In vitro effect of adenosine on the mRNA expression of Kir 2.1 and Kir 4.1 channels in rat retinal Müller cells at elevated hydrostatic pressure

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Abstract. The aim of this study was to investigate the expression of Kir 2.1 and Kir 4.1 channels at an elevated hydrostatic pressure *in vitro*, and to determine whether adenosine may modulate the mRNA expression of Kir 2.1 and Kir 4.1 channels in retinal Müller cells at an elevated hydrostatic pressure *in vitro*. Müller cells treated with 1 μM adenosine at 40 mmHg/24 h, and mRNA expression of Kir 2.1 and Kir 4.1 channels were examined using real-time PCR. Müller cells significantly increased the mRNA expression of Kir 2.1 and Kir 4.1 channels at 40 mmHg/24 h. When further treated with 1 μM adenosine at 40 mmHg/24 h, the mRNA expression of the Kir 2.1 channels decreased, while the mRNA expression of the Kir 4.1 channels continued to increase. When the pressure was elevated, Müller cells were still able to take up K^+ and mediate the potassium concentration of the retina. Adenosine upregulated the expression of the Kir 4.1 channels, but weakly affected the expression of the Kir 2.1 channels.

Introduction

Glaucoma is a member of a group of eye diseases characterized by an increased intraocular pressure (IOP), degeneration of the optic nerve and irreversible retinal ganglion cell (RGC) death (1). Elevated pressure is a significant risk factor (2,3). Although the role of glial cells in glaucomatous retinae remains under debate, certain research has found that the expression of various genes by Müller cells can be altered in glaucomatous models, including the glial fibrillary acidic protein (GFAP), glutamine synthetase (GS) and nestin (1,4-6). However, the situation in inwardly rectifying potassium (Kir) channels remains unclear.

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. One of the key roles of these cells is the spatial buffering of extracellular K^+ ions. Müller cells transport K^+ through their cell bodies away from excited neurons, from extracellular regions of 'high K^+' to those of 'low K^+' in the retina. In Müller cells, the most significant mediators of K^+ buffering are the Kir channels, particularly the Kir 2.1 and Kir 4.1 channels (7-10). Kir 2.1 channels are strongly rectifying Kir channels, which allow inward K^+ currents even at high extracellular K^+ concentrations. However, Kir 4.1 channels are weakly rectifying Kir channels at negative potentials, allowing either 'inward' or 'outward' K^+ currents, depending on the concentration of extracellular K^+. The present study suggests that the acceleration of K^+ clearance through the Kir 2.1 and Kir 4.1 channels in Müller cells can prevent the effects of neuronal information processing by depolarization caused by glia-derived K^+ (8,11,12).

Adenosine is a natural chemical messenger, which binds to four subtypes (A_1, A_{2A}, A_{2B} and A_3) of adenosine receptors (ARs), and regulates the physiological functions of cells. In the retina, adenosine is capable of dilating vessels and serves an autoregulatory role in mediating the compensatory dilation in response to hypoxia, ischemia, hypoglycemia and high hydrostatic pressure (13-15).

The aim of this study was to investigate the expression of Kir 2.1 and Kir 4.1 channels at an elevated hydrostatic pressure *in vitro*, and determine whether adenosine can modulate the expression of Kir 2.1 and Kir 4.1 channels in retinal Müller cells at an elevated hydrostatic pressure *in vitro*.

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Materials and methods

Reagents. Adenosine was purchased from Sangon Biotech Co. Ltd. (Shanghai, China). A total of 1 μM of adenosine was dissolved in serum-free medium prior to usage (16,17).

Pressure system. T75 culture flasks, equipped with manometers, were placed in incubators and maintained at 37˚C, as the pressure mechanism, as described in detail in our previous study (3). A mixture of 95% air and 5% CO_2 was pumped into the flasks to obtain the pressure (2). To determine the short-term effects of elevated pressure on the Müller cells, we exposed our cultures to a wide range of pressures (20, 40, 60 and 80 mmHg). In our previous study, we evaluated the effects of glutamine synthetase (GS) at each pressure for 24 or 48 h and determined that a hydrostatic pressure of 40 mmHg/24 h produced the most reliable and measurable effects on the Müller cells. Thus, we used 40 mmHg/24 h as the experimental condition.

Cell separation and culture. Eyeballs from post-natal 0-3 day Sprague-Dawley rats (Slaccas Laboratory Animal Co. Ltd, Shanghai, China) were enucleated, and the retina of each was freely dissected 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. Following primary initial outgrowth, the cell culture medium was replaced every 48 h, and maintained in DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (FBS) (Sijiqing).

Following 5-8 days, all of the flasks were agitated at 37˚C, 100 rpm for 1 h, and the cell culture medium was refreshed. Through agitation the other cell types (microglial cells and RGCs), which were initially adhered to the surface of the Müller cells, were rinsed off and a purified flat cell population was obtained. For passage, cell cultures were incubated at 37˚C with PBS containing 0.125% trypsin.

