

Effect of SCH442416 on glutamate uptake in retinal Müller cells at increased hydrostatic pressure

YONG LI^{1*}, XIAOHONG LIU^{2*}, SHOUYUE HUANG², PING HUANG³ and YISHENG ZHONG²

¹Department of Ophthalmology, Tongren Hospital Affiliated Medical School, Shanghai Jiaotong University, Shanghai 200336;

²Department of Ophthalmology, Ruijin Hospital Affiliated Medical School; ³Shanghai Key Laboratory for Bone and Joint Diseases, Shanghai Institute of Traumatology and Orthopaedics, Ruijin Hospital Affiliated Medical School, Shanghai Jiaotong University, Shanghai 200025, P.R. China

Received August 27, 2014; Accepted May 13, 2015

DOI: 10.3892/mmr.2015.3882

Abstract. The A_{2A} receptor (A_{2A}R) antagonist has been considered as an attractive option to improve the treatment of neurological disorders, and the function of A_{2A}R antagonist may inhibit the release of glutamate and prevent neuron damage. The aim of the present study was to investigate whether SCH442416 can modulate the glutamate uptake in retinal Müller cells under increased hydrostatic pressure. The levels of glutamine synthetase (GS) and glutamate aspartate transporter (GLAST) were assessed in retinal Müller cells under 40 mmHg pressure for 24 h using reverse transcription-quantitative polymerase chain reaction and western blotting, and a glutamate uptake assay was performed using a scintillation counting method. Following treatment of the Müller cells with 100 nM SCH442416 under 40 mmHg pressure for 24 h, the mRNA and protein expression levels of GS and GLAST, and glutamate uptake activity were investigated. Under 40 mmHg pressure, the expression levels of GS and GLAST in the Müller cells, and glutamate uptake activity were significantly reduced. Treatment with SCH442416 significantly ameliorated the decreased expression levels of GS and GLAST, and improved the glutamate uptake activity in the retinal Müller cells exposed to 40 mmHg pressure,

resulting in increased expression levels of GS and GLAST, and increased glutamate uptake activity in the Müller cells under pressure. These results suggested that SCH442416 may be a potential candidate as a beneficial neuroprotective agent for the treatment of glaucoma by accelerating the clearance of extracellular glutamate.

Introduction

Glaucoma is the leading cause of blindness worldwide and is one of the most common neurodegenerative diseases, which is characterized by the irreversible and progressive loss of retinal ganglion cells (RGCs) and damage to the optic nerve, usually in response to abnormally increased intraocular pressure (1-4).

Müller cells are the principal glia of the retina, and the predominant function of Müller cells is to regulate extracellular glutamate levels (5). Glutamate, a normal constituent of the retina, is taken up by Müller cells and is converted to glutamine, which is taken up by the neurons. The neurons use glutamine to synthesize glutamate for neurotransmission (5). Müller cells are involved in glutamate metabolism via the glutamate aspartate transporter (GLAST) and glutamine synthetase (GS). The GLAST is responsible for the transport of glutamate into Müller cells and GS is the enzyme, which converts glutamate into glutamine inside the Müller cells (6). Increased levels of extracellular glutamate have been reported in a primate model of glaucoma and in human patients with glaucoma (7). This increase in extracellular glutamate levels is predominantly due to the downregulation of GLAST (8). Excess glutamate release is involved in glaucomatous neuropathy, which causes excitotoxic damage to the RGCs through the activation of ionotropic and metabotropic glutamate receptors (9,10). Consequently, the efficient removal of glutamate from the extracellular space is required for the maintenance of a healthy retina.

Adenosine is a ubiquitous local modulator, which regulates various physiological and pathological functions by stimulating membrane receptors. Biochemical, pharmacological, and molecular investigations have identified four adenosine receptor subtypes, A₁, A_{2A}, A_{2B} and A₃ (11). There is increasing evidence that adenosine is an important intracellular mediator in the retina and has considerable potential to protect retinal

Correspondence to: Professor Yisheng Zhong, Department of Ophthalmology, Ruijin Hospital Affiliated Medical School, Shanghai Jiaotong University, 197 Ruijin Er Road, Shanghai 200025, P.R. China
E-mail: yszhong68@126.com

Ms. Ping Huang, Shanghai Key Laboratory for Bone and Joint Diseases, Shanghai Institute of Traumatology and Orthopaedics, Ruijin Hospital Affiliated Medical School, Shanghai Jiaotong University, 197 Ruijin No. 2 Road, Shanghai 200025, P.R. China
E-mail: pinghpingh@126.com

