Effect of A_{2A} receptor antagonist (SCH 442416) on the mRNA expression of glutamate aspartate transporter and glutamine synthetase in rat retinal Müller cells under hypoxic conditions *in vitro*

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Abstract. The purpose of the present study was to investigate the effect of the A_{2A} receptor antagonist (SCH 442416) on the mRNA expression of glutamate aspartate transporter (GLAST) and glutamine synthetase (GS) in rat retinal Müller cells under hypoxic conditions *in vitro*. Immunofluorescent staining of GS and GFAP was used for the identification of Müller cells. The GLAST and GS mRNA expression of Müller cells treated with 0.1, 1 and 10 μM SCH 442416 under hypoxic conditions was examined by real-time PCR. Müller cells increased the mRNA expression of GLAST under hypoxic conditions; those treated with 0.1 μM SCH 442416 showed a further significant increase in the mRNA expression of GLAST *in vitro*. Although the mRNA expression of GS was decreased under hypoxic conditions, the mRNA expression was increased when Müller cells were treated with 0.1 μM SCH 442416. A_{2A} receptor antagonist increased the GLAST and GS expression of Müller cells and accelerated the clearance of extracellular glutamate under hypoxic conditions *in vitro*.

Introduction

Glutamate, a normal constituent of retina, is the primary chemical signal used by ganglion cells, photoreceptors and bipolar cells. However, excessive stimulation by glutamate results in neuronal injury and degeneration (1,2).

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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. Among all the roles, Müller cells play an important role in keeping the extracellular levels of neurotransmitters low and regulating synaptic transmission, such as glutamate (3-6). Müller cells participate in glutamate metabolism by glutamate aspartate transporter (GLAST) and glutamine synthetase (GS). GLAST transports glutamate into Müller cells, and GS is the enzyme which converts glutamate to glutamine inside Müller cells (7-9). In several pathological states, such as hypoxia, edema and injury, Müller cells can be rapidly activated, remove metabolic waste and maintain the balance of the retinal extracellular environment to protect retinal ganglion cells (RGCs).

Adenosine is considered a reactive metabolite involved in cellular communication during periods of certain pathological states. In the eye, adenosine levels have been shown to increase during periods of retinal ischemia and hypoxia (10,11). Four adenosine receptor subtypes, A_1, A_{2A}, A_{2B} and A_3, have been identified on the basis of their molecular and pharmacological characteristics (12,13). It is well known that A_{2A} receptors are promoters of excitotoxicity by directly stimulating glutamate outflow, inhibiting glutamate uptake from neurons and glial cells in the central nervous system (14,15). In recent years, the neuroprotection afforded by A_{2A} blockade has been observed in animal models of several neurodegenerative disorders, such as Huntington's disease, Alzheimer's disease, epilepsy and excitotoxic conditions, including ischemia and trauma (15-17).

The aim of this study was to investigate whether A_{2A} receptor ($A_{2A}R$) antagonist (SCH 442416) modulates the expression of GS and GLAST in retinal Müller cells under hypoxic conditions *in vitro*.

Materials and methods

Drugs. The $A_{2A}R$ (SCH 442416), 2-(2-furanyl)-7-[3-(4-methoxyphernyl) propyl]-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo

[1,5-c]pyrimidin-5-amine was purchased from Tocris Bioscience.

Cell separation and culture. Eye balls from post-natal day 0-3 Sprague-Dawley rats (Slaccas Laboratory Animal Co., Ltd.) were enucleated, and the retina of each was 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 initial primary outgrowth, 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, at 100 rpm for 1 h, and the cell culture medium was refreshed. By shaking, other types of cells (microglial cells, RGCs), which initially adhered to the surface of the Müller cells, were rinsed off, and a purified flat cell population was obtained. For passaging, cell cultures were incubated at 37˚C with PBS containing 0.125% trypsin. Experiments were performed after second passage when the cell confluence was 80-90%.

