In vitro effect of adenosine A_{2A} receptor antagonist SCH 442416 on the expression of glutamine synthetase and glutamate aspartate transporter in rat retinal Müller cells at elevated hydrostatic pressure

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Abstract. The aim of this study was to investigate the effect of an adenosine A_{2A} receptor antagonist on the expression of glutamine synthetase (GS) and glutamate aspartate transporter (GLAST) in rat retinal Müller cells at elevated hydrostatic pressure *in vitro*. Immunofluorescence staining of GS and GFAP was used for the identification of Müller cells. The expression of GS and GLAST in different hydrostatic pressure (0, 20, 40, 60, 80 mmHg/24 h) was examined by real-time PCR and Western blotting to identify the most suitable pressure. Müller cells treated with 0.1, 1, 10 μM SCH 442416 (A_{2A} receptor antagonist) in the most suitable pressure, and the levels of GS and GLAST were examined by real-time PCR and Western blotting. Significantly increased expression of GS and GLAST at 40 mmHg pressure was observed in Müller cells and treatment with 10 μM SCH 442416 in 40 mmHg pressure further promoted the expression of GS and GLAST. A_{2A} receptor antagonist increased the expression of GLAST and GS of Müller cells and accelerated the clearance of extracellular glutamate.

Introduction

Glaucoma is the leading cause of blindness in the world and one of the most common neurodegenerative diseases, which is characterized by irreversible and progressive retinal ganglion cell (RGC) loss and damage the optic nerve, usually in response to abnormally elevated intraocular pressure (IOP) (1-4). In recent years, glial cell has emerged as the significant player in RGCs apoptosis and survival (2,5,6).

Müller cells, the major glial cells of the retina, provide functional and structural support to the retinal neurons and constitute a functional link between neurons and vessels. Additionally, Müller cells keep the integrity of the blood-retinal barrier, and play an important role in keeping extracellular level of neurotransmitters low and regulating synaptic transmission such as glutamate (7-10).

Glutamate, a normal constituent of retina, is taken up by Müller cells and then is converted to glutamine. It is released and taken up by the neurons. The neurons use gultamate to synthesize glutamate for neurotransmission. In the high non-physiological concentration, glutamate can cause neuronal injury and degeneration. Müller cells participate in glutamate metabolism by glutamate aspartate transporter (GLAST) and glutamine synthetase (GS). GLAST transport glutamate into Müller cells and GS is the enzyme that converts glutamate to glutamine the inside of Müller cells (11-13).

In some pathological state (hypoxia, edema, injury), Müller cells can be active rapidly and remove metabolic waste and maintain the balance of the retinal extracellular environment, to protect RGCs (8,14-16). Recently, we demonstrated that Müller cells were actived by GS and GLAST in the elevated moderate hydrostatic pressure *in vitro* (17).

Adenosine, is a natural chemical messenger binding to four subtypes (A_1, A_{2A}, A_{2B}, A_3) of adenosine receptors (ARs) and regulates physiological functions of the cell (18). It has been shown that adenosine receptors were expressed in the rat eye. In retina, adenosine can dilate vessels and serve an auto-regulatory role in mediating compensatory dilation in response to hypoxia, ischemia, and hypoglycemia (19,20). In recent years, A_{2A} receptor ($A_{2A}R$) antagonist has been seen as an attractive option to improve the treatment of neurological disorders such as Parkinson, Huntington and Alzheimer. The function of $A_{2A}R$ antagonist may inhibit the release of glutamate and prevent the damage of neuron (21-23).

The aim of this study is to investigate whether $A_{2A}R$ antagonist (SCH442416) may modulate the expression of

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GS and GLAST in retinal Müller cells on elevated hydrostatic pressure *in vitro,* employing a novel pressure mechanism, which is simpler and cheaper compared with the traditional pressure model.

Materials and methods

Drugs. The A_{2A} receptor antagonist (SCH 442416), 2-(2-Furanyl)-7-[3-(4-methoxyphernyl)propyl]-7H-pyrazolo[4,3-e][1,2,4] triazolo[1,5-c]pyrimidin-5-amine, was purchased from Tocris Buoscience.

