham-operated animals exhibited no significant difference between the right (19.03±0.25 mmHg) and left (19.47±0.69 mmHg) eye in the 8-week observation period
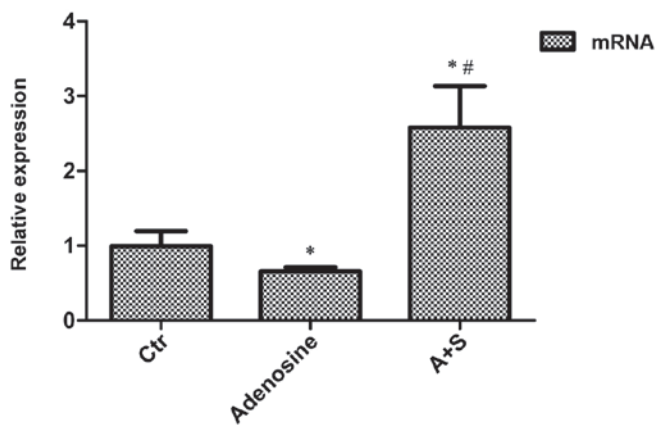
Figure 1. Effects of adenosine and SCH442416 on GLAST mRNA expression in rat models of COH. Adenosine and/or SCH442416 were injected into the rat vitreous space 2 weeks before the surgery. The relative expression of GLAST mRNA in the right eyes of the control (COH), adenosine- and SCH442416-treated rats is shown. Data are presented as the mean ± standard deviation, n=6. *P<0.05 versus control; #P<0.05 versus the adenosine group. A+S, adenosine + SCH442416; ctr, control; COH, chronic ocular hypertension; GLAST, L-glutamate/L-aspartate transporter.
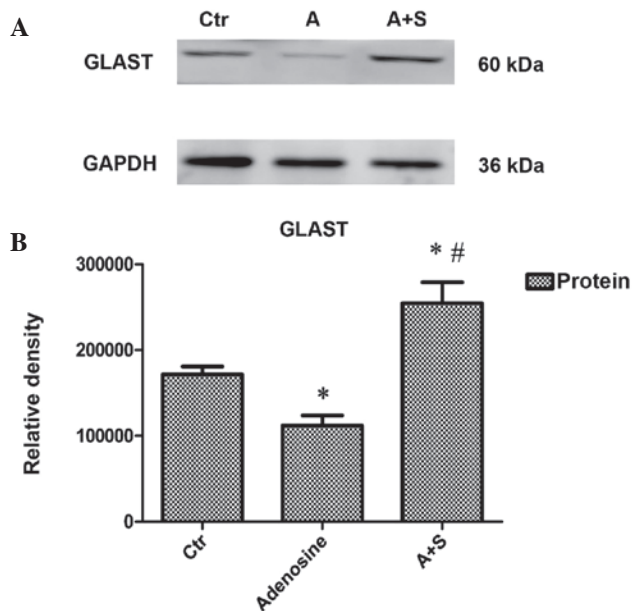

Figure 2. Effect of adenosine and SCH442416 on GLAST protein expression in the retinas of COH rat models. Adenosine and/or SCH442416 were injected into the rat vitreous space 2 weeks before the surgery. (A) GLAST protein expression in the right eyes of the control (COH), adenosine- and SCH442416-treated rats. (B) Bar chart summarizing the average densitometric quantification of the GLAST western blot bands for the control (COH), adenosine- and SCH442416-treated rats. Data are presented as the mean ± standard deviation, n=6. *P<0.05 versus control, #P<0.05 versus the adenosine group. A+S, adenosine + SCH442416; ctr, control; COH, chronic ocular hypertension; GLAST, L-glutamate/L-aspartate transporter.
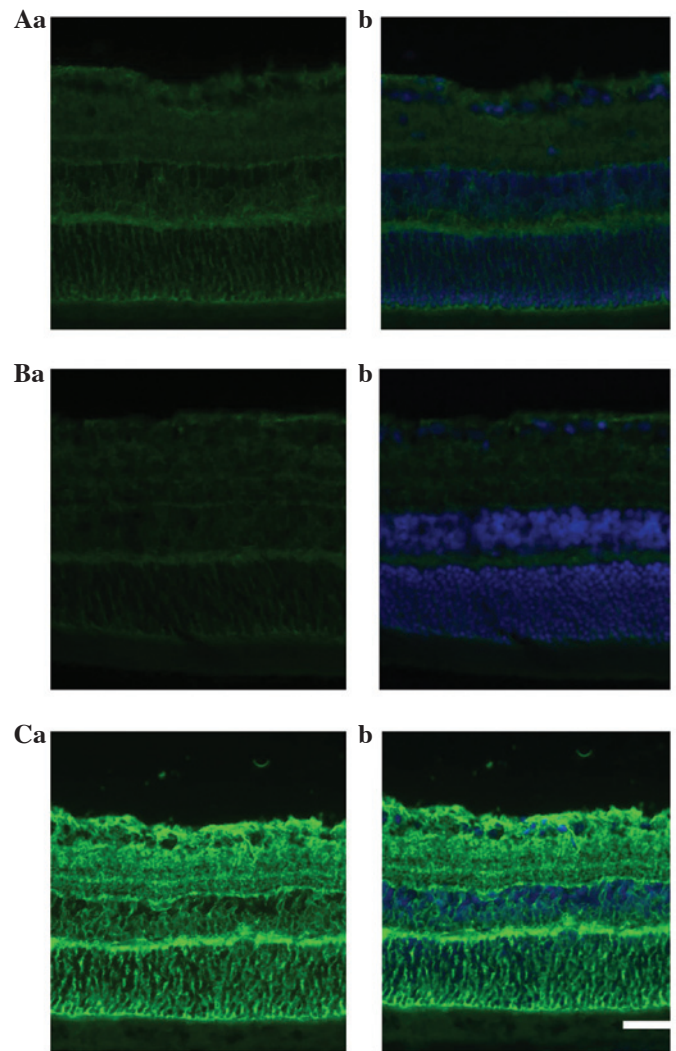