Cell proliferation in normoxia vs. hypoxia. SCH 442416 was added to the cell culture medium at the final concentrations of 0.1, 1 and 10 μM.

Müller cells were spread out in 6-well plates at $5x10^5$/ml 24 h prior to the induction of hypoxia. For hypoxia, the medium was changed to serum- and SCH 442416-free DMEM, and the cultures were transferred to a humidified hypoxia chamber (37˚C, 94% nitrogen, 1% O_2, 5% CO_2) for 24 h. For normoxia, the medium was changed to serum-free DMEM and the cultures were placed in a humidified hypoxia chamber (37˚C, 20% O_2, 5% CO_2) for 24 h. After 24 h of incubation, the cells were analyzed.

Immunocytochemistry. Müller cells, which were cultured under hypoxic conditions for 24 h, were fixed in 4% paraformaldehyde for 10 min. The coverslips were incubated overnight in the primary antibodies anti-GFAP (1:200, polyclonal mouse anti-GFAP antibody; Abcam), anti-GS (1:5,000, polyclonal rabbit anti-GS antibody; Abcam) at 4˚C. Then, the coverslips were immunolabeled with fluoroscein isothiocyanate (FITC; 1:200; Invitrogen) or Cy3 (1:200; Biolegend)-linked anti-mouse or anti-rabbit IgG. The labeled cells were visualized and processed by laser confocal microscopy (Leica).

Gene expression analysis by quantitative real-time PCR. Total RNA was isolated from the cells using TRIzol reagent (Invitrogen). Each RNA sample was quality-controlled for DNA and protein contamination. The cDNAs were reverse-transcribed according to the manufacturer's instructions. To analyze GS and GLAST mRNA expression, the quantitative real-time PCR method was used. The primer sequences were as follows: GS, sense 5'-ccgctcttcgtctcgttc-3', antisense 5'-ctgcttgatgcctttgtt-3'; GLAST, sense 5'-cctatgtggcagtcgttt-3', antisense 5'-ctgtgatgggctggctaa-3'; β-actin, sense 5'-cccatctatgagggttacgc-3', antisense 5'-tttaatgtcacgcacgatttc-3'. Different mRNA levels were subsequently normalized to β-actin mRNA levels.

Statistical analysis. Data are reported as the means ± SEM, and were analyzed by one-way ANOVA followed by LSD test for multiple comparison. Differences were considered statistically significant at $P<0.05$.

Results

Expression of cytoskeletal proteins in the cultured Müller cells. The cultured Müller cells under hypoxic conditions showed positive labeling for GS and GFAP, the molecular markers for Müller cells in the retina (Fig. 1).

GS and GFAP are two important cytoskeletal proteins in retinal Müller cells. Fig. 1 shows the expression of these proteins by immunocytochemical staining. In normal retina, Müller cells express little or no GFAP, but become strong when the retina is damaged. GS is predominantly expressed in the retina and has been used as a specific marker for Müller cells (18-20). In our study, >90% of cells in the culture system showed positive markers for GS and GFAP, therefore these cells were identified as Müller cells.

Effect of SCH 442416 on the mRNA expression of GLAST in the cultured Müller cells. In the present study, we chose different concentrations of SCH 442416 to carry out the experiment (0.1, 1 and 10 μM). The mRNA expression of GS was compared among Müller cells incubated with the different concentrations of SCH 442416 cultured under hypoxic conditions. Real-time PCR showed that the mRNA expression of GLAST was increased significantly when Müller cells were cultured with 0.1 μM SCH 442416 under hypoxic conditions, compared to the normoxia control and hypoxia control (Fig. 2).

Effect of SCH 442416 on the mRNA expression of GS in the cultured Müller cells. Real-time PCR showed that the mRNA levels of GS were decreased in the hypoxia control compared to the normoxia control. However, the mRNA expression of GS was increased significantly when Müller cells were cultured with 0.1 μM SCH 442416 under hypoxic conditions, compared to the normoxia control and hypoxia control (Fig. 3).

