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The Effect of A_{2A} Receptor Antagonist (SCH 442416) on the mRNA Expression of Kir 2.1 and Kir 4.1 Channels in Rat Retinal Müller Cells Under Hypoxic Conditions *in Vitro*

Wpływ antagonisty receptora A_{2A} (SCH 442416) na ekspresję mRNA kanałów Kir 2.1 i Kir 4.1 w komórkach Müllera u szczurów w warunkach hipoksji *in vitro*

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Objectives. To investigate the situation of inwardly rectifying potassium channels Kir 2.1 and Kir 4.1 under hypoxic conditions, and whether the A_{2A} receptor antagonist could modulate the mRNA expression of Kir 2.1 and Kir 4.1 channels in retinal Müller cells *in vitro* under hypoxic conditions.

Material and Methods. Müller cells were treated with 0.1 1, 10 and 100 μM of $A2_A$ receptor antagonist (SCH 442416) under hypoxic conditions for 24 h, and the expression of the Kir 2.1 and Kir 4.1 mRNA channels was examined using real-time polymerase chain reaction (qPCR).

Results. There were no significant changes in the mRNA expression of the Kir 2.1 or Kir 4.1 channels under hypoxic conditions compared with normal conditions after 24 h cultured *in vitro*. The mRNA expression of Kir 2.1 and Kir 4.1 channels treated with 0.1 μM SCH 442416 under hypoxic conditions were increased, but at higher concentrations of SCH 442416, the mRNA expression of the Kir 2.1 and Kir 4.1 channels decreased.

Conclusions. The $A2_A$ receptor antagonist (SCH 442416) could increase mRNA expression of the Kir 2.1and Kir 4.1 channels in Müller cells to protect the retinal neurons *in vitro* under hypoxic conditions **(Adv Clin Exp Med 2013, 22, 6, 825–829).**

Key words: Müller cells, Kir 4.1 channels, Kir 2.1 channels, A_{2A} receptor antagonist, hypoxia.

Hypoxia certainly plays a critical role in retinal disease and is a primary cause of central neuronal damage. Local hypoxia can cause hyperpermeability and neovascularization of the retinal vasculature [1–3].

Müller cells are the major glial cells of the retina. They have a wide array of responses to maintain homeostasis for neuronal and vascular elements [4, 5]. They can maintain the integrity of the blood-retinal barrier and clear metabolic waste via regulation of the homeostasis of extracellular pH and K^+ ions. To avoid high K^+ levels, which can induce depolarization of retinal neurons, Müller cells can take up excess K^+ from the extracellular space, especially in the synaptic layers of the retina, and release a similar amount of K^+ into spaces outside of the neural retina, especially into the blood and the vitreous humor [6].

Müller cells mediate the mechanisms of K^+ buffering through rectifying potassium channels, especially the inwardly rectifying potassium channels Kir 2.1 and Kir 4.1. The Kir 2.1 channels are the strongest inwardly rectifying Kir channels, and can allow inward K^+ currents even when there are

high levels of extracellular K^+. The Kir 4.1 channels are weaker Kir channels, allowing either "inward" or "outward" K^+ currents depending on the concentrations of extracellular K^+ [7–11].

Adenosine is a reactive metabolite involved in cellular communication during periods of some pathological state. In the eyes, the levels of adenosine increase due to retinal ischemia and/or hypoxia. Four adenosine receptor subtypes (A_1, A_{2A}, A_{2B}, and A_3) have been identified. Recently, neuroprotection of the A_{2A} blockade has been found in some animal models of some neurodegenerative disorders, such as epilepsy and Huntington's disease, and some excitotoxic conditions, such as ischemia and trauma [12–14]. The current authors' previous studies also found that A_{2A} receptor ($A_{2A}R$) antagonist could significantly upregulate the expression of glutamine synthetase and glutamate aspartate transporter, and maintain glutamate homeostasis under hypoxic conditions [15]. However, it was still not clear whether $A_{2A}R$ antagonist also could regulate the expression of the Kir channels in Müller cells in hypoxia.

The current study focusses on investigating the situation of the Kir 2.1 and Kir 4.1 channels under hypoxic conditions, and whether $A_{2A}R$ antagonist (SCH 442416) could regulate the mRNA expression of Kir 2.1 and Kir 4.1 channels in retinal Müller cells *in vitro* under hypoxic conditions.