*Contributed equally

Key words: Müller cells, SCH442416, glutamine synthetase, glutamate aspartate transporter, glutamate uptake

neurons (12-14). Previously, an A_{2A} receptor ($A_{2A}R$) antagonist has been suggested as an attractive option to improve the treatment of neurological disorders, including Parkinson's disease, Huntington's disease and Alzheimer's disease (15,16). The function of the $A_{2A}R$ antagonist may be to inhibit the release of glutamate and prevent damage of the neuron (17). The aim of the present study was to investigate whether the $A_{2A}R$ antagonist, SCH442416, modulates the expression levels of GS and GLAST, and the uptake of glutamate in retinal Müller cells exposed to increased hydrostatic pressure.

Materials and methods

Pressure device. The pressure device used in the present study was described in detail in our previous study (18). Briefly, a T75 culture flask (Shanghai Jun Sheng Biological Technology Co., Ltd., Shanghai, China) was equipped with a manometer (Fang Jun Instrument Co., Ltd., Shanghai, China) and placed in an incubator, maintained at 37°C, as the pressure device. An air mixture of 95% air and 5% CO₂ was pumped into the flasks to obtain pressure. The pressure level of the model was 40 mmHg, as in our previous investigation (18), which was adjusted every 4 h. The total duration of the induced pressure was 24 h. In the experiments, several precautions were made to limit artifacts from the experimental method. Laboratory film (Pechiney, Stamford, CT, USA) was used to seal the interfaces and, to avoid artifacts caused by 'on-off' changes in pressure, all operations involving the refreshment of medium or adjustment of pressure were performed within a 5 min period.

Müller cell culture. All investigations involving animals in the present study were performed in strict accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research (19). The present study was approved by the Ethics Committee of Ruijin Hospital, Shanghai Jiaotong University (Shanghai, China). The primary culture of retinal Müller cells was generated, as previously described (18). Briefly, the retinas of 80 newborn (2-5 days old, male and females) Sprague-Dawley rats, obtained from Shanghai Slack Laboratory Animal Co., Ltd. (Shanghai, China) were collected following sacrifice by intraperitoneal of 30% chloral hydrate (500 mg/kg; Chemical Reagent Co., Ltd., Shanghai, China). For each experiment, the retinas (n=20) were dissected and stored on ice in D-Hank's solution (Anresco LLC, Solon, OH, USA). The tissue was dissociated by centrifugation at room temperature for 5 min at 600 x g and was incubated for 15 min at 37°C in phosphate-buffered saline (PBS), containing 0.125% trypsin (Anresco LLC). Finally, the cell suspension was cultured in T75 culture flasks at 37°C in humidified air containing 5% CO₂. Following the initial outgrowth, the cell culture medium was replaced every 48 h and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Carlsbad, CA, USA), supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (Sijiqing, Zhejiang, China).

Following culture for 5-8 days, the flasks were agitated at 37°C for 1 h at 100 rpm and the cell culture medium was refreshed. By agitating the plates, other types of cell, including microglial cells and RGCs, which were initially adhered to

the surface of the Müller cells, were rinsed off with DMEM to obtain a purified cell population. For passage, the cell cultures were incubated at 37°C with PBS, containing 0.125% trypsin. The Müller cells were identified via GS and glial fibrillary acidic protein (GFAP) staining using indirect immunofluorescence. The cells were fixed with 4% paraformaldehyde at room temperature for 10 min and were incubated with 0.3% Triton X-100 at 37°C for 10 min. The cells were washed three times (10 min/wash) with PBS, blocked with 10% goat serum in PBS and subsequently incubated with the rabbit anti-rat polyclonal antibody against GS (1:5,000; ab49873; Abcam, Cambridge, MA, USA) and the mouse anti-rat monoclonal antibody against GFAP (1:200; ab4648, Abcam) as an identity marker for Müller cells. The cells were then incubated overnight at 4°C. The following day, the cells were incubated with the secondary donkey anti-rabbit IgG-Cy3 polyclonal antibody (1:200; 406402; BioLegend, Inc., San Diego, CA, USA) at 37°C in darkness for 1 h. Following three washes with PBS, the cells on the coverslips were mounted on glass slides with Histomount (Invitrogen Life Technologies, Carlsbad, CA, USA). The cells were viewed under an Axio microscope (Zeiss, Oberkochen, Germany), and images were acquired with a digital camera (Canon, Tokyo, Japan).