Discussion

The results of the present study showed that Müller cells increased the mRNA expression of GLAST under hypoxic conditions. Cells treated with 0.1 μM SCH 442416 showed a further significant increase in the mRNA expression of GLAST *in vitro* (2). Although the mRNA expression of GS was decreased under hypoxic conditions, the mRNA expression was increased when Müller cells were treated with 0.1 μM SCH 442416.

Hypoxia certainly plays a central role in retinal diseases, such as diabetes, retinal vascular occlusion and glaucoma. It is an important cause of central neuronal damage (21,22). In normal retina, Müller cells express little or no GFAP, but the expression becomes strong when the retina was damaged. Our study also confirmed that hypoxia results in the increased expression of GFAP.

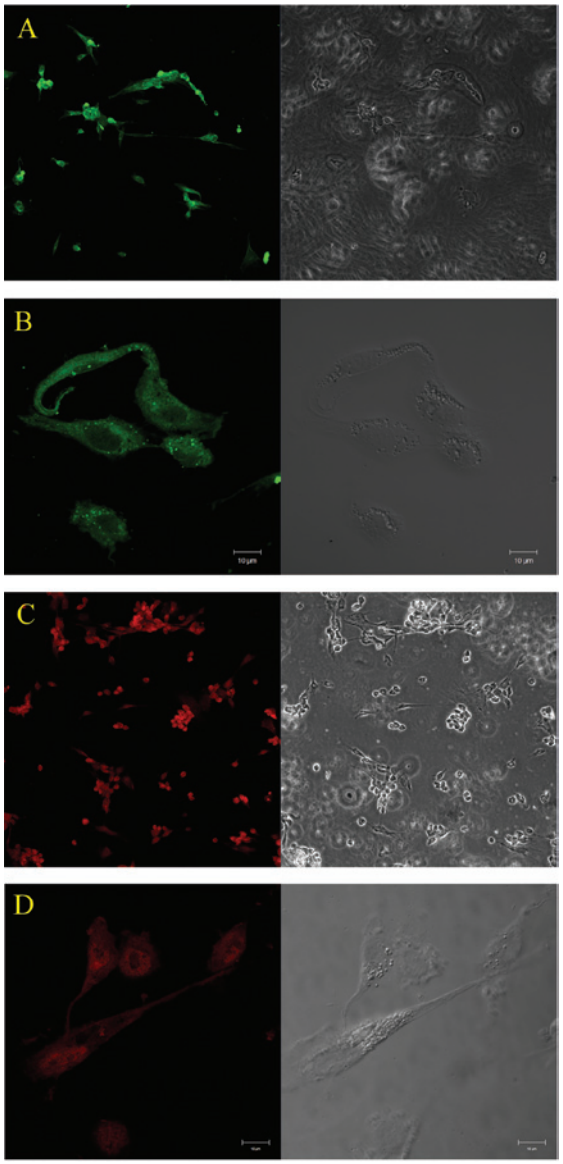

Figure 1. Identification of Müller cells. (A and B) GFAP (green) was used to label for Müller cells. (C and D) GS (red) was used to label for Müller cells.