Application of hydrostatic pressure. T75 culture flasks, described in detail in our previous studies (17), were equipped with a manometer, and placed in an incubator and maintained at 37˚C, as the pressure mechanism. An air mixture of 95% air and 5% CO_2 was pumped into the flasks to obtain pressure.

The pressure of the initial models were 0, 20, 40, 60, 80 mmHg, respectively. We selected the most suitable pressure to continue the experiments, by the comparison of the expression of GS and GLAST.

In our experiments, many precautions and design considerations were taken to limit artifact from the experimental method. Laboratory film (Pechiney) was used to seal the interfaces. To avoid artifacts due to 'on-off' changes in pressure, all the operations of refreshing the medium or adjustment of pressure were completed without delay.

Cell culture and purification. Primary culture of retinal Müller cells was generated as previously described (17). Briefly, retina from newborn (0-3 days) Sprague-Dawley rats (Slaccas Laboratory Animal Co., Ltd.) were dissected free and stored on ice in D-Hank's solution (Anresco). Tissue was dissociated by centrifugation and incubated for 15 min at 37˚C in phosphate-buffered saline (PBS) containing 0.125% trypsin (Anresco). Finally, the cell suspension was cultured in T75 culture flasks at 37˚C in humidified air containing 5% CO_2. After the initial outgrowth primary, the cell culture medium was replaced every 48 h, and maintained in DMEM/F12 medium (Gibco) supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (FBS) (Sijiqing).

After 5-8 days, all the flasks were shaken at 37˚C, 100 r/min in 1 h, and the cell culture medium was refreshed. By shaking the other types of cells (microgilal cells, RGCs), which were initially adhered to the surface of Müller cells, were rinsed off and a purified cell population was obtained. For passage, cell cultures were incubated at 37˚C with PBS containing 0.125% trypsin.

Experiments were performed after second passage when cell confluence was 80-90%. The cells were cultured at different pressure (0, 20, 40, 60, 80 mmHg) for 24 h to chose the most suitable pressure. Then the cells were cultured in serum-free medium in the presence of 0.1, 1, 10 μM SCH 442416, respectively, in the most suitable pressure for 24 h.

Immunofluorescence. The cultured cells grown to 80% confluence on the coverslips were fixed in sodium phosphate buffer (100 mM, pH 7.4) containing 4% paraformaldehyde for 10 min. The cells were washed in PBS, then incubated with different primary antibodies GS (Abcam, 1:5000, polyclonal rabbit anti-GS antibody), GFAP (Abcam, 1:200, polyclonal mouse anti-GFAP antibody) overnight at 4˚C. After the cells were washed three times (5 min each) in PBS, they were immunolabeled with fluorescein isothiocyanate Cy3 (Biolegend, 1:200) or FITC (Invitrogen, 1:200) linked anti-mouse or anti-rabbit IgG. The labeled cells were visualized and processed using an Axio (Zeiss) microscope.

Western blotting. The cultured cells with different samples were washed twice in PBS. Protein concentration was determined by the radio immunoprecipitation assay, and lysated in 2X Laemmli buffer. Protein extracts were boiled for 10 min and centrifuged at 16,000 x g. Proteins were separated on 12% SDS-PAGE and transferred to PVDF membranes (Millipore). The membranes were soaked in Tris-buffered saline (20 mmol/l Tris-Cl, 140 mmol/l NaCl, pH 7.5) containing 5% skimmed milk and 0.1% Tween-20 for 1 h at room temperature. Primary antibodies used were GS (Abcam, 1:5000), GLAST (Abcam, 1:3000), respectively. Blots were incubated with primary antibodies overnight at 4˚C. Anti-β actin antibody (Abcam, 1:3000) was used as a reference to normalize the intensities of immunoreactions with different antibodies. Then after several washed, the membranes were incubated with secondary antibody (Invitrogen, 1:10000) for 1 h at room temperature in darkness. Bands intensites were quantified by scanning and densitometry with Odyssey (Li-CDR).