Using immunocytochemisty, early passage Müller cells and the Müller cell marker, glutamine synthetase (GS), were characterized (3,18). Contamination from other cell types was also tested and reported, and revealed <10% of cells expressing specific makers for other cell types, including astrocytes and microglial cells.

Experiments were performed following the second passage when cell confluence was 80-90%. Cells were cultured in serum-free medium for 16 h. Then, the cells were cultured in 0 mmHg (the control) or 40 mmHg in the presence or absence of 1 μM adenosine for 24 h.

Immunofluorescence. The cultured cells which had 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 various primary GS antibodies (Abcam, 1:5000, polyclonal rabbit anti-GS antibody) overnight at 4˚C. Subsequently, the cells were washed three times (5 min each) in PBS, and immunolabeled with fluorescein isothiocyanate Cy3 (BioLegend,1:200) linked with anti-mouse or anti-rabbit IgG. The labeled cells were visualized and processed using an Axio microscope (Zeiss).

RNA extraction. Total RNA from cultures was isolated using TRIzol reagent (Gibco) according to the manufacturer's instructions. RNA was treated with RNase-free DNase (Sangon Biotech) to remove any genomic DNA contamination. The isolated RNA had an optical density (OD) 260/280 ratio of ≥2.0.

Real-time PCR. To synthesize a cDNA template for PCR, we reverse-transcribed 2 μg total RNA to a cDNA probe. The primer sequences were as follows: Kir 2.1 channels: sense, 5'-gcctcctggttgctgttc-3' and antisense, 5'-tggtggtctgcgtctcaat-3'; Kir 4.1 channels: sense, 5'-agttcgcacttcctatctaccg-3' and antisense, 5'-gggacgccactttcacaa-3'; β-actin: sense, 5'-cccatctat gagggttacgc-3' and antisense, 5'-tttaatgtcacgcacgatttc-3'. Real-time PCR was performed using a LightCycler instrument (Rotor-Gene), with a SYBR-Green PCR Master mix (Shuiyuan Biotech), according to the manufacturer's instructions. The PCR conditions were as follows: initial denaturation at 94˚C for 5 min and 40 cycles performed at 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 30 sec.

Statistical analysis. Data were reported as the means ± standard error of the mean (each group, n=3-4). All analyses were performed with the SPSS statistical package. Data were analyzed using one-way analysis with a $P<0.05$ used to indicate a statistically significant difference.

Results

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

Effect of adenosine on the expression of Kir 2.1 channels in the cultured retinal Müller cells. In our study, the real-time PCR data revealed that the mRNA expression of Kir 2.1 channels was significantly increased in the Müller cells cultured in the presence or absence of 1 μM adenosine at 40 mmHg for 24 h, compared with the normoxia control. However, there were no significant changes between the groups in which Müller cells were cultured in the presence or absence of 1 μM adenosine at 40 mmHg pressure for 24 h; there was even a decline (Fig. 3).

Effect of adenosine on the expression of Kir 4.1 channels in the cultured retinal Müller cells. The real-time PCR data revealed that the mRNA expression of Kir 4.1 channels was significantly increased in the Müller cells cultured with 1 μM adenosine at 40 mmHg pressure for 24 h, compared with the normoxia control or at 40 mmHg pressure, in the absence of adenosine (Fig. 4).

Discussion

The results of this study demonstrated that in Müller cells, the mRNA expression of Kir 2.1 and Kir 4.1 channels significantly

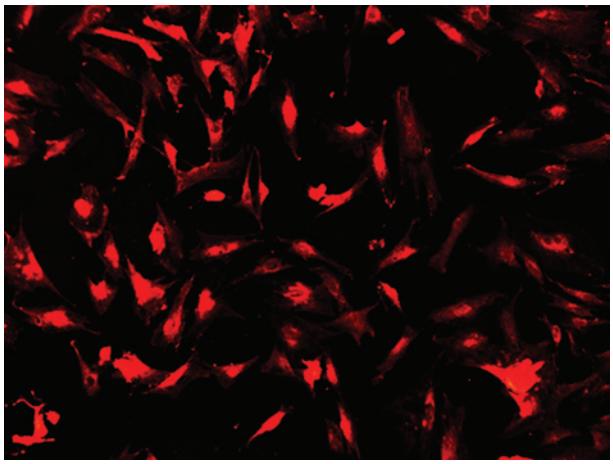

Figure 1. Identification of Müller cells. GS (red) was used to label the Müller cells. Magnification, x100. GS, glutamine synthetase.

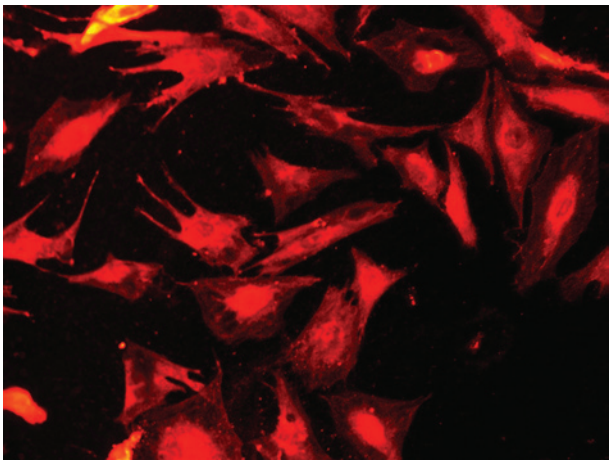

Figure 2. Identification of Müller cells. GS (red) was used to label the Müller cells. Magnification, x200. GS, glutamine synthetase.