Drug treatment. The A_{2A} receptor antagonist, 2-(2-Furan yl)-7-[3-(4-methoxyphenyl)propyl]-7H-pyrazolo[4,3-e] (1,2,4) triazolo[1,5-c]pyrimidin-5-amine (SCH442416), was purchased from Tocris Bioscience (Ellisville, MO, USA). The experiments were performed following the second passage, when cell confluence was 80-90%. The cells were cultured in serum-free medium and divided into the following three groups: Normal culture group; 40 mmHg pressure culture group; 40 mmHg pressure + 100 nM SCH442416 culture group. The Müller cells in the three groups were continually cultured at 37°C for another 24 h. The concentration of SCH442416 used in the present study was selected, according to preliminary experiments (Data not shown).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). The cells were collected and used for total RNA preparations. The total RNA was reverse-transcribed into cDNA using a previously described method (20) and the Invitrogen Reverse Transcription kit (Invitrogen Life Technologies). The PCR solution contained 2 µl cDNA, specific primers (1 µM each) and 10 µl QuantiTect SYBR Green PCR kit reagent (Qiagen, Hilden, Germany) in a final volume of 20 µl. The following primer pairs from Sangon Biotech Co., Ltd. (Shanghai, China) were used: GS, sense 5'-CCGCTCTTCGTCTCGTTC-3' and anti-sense 5'-CTGCCTGATGCCTTTGTT-3'; GLAST, sense 5'-CCTATGTGGCAGTCGTTT-3' and anti-sense 5'-CTGTGATGGGCTGGCTAA-3'; and β-actin, sense 5'-GCGCTCGTCTCGTACACACGG-3' and anti-sense 5'-GTGTGGTGCCAAATCTTCTCC-3'. The PCR parameters were as follows: Initial denaturation at 94°C for 5 min; amplification and quantification, 40 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec; melting curve, 55°C with the temperature gradually increased up to 95°C. The mRNA expression levels were normalized against the levels of β-actin, as described previously (20).

Western blot analysis. The cultured cells in the samples from the different groups were washed twice with PBS. The total protein was extracted with the EpiQuik Whole Cell Extraction kit (Epigentek, Farmingdale, NY, USA) according to the manufacturer's instructions. Protein concentration was determined by the radioimmunoprecipitation buffer assay (Cell Signaling Technology, Inc., Danvers, MA, USA) and lysed in 2X Laemmli buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The protein extracts (40 μ g) were boiled for 10 min and centrifuged at 14,000 \times g. The proteins were separated on 12% SDS-PAGE gels (Sigma-Aldrich, St. Louis, MO, USA) and were transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were soaked in Tris-buffered saline (Sigma-Aldrich), containing 20 mmol/l Tris-Cl, 140 mmol/l NaCl (pH 7.5), with 5% non-fat milk and 0.1% Tween-20 (Sigma-Aldrich) for 1 h at room temperature. The membranes were incubated with primary rabbit anti-rat polyclonal antibodies against GS (ab49873; 1:10,000; Abcam) and GLAST (ab416; 1:200; Abcam) overnight at 4°C. Rabbit anti-rat polyclonal anti-GAPDH antibody (ab37168; 1:10,000; Abcam) was used as a reference to normalize the intensities of the immunoreactions with different antibodies. Following several washes with PBS, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (A20019; 1:2,000; Invitrogen Life Technologies) for 1 h at room temperature and visualized using enhanced chemofluorescence reagent (Beyotime Institute of Biotechnology, Haimen, China). Images were captured using ImageQuant Las 4000 mini (GE Healthcare Life Sciences, Kochi, Japan) and the protein bands were quantitatively analyzed using ImagePro Plus image analysis software v.7.0 (Zeiss).

Glutamate uptake assay. The cultured Müller cells in the treatment groups were washed in PBS and pre-incubated in Krebs's solution (Sigma-Aldrich), containing 119 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4 , 25 mM NaHCO_3 , 2.5 mM CaCl_2 and 1 mM MgCl_2 , for 30 min at 37°C. The Müller cells were then exposed to 0.5 $\mu\text{Ci/ml}$ L-[2,3- ^3H] glutamate (New England Nuclear, Boston, MA, USA) and 10 mmol/l unlabeled glutamate for 60 min at 37°C. The reaction was terminated by washing the cells three times with ice-cold PBS. The Müller cells were subsequently lysed in PBS and small aliquots (20 μl) were removed from each well for the determination of protein content. The L-[2,3- ^3H] glutamate content of the lysates were determined by scintillation counting (Triathler Scintillator; Beijing Huaruisen Science and Technology Development Co., Ltd., Beijing, China). All experiments were performed in triplicate for each of the four separate cell preparations.