Real-time PCR analysis. The cells were collected and used for total RNA preparations. Total RNA was reverse-transcribed into cDNA using a previously described method (24). The cDNA was used in each 20 μl PCR (40 cycles of 94˚C for 30 sec, 55˚C for 30 sec, and 72˚C for 30 sec) with gene-specific primers. The primer sequences used for RT-PCR were as follows: GS, sense 5'-ccgctcttcgtctcgttc-3', antisense 5'-ctgcttgatgcctttgtt-3'; Glast, sense 5'-cctatgtggcagtcgttt3', antisense 5'-ctgtgatgggct ggctaa3'; β-actin, sense 5'-cccatctatgagggttacgc-3', antisense 5'-tttaatgtcacgcacgatttc-3'. Real-time PCRs were performed on a LightCycler instrument (Rotor Gene) and with SYBR Green I, according to the manufacturer's recommendations.

Statistical analysis. Data are reported as the mean ± SD (n=3-6 each group). All analyses were performed with the SPSS statistical package. Data were analyzed using one-way analysis of variance followed by LSD test with a P-value of <0.05 accepted as statistically significant.

Results

Identification of cultured retinal Müller cells. We used immunofluorescence to identify the cultured Müller cells. The cultured cells showed positive labeling for GS and GFAP, molecular markers for Müller cells in the retina. By this immunocytochemical labeling, the cultured cells were considered to be Müller cells (Figs. 1 and 2).

The choice of the most suitable pressure. Western blotting and real-time PCR showed that the expression of GS and GLAST fluctuated in the different pressure samples.

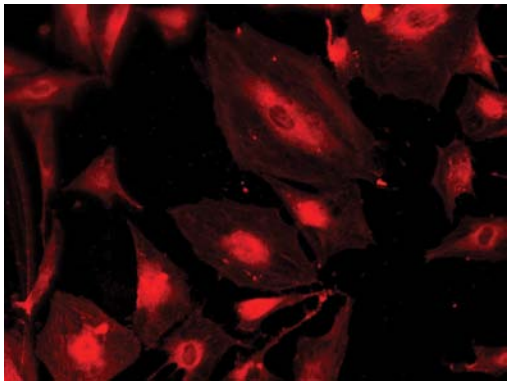

Figure 1. Identification of Müller cells. GS (red) was used to label Müller cells.

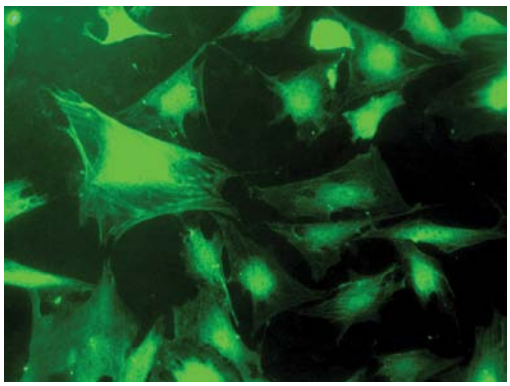

Figure 2. Identification of Müller cells. GFAP (green) was used to label Müller cells.

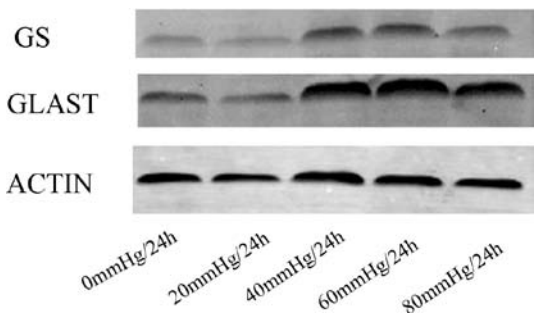

Figure 3. GS and GLAST protein expression of Müller cells at different pressures.

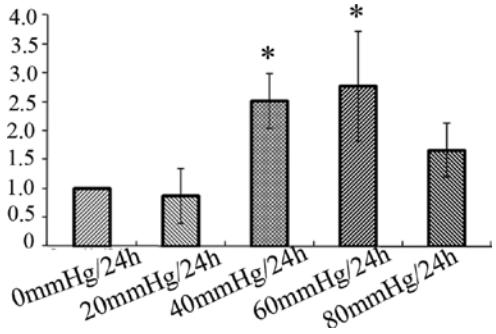

Figure 4. GS mRNA expression of Müller cells at different pressures. The expression of GS in 40 mmHg/24 h group, and 60 mmHg/24 h group, was increased significantly compared with that at 0 mmHg/24 h (*P<0.05).