Material and Methods

Drugs

$A_{2A}R$ antagonist (SCH 442416), 2-(2-Furanyl)--7-[3-(4-methoxyphernyl)propyl]-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5c]pyrimidin-5-amine was purchased from Tocris Bioscience. The SCH 442416 was dissolved in a serum-free medium to make final concentrations of 0.1, 1, 10 and 100 μM.

Cell Culture

The eyeballs of postnatal day 0–3 Sprague--Dawley rats were purchased from Shanghai Laboratory Animal Center CAS (SLACCAS), and each retina was dissected and stored in D-Hank's solution (Anresco) on ice. All of the tissues were dissociated and incubated for 15 min in PBS, which contained 0.125% trypsin (Anresco) at 37°C.

The tissues were then cultured in T75 flasks (in air containing 5% carbon dioxide, at 37°C). After the 1st outgrowth, the medium was refreshed every 48 h, and maintained in DMEM/F12 medium (Gibco) containing glutamine (2 mM), streptomycin (100 μg/mL), penicillin (100 U/mL) and 10% fetal bovine serum (FBS) (Gibco).

After 8–11 days, the flasks were shaken at 37°C, 100 r/min for 1 h. By shaking, other kinds of cells (such as microgilal cells and retinal ganglion cells) were rinsed off, so that a purified Müller cell population was obtained.

The cultures were incubated at 37°C for a 2nd passage. The experiments were performed after the 2nd passage when the confluence was 75–80 %.

Müller Cell Proliferation in Normoxia or Hypoxia

Firstly, Müller cells were planted in six-well plates at 5×10^5/mL for 24 h. Secondly, the medium was replaced with serum and various concentrations of SCH 442416 (0.1, 1, 10 and 100 μM) and the cultures were placed in hypoxic conditions (37°C, 94% nitrogen, 1% oxygen, 5% carbon dioxide) for 24 hours as different hypoxia groups. For the normoxia group, the medium was changed to serum-free DMEM and the cultures were placed in normal conditions (37°C, 20% oxygen, 5% carbon dioxide) for 24 h. After 24 h of incubation, the cells were analyzed.

Immunocytochemistry

Müller cells cultured under hypoxic condition for 24 h were fixed with 4% paraformaldehyde for 10 min. The cover slips were incubated in primary antibodies: anti-GFAP (Abcam, 1 : 200, polyclonal mouse antibody) and anti-GS (Abcam, 1 : 5000, polyclonal rabbit antibody) overnight at 4°C. Then the cover slips were immunolabeled with fluorescein isothiocyanate FITC (Invitrogen, 1 : 200) or Cy3 (Biolegend, 1 : 200). The cells were then observed by laser confocal microscopy (Leica).

Analysis of mRNA Expression by Real-Time PCR

RNA was isolated from Müller cells with Trizol reagent (Invitrogen). The cDNAs were reverse transcribed according to the manufacturer's instructions. Real-time PCR was used to analyze the mRNA expression of the Kir2.1 and Kir4.1 channels.

The primer sequences were as follows: Kir2.1 channels: sense 5'- gcctcctggttgctgttc-3', antisense 5'-tggtggtctgcgtctcaat-3'; Kir4.1channels: sense 5'-agttcgcacttcctatctaccg-3', antisense 5'- gggacgccactttcacaa-3'; β-actin, sense 5'-cccatctatgagggttacgc-3', antisense 5'-tttaatgtcacgcacgatttc-3'.

Real-time PCR was performed in a LightCycler instrument (Rotor Gene), with using the SYBR Green PCR Master Mix (Shuiyuan Biotech). The conditions were: 1) initial denaturation at 94°C for 5 min; 2) 40 cycles performed at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s.