Statistical analysis. The data are expressed as the mean \pm standard deviation. All analyses were performed using SPSS 19.0 statistical software (IBM SPSS, Chicago, IL, USA). The data were analyzed using one-way analysis of variance, followed by a least significant difference test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effect of SCH442416 on the mRNA expression levels of GS and GLAST in the cultured retinal Müller cells under pressure

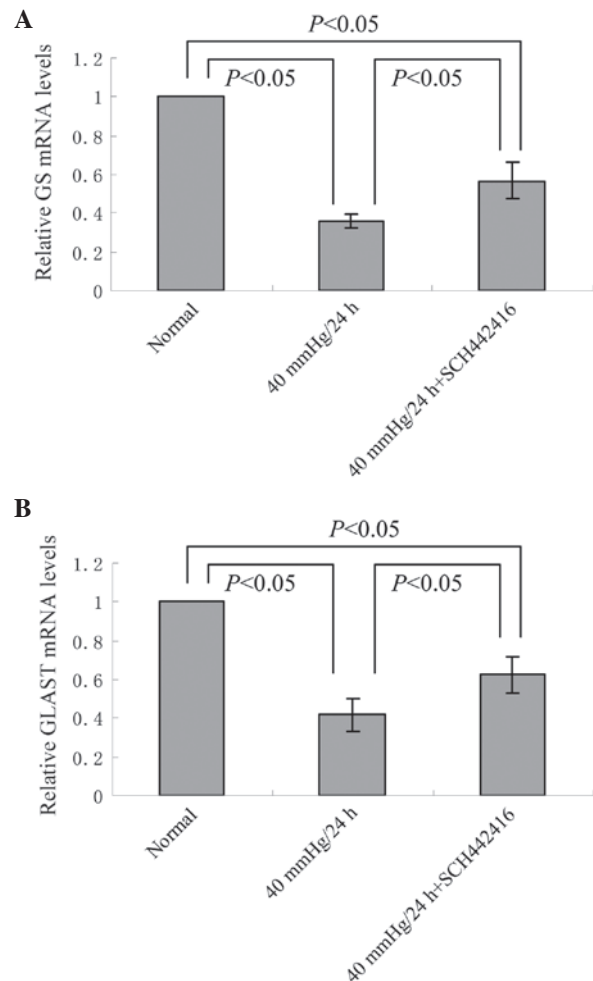


Figure 1. Reverse transcription-quantitative polymerase chain reaction analysis of the mRNA expression levels of (A) GS and (B) GLAST in retinal Müller cells cultured with or without SCH 442416, subjected to 40 mmHg pressure conditions for 24 h. Each measurement was performed in triplicate for each of four separate cell preparations. The mRNA expression levels of GS and GLAST were significantly decreased in the Müller cells cultured with or without SCH442416 under 40 mmHg pressure, compared with the normal control. The mRNA expression levels of GS and GLAST in the 40 mmHg pressure + SCH442416 culture group were significantly higher, compared with those in the 40 mmHg pressure culture group. The data are expressed as the mean \pm standard deviation. GS, glutamine synthetase; GLAST, glutamate aspartate transporter.

conditions. The mRNA expression levels of GS and GLAST of the retinal Müller cells incubated in serum-free medium, in the presence or absence of SCH442416, under 40 mmHg pressure for 24 h was analyzed using RT-qPCR. Compared with the normal culture group, the mRNA expression levels of GS and GLAST were significantly decreased in the Müller cells cultured with or without SCH442416 under 40 mmHg pressure conditions ($P < 0.05$; Fig. 1). However, the mRNA expression levels of GS and GLAST in the 40 mmHg pressure + 100 nM SCH442416 culture group were significantly higher, compared with those in the 40 mmHg pressure culture group ($P < 0.05$; Fig. 1).