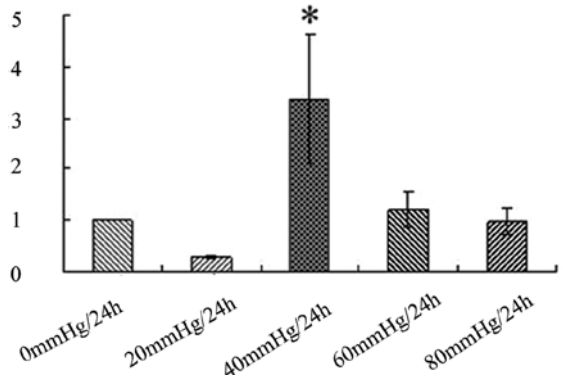

Figure 5. GLAST mRNA expression of Müller cells at different pressures. The expression of GLAST in 40 mmHg/24 h group, was increased significantly compared with that at 0 mmHg/24 h (*P<0.05).

The expression of GS in the 20 mmHg/24 h group was decreased compared with the control (0 mmHg/24 h) group and were increased in 40 mmHg/24 h group (p<0.05) and 60 mmHg/24 h group (P<0.05), and then the expression of GS was decreased slightly in 80 mmHg/24 h group (Figs. 3 and 4).

The expression of GLAST was similar to that of GS, but there was only a statistical significant increase in the 40 mmHg/24 h group, compared with the control (0 mmHg/24 h) group (P<0.05) (Figs. 3 and 5). Therefore, the pressure of 40 mmHg was chosen to make the subsequent experiment.

Effect of SCH 442416 on the expression of GS and GLAST in the cultured retinal Müller cells. In the present study, we chose different concentrations of SCH 442416 to make the experiment including 0.1, 1 and 10 μM. The expression of GS and GLAST was compared between the different concentrations to incubate Müller cells cultured in 40 mmHg/24 h.

The expression of GS and GLAST was compared in control and in 40 mmHg pressure retinal Müller cells cultured in the presence or absence of SCH 442416 treated (0.1, 1 and 10 μM) for 24 h. Western blotting and real-time PCR showed that the level of GS and GLAST was significantly increased in the Müller cells cultured with 10 μM SCH 442416 at 40 mmHg pressure for 24 h, compared with in control or at 40 mmHg pressure, absent of SCH 442416 (Figs. 6-8).

Discussion

The result of this study showed that Müller cells increased the expression of GS and GLAST in 40 mmHg pressure significantly and was treated with 10 μM SCH 442416 in 40 mmHg pressure showed a further significant increase in the expression of GS and GLAST.

GFAP is the traditionally maker for astrocytes and is also expressed in Müller cells (25,26). GS is predominantly expressed in the retina and has been used as a specific marker for Müller cells (27-29). In our study, >90% of cells in this culture system showed the positive markers for GFAP and GS, so these cells were identified as Müller cells.

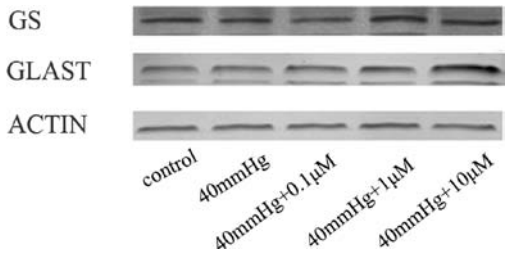

Figure 6. GS and GLAST protein expression of Müller cells treated with the different concentrations of SCH 442416.