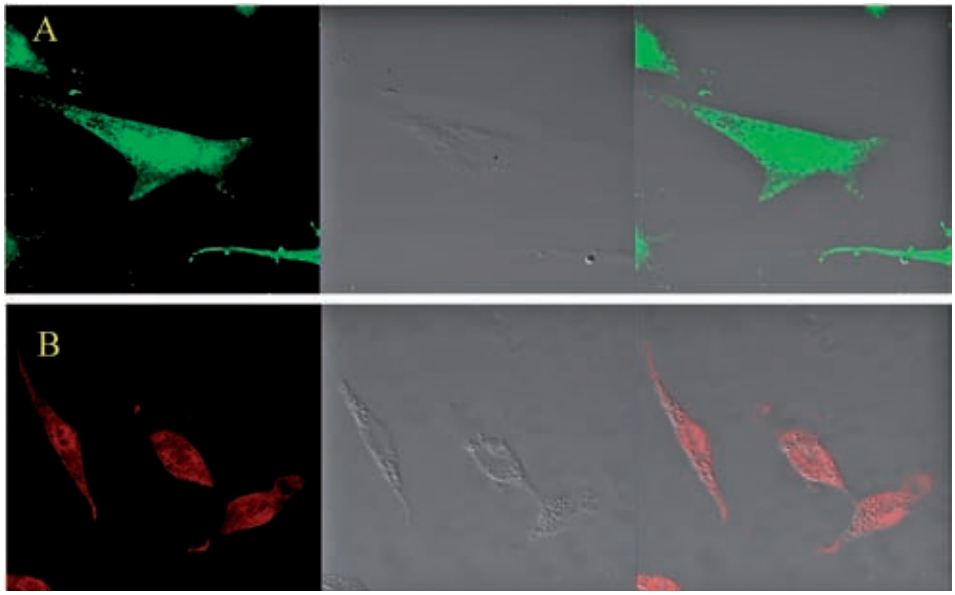

Fig. 1. Immunocytochemistry of Müller cells in hypoxia

Statistical Analysis

Data are shown as the mean ± standard error (n = 4 for each group). The analyses were executed with SPSS 13.0 software. The data were analyzed by a one-way ANOVA test followed by an LSD test for multiple comparison. Differences were considered statistically significant when $P < 0.05$.

Results

Immunocytochemistry of Retinal Müller Cells in Hypoxia

The figures showed positive labeling for GFAP and GS under hypoxic conditions; GFAP and GS were the molecular markers for Müller cells in the retina (Fig. 1), so those cells were identified as Müller cells.

GS, a molecular marker for Müller cells in the retina, has usually been used as a specific label for Müller cells. Fig. 1B shows the expressions of GS by immunocytochemistry staining. Müller cells scarcely express GFAP in a normal retina, but the expression gets stronger when the retina is damaged [15–17]. In the current study, more than 90% of the cells showed positive markers for GFAP (Fig. 1A). The cells can be identified as Müller cells, and hypoxia induced the activation of Müller cells.

GFAP (which is green) was labeled for Müller cells (A); GS (which is red) was labeled for Müller cells (B).

The effect of SCH 442416 on mRNA

Expression of Kir 2.1 Channels in Müller Cells

The PCR data showed that the mRNA expression of Kir 2.1 channels was significantly increased when Müller cells were treated with 0.1 μM SCH 442416 in hypoxia, compared with the normoxia control, the hypoxia control, and even with any another SCH 442416 concentration (1, 10 or 100 μM) (Fig. 2).

The mRNA levels of Kir 2.1 channels were significantly increased when Müller cells were treated with 0.1 μM SCH 442416 in hypoxia, compared with the normoxia control, the hypoxia control, and even with any another SCH 442416 concentration (1, 10 or 100 μM) (* $p < 0.05$).

The Effect of SCH 442416 on the mRNA Expression of Kir 4.1 Channels in the Cultured Retinal Müller Cells

The PCR data showed that the mRNA expression of Kir 4.1 channels was increased when Müller cells were treated with 0.1 μM SCH 442416 in hypoxia, compared with the normoxia control, the hypoxia control, and even any another SCH 442416 concentration (1, 10 and 100 μM) (Fig. 3).

The mRNA levels of Kir 4.1 channels were significantly increased when Müller cells were treated with 0.1 μM SCH 442416 in hypoxia, compared with the normoxia control, the hypoxia control, and even any another SCH 442416 concentration (1, 10 and 100 μM) (* $p < 0.05$).