Effect of SCH442416 on the protein expression levels of GS and GLAST in the cultured retinal Müller cells under pressure conditions. The protein expression levels of GS and GLAST in retinal Müller cells were compared between the normal control group and the groups under 40 mmHg pres-

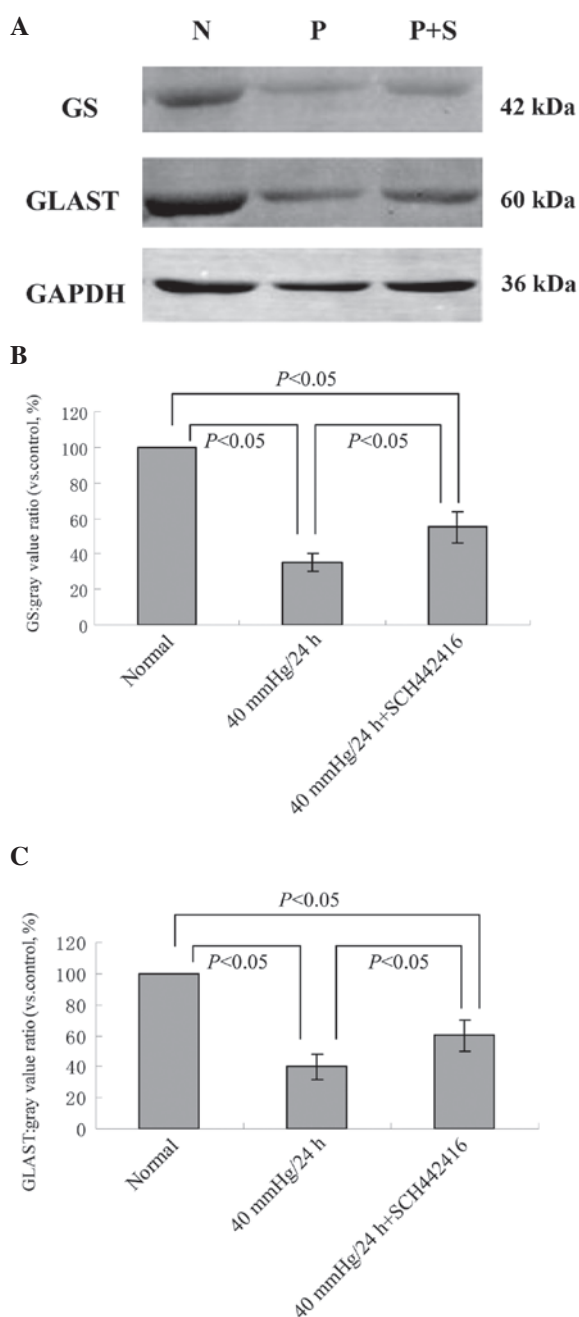


Figure 2. (A) Western blot analysis of the protein expression levels of GS and GLAST in retinal Müller cells cultured with or without SCH 442416 subjected to 40 mmHg pressure for 24 h. Each measurement was performed in triplicate for each of four separate cell preparations. (B and C) Protein expression levels of GS and GLAST were significantly decreased in the Müller cells cultured with or without SCH442416 under 40 mmHg pressure. The protein expression levels of GS and GLAST in the 40 mmHg pressure + SCH442416 culture group were significantly higher, compared with the 40 mmHg pressure culture group. The data are expressed as the mean \pm standard deviation. N, normal culture group; P, 40 mmHg pressure culture group; P + S, 40 mmHg pressure + 100 nM SCH442416 culture group. GS, glutamine synthetase; GLAST, glutamate aspartate transporter.

sure for 24 h, in the presence or absence of SCH442416. Western blotting revealed that the expression levels of GS and GLAST were significantly decreased in the Müller cells cultured with or without SCH442416 under 40 mmHg pressure, compared with the normal culture ($P<0.05$; Fig. 2). However, the protein expression levels of GS and GLAST in