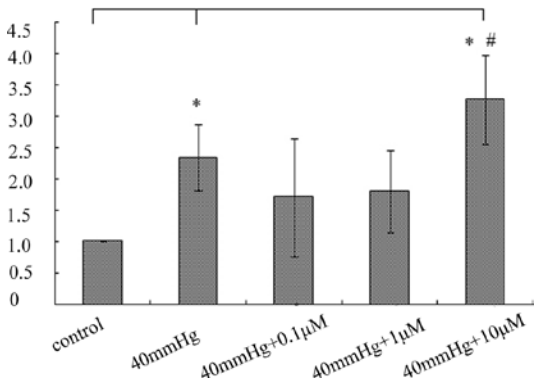

Figure 7. GS mRNA expression of Müller cells treated with SCH 442416 (0, 0.1, 1, 10 μM). The mRNA levels of GS were significantly increased in Müller cells cultured with 10 μM SCH 442416 treated at 40 mmHg pressure for 24 h, compared with in control (*P<0.05), and at 40 mmHg pressure, absent of SCH 442416 treated ($^{\#}$P<0.05).

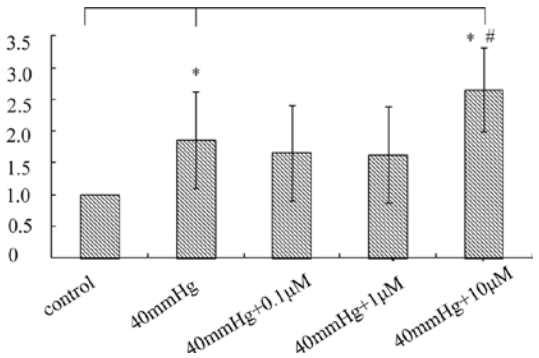

Figure 8. GLAST mRNA expression of Müller cells treated with SCH 442416 (0, 0.1, 1, 10 μM). The mRNA levels of GLAST were significantly increased in Müller cells cultured with 10 μM SCH 442416 treated at 40 mmHg pressure for 24 h, compared with the control (*P<0.05), and at 40 mmHg pressure absent of SCH 442416 treated ($^{\#}$P<0.05).

Holcombe *et al* reported that IOP threshold of ~70 mmHg for the maintenance of retinal perfusion and IOPs elevated ≤70 mmHg was consistent with the maintenance of GLAST activity in rat glaucomatous model *in vivo* (30), which was consistent with our result (40 mmHg pressure). Woldemussie *et al* found that the expression of GLAST increased and remained high for 2 months, following the elevation of IOP (30-40 mmHg) in rat glaucomatous models *in vivo*, however, the expression of GS showed little change for the first 3 weeks (13). Nonetheless, Zhang *et al* reported that the induced expression of GS was observed as early as 24 h following the IOP in elevation in the rat models of acute intraocular hypertension (31). Ishikawa *et al* also reported that the pressure was associated with the GS activity in rat glaucomatous model *in vivo* (32). In our study, Western blotting and real-time PCR demonstrated that the expression of GS and GLAST was obviously increased at 40 mmHg pressure. On the basis of the previous research and our result (33,34), we demonstrated that Müller cells were activated to mediate the extracellular glutamate by the upregulated expression of GLAST and GS in the elevated moderate hydrostatic pressure.

The progressive loss of RGCs is a central feature of the glaucoma. RGCs and neighboring nutritional cells, Müller cells maintain a balance between the intrinsic cell survival signal and the neurotoxic signal. When the balance was broken, because of a risk such as ischemia, hypoxia and elevated IOP, RGCs would enter a programmed cell death pathway (apoptosis). Glutamate excitotoxicity is thought to contribute to a broad variety of neurological diseases including glaucoma (35-39). The previous studies showed that $A_{2A}R$ inactivation could protect the nerve against various insults including ischemia, excitotoxicity, and mitochondrial toxicity (40,41). Our data showed that A_{2A} receptor antagonist took up the expression of GLAST and GS in Müller cells to accelerate the clearance of extracellular glutamate. The result suggested that A_{2A} receptor antagonist may play a protective role in RGCs by mediating the level of glutamate in retina.

A perfect glaucomatous drug should focus not only on the decrease of IOP but also on the neuroprotection of RGCs. Previous studies had demonstrated that the activation of A_{2A} receptor did not contribute to the ocular hypotensive response (42-44). Therefore, we presumed that A_{2A} receptor antagonist lowered the IOP, and did not elevate IOP. Further research is required.

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