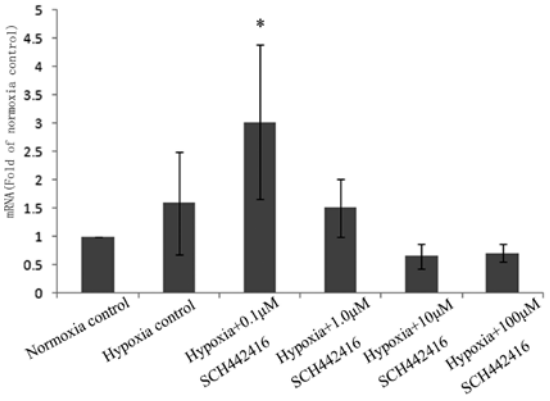

Fig. 2. The mRNA expression of Kir 2.1 channels of Müller cells cultured with SCH 442416 0.1, 1, 10 and100 μM)

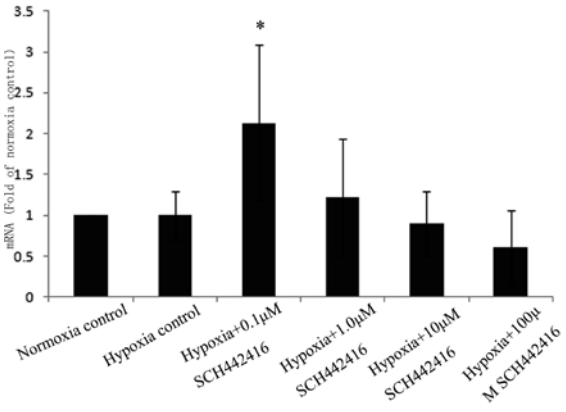

Fig. 3. The mRNA expression of Kir 4.1 channels of Müller cells treated with SCH 442416 (0.1, 1, 10 and 100 μM)

Discussion

The results of this study showed that: 1) hypoxia could induce the activation of Müller cells; 2) there were no significant changes in the mRNA expression of Kir 2.1 and Kir 4.1 channels in hypoxia, compared with normal conditions after 24 h of culturing *in vitro*; 3) Müller cells had increased mRNA expression of Kir 2.1, Kir 4.1 channels cultured with 0.1 μM SCH 442416 under hypoxic conditions, but when the SCH 442416 concentration was further increased, the mRNA expression of the Kir 2.1 and Kir 4.1channels decreased (Fig. 2 and 3).

Hypoxia certainly plays a crucial role in retinal diseases like retinal vascular occlusion, diabetes and glaucoma [3, 18]. In a normal retina, there is little or no GFAP in the Müller cells, but it becomes strong when the retina is damaged. In the present study, it was found that hypoxia could result in a high expression of GFAP, from which it can be inferred that hypoxia could induce the activation of Müller cells.

In hypoxic conditions, activated neurons release K^+, which can induce the depolarization of neurons, thereby causing neuronal hyperexcitation resulting in excess release of neurotransmitters and glutamate toxicity. Müller cells can take up excess K^+ from the extracellular space, and release an appropriate amount of K^+ into spaces outside of the retina. Kir channels localized in Müller cell membranes are used to mediate extracelluar K^+ [6].

Müller cells express different types of K^+ channels. Kir 2.1 channels are found in neuron-abutting membranes, and Kir 4.1 channels are found in membranes adjacent to the space outside the neural retina [6]. The results of the present study indicate that the response of Kir 2.1and Kir 4.1 channels of Müller cells to hypoxia was not especially sensitive *in vitro* (Fig. 2 and 3). The data also showed that the mRNA expression of Kir 2.1 channels increased slightly under hypoxic conditions, compared with normal conditions (Fig. 2).

On the basis of the results, the authors propose 2 surmises. 1st: Kir 2.1 and Kir 4.1 channels were not the key regulatory proteins in the Müller cells under hypoxic conditions. 2nd: in the present study, the experimental hypoxic time was 24 h; maybe this was too short for the activation of Kir 2.1 and Kir 4.1 channels. The question needs to be investigated in further experiments.

The results of the study showed that 0.1 μM SCH 442416 could up-regulate the mRNA expression of the Kir 2.1and Kir 4.1 channels of Müller cells in hypoxia. But when the SCH 442416 concentration was increased, the mRNA expression of the Kir 2.1 and Kir 4.1channels decreased. So the authors concluded that low concentrations of SCH 442416 could up-regulate the mRNA expression of Kir 2.1 and Kir 4.1 channels, which may accelerate the clearance of K+ to protect the retinal neurons.

In recent years, $A_{2A}R$ antagonist has been viewed as an attractive drug to treat neurological disorders [19, 20]. On the basis of the current results, the authors regard $A_{2A}R$ antagonist as a novel choice for neuro-protection under hypoxic conditions. Of course, there are some problems that need to be resolved, such as the appropriate drug concentration and the best hypoxic time. The authors plan to study these problems in future experiments.

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