Figure 3. Immunofluorescence images of adenosine- and adenosine antagonist-induced changes in GLAST expression. Adenosine and/or SCH442416 were injected into the vitreous space 2 weeks before the surgery. (A-C) Immunofluorescence labeling showing the (Aa, Ba and Ca) GLAST protein expression and (Ab, Bb and Cb) GLAST with DAPI in retinal slices taken from the (A) control, (B) adenosine-injected or (C) adenosine + SCH442416-injected rat eyes. Treatment with adenosine decreased the GLAST protein expression compared with the control, while the administration of adenosine + SCH442416 markedly increased GLAST fluorescence compared with the control and adenosine groups (n=6). Scale bar, 20 μm. GLAST, L-glutamate/L-aspartate transporter.

(n=6, P=0.329). In the episcleral vein cauterization group the IOP of the right eye showed a significant increase from the first week of the study and continued to show a steady increase until the eighth week. The mean IOP of the right eye in the episcleral vein cauterization group was 27.3±1.83 mmHg, compared with 19.03±0.25 mmHg in the sham surgery group (n=6, P=0.001).

Adenosine- and adenosine A$_{2A}$ receptor antagonist-induced changes in GLAST mRNA expression in the rat COH models. Adenosine or adenosine + SCH442416 solution was intravitreally injected into the right eye of the rats. The rats were sacrificed two weeks later by cervical dislocation and GLAST mRNA expression was evaluated through RT-qPCR analysis. Compared with the control (COH) group, the GLAST mRNA expression was observed to decrease by 33.6% in the adenosine treatment group (n=6, P=0.020) and to increase by 159.6% in the group treated with SCH442416 (n=6, P=0.001) (Fig. 1).

Adenosine- and adenosine A$_{2A}$ receptor antagonist-induced changes in GLAST protein expression in the rat COH models. Adenosine or adenosine + SCH442416 solution was intravitreally injected into the right eye of the rats. The rats were

sacrificed two weeks later, and the retinas were dissected and used for the experiment. Compared with the control (COH) group, the administration of adenosine decreased GLAST protein expression in the retina by 34.7% (n=6, P<0.001), whereas treatment with the adenosine A_{2A} receptor antagonist SCH442416 increased GLAST protein expression by 48.3% (n=6, P<0.001) (Fig. 2A and B).

In the immunohistochemical experiments, the administration of adenosine was shown to decrease GLAST protein expression relative to that in the control rat retina. By contrast, the administration of SCH442416 markedly increased the GLAST protein expression compared with that in the control and adenosine groups (Fig. 3A-C).

Discussion

Retinal Müller cells release adenosine and glutamate in pathological conditions. Glutamate is a major excitatory neurotransmitter in the mammalian retina and its presence at high concentrations plays a key role in neuronal damage. Extracellular glutamate is primarily taken up into the cell via GLAST (~50% of extracellular glutamate) (11); however, the effect of adenosine on GLAST function and the underlying mechanism have yet to be fully elucidated. The results of the present study have provided the first evidence, to the best of our knowledge, that treatment with adenosine (10 μM) can decrease the retinal GLAST protein and mRNA expression. It has been reported that elevations in IOP to <70 mmHg are conducive to the maintenance of GLAST activity in rats (12). The present study results were consistent with this finding. Decreases in the expression of GLAST protein were accompanied by decreases in the GLAST mRNA level, suggesting that decreased transcription plays an important role in the downregulation of GLAST protein expression.

Adenosine, one of the most bioactive substances, is widely present in intracellular and extracellular fluid (13). Adenosine produces its biological effect through adenosine receptors. All adenosine receptors are G-protein-coupled receptors and can be grouped into four subtypes: A_1, A_{2A}, A_{2B} and A_3. Biological and pharmacological studies have found that all four adenosine receptor are located in the retina. The A_1 receptor (A_1R), A_{2A}R and A_{2B}R have been studied extensively, while less focus has been placed on the A_3R. It has been demonstrated that A_1R activation has a protective function *in vivo*, inhibiting inflammation and apoptosis in the heart, brain and kidneys (14). The release of neurotransmitters, including glutamate, from the synaptic terminals is inhibited by activation of the A_1R. By contrast, activation of the A_{2A}R promotes the release of neurotransmitters, including glutamate. The A_{2B}R is expressed at a low density in almost all tissues and exhibits low-affinity ligand binding (15). Less is known about the A_3R than about the A_1R, A_{2A}R and A_{2B}R. With regard to the A_{2A}R, early studies have indicated that A_{2A}R antagonists can induce a decrease in glutamate uptake in a mouse model of Alzheimer's disease (15,16). In the present study, an A_{2A}R antagonist was selected for the study, since none of the other adenosine receptors tested (A_1R or A_3R) modified glutamate uptake. Although a number of previous studies have shown that A_{2A}R antagonists increase GLAST expression in cultured cells (17,18), the results of the present study provide the first evidence, to the best of our knowledge, that the blockage of A_{2A}Rs can increase the GLAST expression in the rat retina

in vivo. The results of the present study may provide a novel method for retinal neuron protection.

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