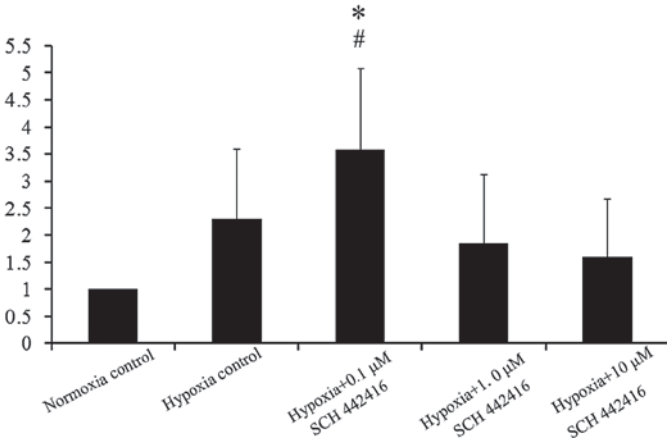

Figure 2. GLAST mRNA expression of Müller cells treated with SCH 442416 (0, 0.1, 1 and 10 μM). The mRNA levels of GLAST were significantly increased in Müller cells cultured with 0.1 μM SCH 442416 treated under hypoxic conditions for 24 h, compared to the normoxia control (*P<0.05), and the hypoxia control, where SCH 442416 treatment was absent ($^{\#}$P<0.05).

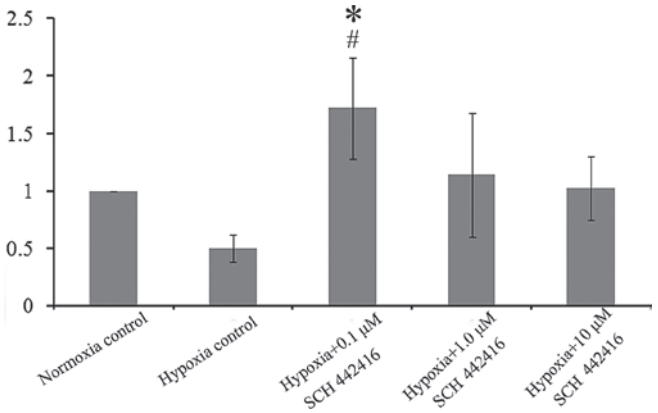

Figure 3. GS mRNA expression of Müller cells treated with SCH 442416 (0, 0.1, 1 and 10 μM). The mRNA levels of GS were decreased in the hypoxia control compared to the normoxia control, nevertheless, there was no statistical significance. The mRNA expression of GS was increased significantly when Müller cells were cultured with 0.1 μM SCH 442416 under hypoxic conditions compared to normoxia control (*P<0.05) and the hypoxia control ($^{\#}$P<0.05).

GS is a major enzyme involved in the metabolism of glutamate in glial cells. GS catalyzes the amidation of glutamate to glutamine, which is an essential part of the cycling of the transmitter pool of glutamate between neurons and glia. Decreased GS activity leads to neuronal damage by allowing extracellular glutamate to accumulate. Decreased GS activity has also been reported after hypoxia or ischemia in the brain (24,25). However, research has revealed that there is a slight change in the expression of GS and even increased GS activity in some retinal damage (26-28). In our study, we found that the mRNA expression of GS was decreased in hypoxia, nevertheless, there was no statistical significance. A low concentration of $A_{2A}R$ antagonist reversed these changes (Fig. 3).

GLAST is the predominant glutamate transporter in Müller cells. Several studies have indicated that GLAST is upregulated by hypoxia (29,30), while others observed the opposite phenomenon (31,32). In our experiment, we detected GLAST mRNA upregulation in hypoxia, and a low concentration of the $A_{2A}R$ antagonist caused a further increase.

According to the glutamate cycle, we presume that the increase in GS and GLAST accelerates the transport and clearance of glutamate in the retina to protect the neurons. Our results suggest that the $A_{2A}R$ antagonist was capable of significantly upregulating GS and GLAST, and maintained glutamate homeostasis by regulating the glutamate uptake and metabolism of Müller cells under hypoxic conditions.

In recent years, the $A_{2A}R$ antagonist has been viewed as an attractive option to improve the treatment of neurological disorders (33,34). Based on the results described here, we regard the $A_{2A}R$ antagonist as a new option for the neuroprotection of retinal Müller cells under hypoxic conditions. These data provide additional evidence for constructing a model of protection against hypoxia in the retina, which invites future studies to explore the function of the $A_{2A}R$ antagonist in ophthalmology.

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