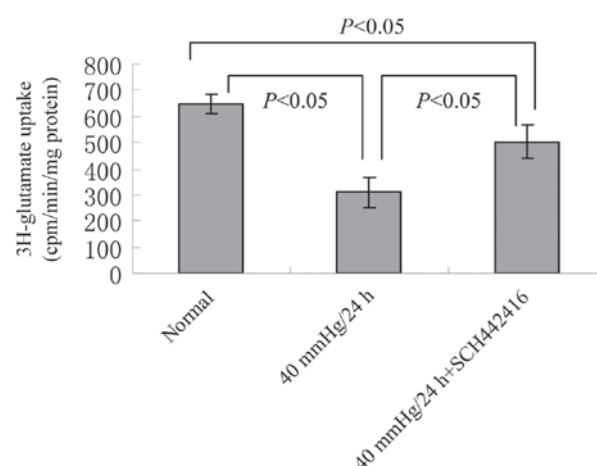


Figure 3. Effect of SCH442416 on the activity of glutamate uptake in cultured retinal Müller cells under pressure conditions. Each measurement was performed in triplicate for each of the four separate cell preparations. The glutamate uptake activity was significantly decreased in the Müller cells cultured with or without SCH442416 under 40 mmHg pressure. The glutamate uptake activity in the 40 mmHg pressure + SCH442416 culture group was significantly higher, compared with the 40 mmHg pressure culture group. The data are expressed as the mean \pm standard deviation.

the 40 mmHg pressure + 100 nM SCH442416 group were significantly higher, compared with the 40 mmHg pressure group ($P<0.05$; Fig. 2).

Effect of SCH442416 on glutamate uptake activity in the cultured retinal Müller cells under pressure conditions. A glutamate uptake assay was performed using a scintillation counting method to determine the ^3H -glutamate content in the lysates. Compared with the normal culture group, the glutamate uptake activity was significantly decreased in the Müller cells cultured with or without SCH442416 under 40 mmHg pressure ($P<0.05$; Fig. 3). However, the glutamate uptake activity in the 40 mmHg pressure + 100 nM SCH442416 culture group was significantly higher, compared with that in the 40 mmHg pressure culture group ($P<0.05$; Fig. 3).

Discussion

The present study used a novel pressure model, which involved the culture of retinal Müller cells under hydrostatic pressure. The hydrostatic pressure used in this model was adjusted to 40 mmHg, a moderately elevated pressure, which often occurs in chronic glaucoma models (21). In the present study, several precautions and design considerations were made to limit artifacts from the experimental procedure. Laboratory film was used to seal interfaces and, to avoid artifacts from 'on-off' changes in pressure, replacements of media or adjustments of pressure were completed without delay. Our previous study also revealed that this pressure model was effective (18).

Glutamate acts as a neurotransmitter in the normal retina. However, excessive stimulation of glutamate receptors can result in excitotoxicity (22). Intraocular glutamate can cause severe degeneration of the inner retinal layers, particularly the RGC layer (23). These findings support the hypothesis that increased extracellular glutamate concentration or decreased glutamate clearance results in excitotoxic damage and may contribute to

the pathogenesis of glaucoma (24-26). Müller cells maintain an close association with retinal neurons and are important in regulating extracellular glutamate levels. Glutamate is transported into the Müller cells via GLAST and is catalyzed by GS to the non-toxic amino acid, glutamine. Glutamate transport is the only mechanism for removing glutamate from the extracellular fluid (27). It has been suggested that functional impairment of glutamate transporters may be involved in excitotoxicity and contribute to the pathogenesis of glaucoma (28,29). The present study indicated that Müller cells treated with 40 mmHg pressure decreased the expression levels of GS and GLAST, and reduced the L-[2,3-³H] glutamate uptake activity, which was consistent with the results of previous studies (30,31).

A_{2A}R is expressed in the inner nuclear layer, RGC layer and, less prominently, in the outer nuclear layer (32-34). Previous studies have demonstrated that A_{2A}R antagonists can enhance the recovery of retinal function following ischemia attack (35,36). The present study demonstrated that the A_{2A}R antagonist, SCH442416, increased the expression levels of GS and GLAST, and increased the L-[2,3-³H] glutamate uptake activity in Müller cells subjected to 40 mmHg pressure. This suggested that the A_{2A}R antagonist may protect RGCs by accelerating the clearance of extracellular glutamate in retina.

Collectively, the data of the present study suggested that Müller cells treated with 40 mmHg pressure decreased the expression levels of GS and GLAST, and reduced glutamate uptake activity. By contrast, SCH442416 increased the expression levels of GS and GLAST, and increased glutamate uptake activity in the Müller cells under pressure, therefore, the SCH442416 A_{2A}R antagonist may be a potential candidate as a neuroprotective agent for the treatment of glaucoma by accelerating the clearance of extracellular glutamate. Further investigations are required to confirm these effects in animal experiments.

Acknowledgements

This study was funded by the National Natural Science Foundation of China (grant no. 81371014) and the Shanghai 'Science and Technology Innovation Action Plan' Basic Research Key Project (grant nos. 11JC1407700 and 11JC1407701).

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