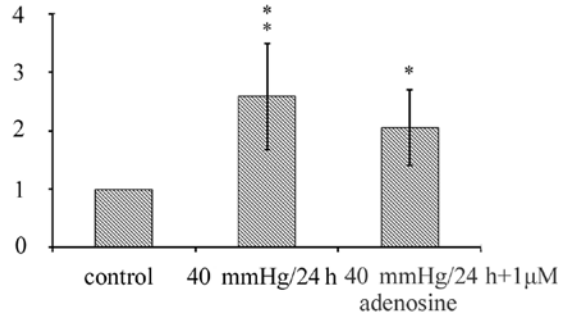

Figure 3. Kir 2.1 channel mRNA expression in Müller cells treated with adenosine. The mRNA expression of Kir 2.1 channels was significantly increased in Müller cells cultured in the presence or absence of 1 μM adenosine at 40 mmHg pressure for 24 h, compared with the normoxia control. However, there were no significant changes between the groups in which Müller cells were cultured in the presence or absence of 1 μM adenosine at 40 mmHg pressure for 24 h. (*p<0.05, **p<0.01, compared with the normoxia control).

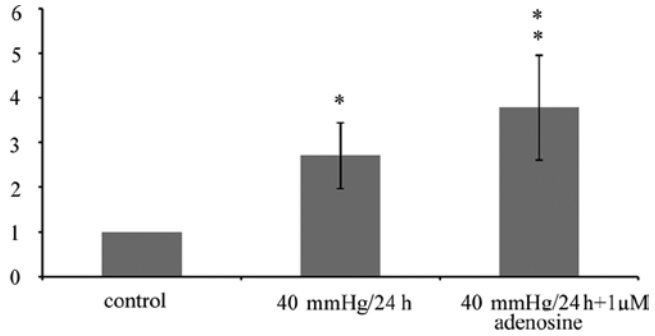

Figure 4. Kir 4.1 channel mRNA expression of Müller cells treated with adenosine. The mRNA expression of Kir 4.1 channels was significantly increased in the Müller cells cultured with 1 μM adenosine at 40 mmHg pressure for 24 h, compared with the normoxia control or at 40 mmHg pressure in the absence of adenosine. (*p<0.05, **p<0.01, compared with the normoxia control).

increased at 40 mmHg *in vitro*. Continually, when cells were further treated with 1 μM adenosine at 40 mmHg pressure, the mRNA expression of Kir 2.1 channels decreased; however, the mRNA expression of Kir 4.1 channels continued to increase, compared to when treated without adenosine at 40 mmHg pressure (Figs. 3 and 4).

GS is predominantly expressed in the retina and has been used as a specific marker for Müller cells. In our study, more than 90% of cells in this culture system demonstrated positive markers for GS, therefore these cells were identified to be Müller cells.

The degeneration of RGCs in glaucoma is accompanied by morphological and functional changes in Müller cells, the main type of glial cell in the retina (18). Müller cells are responsible for the maintenance of homeostasis in the extracellular medium of the retina and protection of the neurons through the release of neurotrophins (19-22). Moreover, Müller cells can also have altered expression and functioning potassium channels, with consequential alteration in ion homeostasis and development of edema in the retina. Activated neurons release potassium ions. To avoid potassium-induced depolarization of neurons, Müller cells take up excess potassium from the extracellular space, particularly in the plexiform layers of the retina, and release an appropriate amount of potassium into the spaces outside the retina. Kir channels localized in Müller cell membranes are used to mediate extracellular potassium. Müller cells express various types of potassium channels. Kir 2.1 channels are expressed in neuron-abutting membranes, through which Müller cells take up excess potassium. Kir 4.1 channels are expressed in membranes which are in close contact with spaces outside the neural retina. On the basis of the present results, we presumed that when the pressure elevated rapidly, Müller cells would remain capable of taking up K^+, and mediate the potassium concentration of the retina.

Kir 2.1 channels are strongly rectifying Kir channels, which mediate only inward potassium currents into Müller cells, while Kir 4.1 channels are weakly rectifying Kir channels, which mediate bidirectional currents between the extraretinal tissues and the interior of the Müller cells (23,24). Based on the present results it has been suggested that adenosine can upregulate the expression of Kir 4.1 channels, but weakly affect the expression of Kir 2.1 channels. The upregulation

of Kir 4.1 channels should accelerate retinal K^+ clearance to prevent neuronal hyperexcitation and excessive release of glutamate.

There are still certain problems which require resolution, including whether adenosine is capable of affecting the expression of Kir 2.1 channels, and what would happen if the concentration of adenosine was changed. Moreover, the effect of adenosine on the protein expression of Kir 2.1 and Kir 4.1 channels require further study.

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