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Prevention of extracellular ADP-induced ATP accumulation of the cultured rat spinal astrocytes via P2Y(1)-mediated inhibition of AMPK

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ABSTRACT

P2Y(1) is probably an important subtype of purinergic receptors (P2Rs) in modulation of the astrocyte activation in spinal cord. The aim of this study was to observe the effect of P2Y(1) receptor on the abnormal energy metabolism of the cultured rat spinal astrocyte induced by extracellular adenosine diphosphate (ADP). The results showed that adenosine triphosphate (ATP) and mitochondrial membrane potential (MMP) in the astrocytes were up-regulated in the presence of ADP, which could be enhanced by MRS2179, a specific antagonist for P2Y(1) receptor. A higher level of expression of the AMP-activated protein kinase (AMPK) was found in the presence of MRS2179 and ADP together than that ADP alone. Blocking of AMPK with Compound C could effectively inhibit the enhancing effect of MRS2179 on ADP-induced astrocyte proliferation and ATP accumulation. Our results suggested that the P2Y(1) receptor mediated inhibition of AMPK may help to prevent the astrocytes from over activation induced by extracellular ADP.

As the major cell population in the nervous system, the astrocytes are indispensable partners of the neurons, either in physiological or in pathological condition. The astrocytes in the spinal dorsal horn exhibit a hyperactive phenotype in response to peripheral nerve injury that initiates neuropathic pain [9,14]. Activation of the astrocytes is accompanied by initiation of cellular signal transduction pathways induced by a variety of chemical mediators or gliotransmitters, ATP for instance [11,12].

The energy for functional responses and metabolic events of the astrocytes was from intracellular ATP, and many communications between the nerve cells required extracellular ATP for transmission [5]. Under normal conditions, ATP is biosynthesized in the mitochondria and is regulated by AMP/ATP ratio in cells. Because of the barrier created by the cell membrane, it still remains unclear whether and how did the extracellular ATP and its derivates affect ATP metabolism in the astrocytes.

Extracellular ATP acted as an important neurotransmitter in certain neuropathic pain states resulted from peripheral nerve injury [3,4]. Extracellular ATP was unstable and easy to be hydrolyzed in the intercellular space. The breakdown of ATP by ectonucleotidases not only terminated its extracellular messenger functions, but also provided a pathway for the generation of ADP, acting via certain P2Y receptors [18]. P2Y was a subfamily of G-protein-coupled P2Rs and expressed in many kinds of nerve cells [16]. The P2Y family is composed out of 8 subtypes and half of subtypes can be activated by ADP or its analogues including ADPbetaS. Effects of P2Y receptors can be blocked by Guanosine 5′-O-(2-thiodiphosphate) (GDPβS), a broad G-protein inhibitor [17]. It was found in previous studies that ADP and one of the P2Y receptors, P2Y(1), played an important role in transmitting messages from the astrocytes to the neurons as well as from the extracellular space to the intracellular organelles [19,20]. Before employment as a therapeutic target for neuropathic pain, the effect and action mechanism of the P2Y(1) receptor on cell survival needed deep investigation, which was exactly the purpose of the present study.

AMPK is an important regulator of physiological energy dynamics. In general, activation of AMPK acts to maintain cellular energy stores, switching on catabolic pathways that produce ATP, while switching off anabolic pathways that consume ATP [13]. Such effects can be reduced by Compound C, a pharmacological inhibitor of AMPK [2]. It still remains unclear on AMPK involving or not in the modulation of extracellular ADP on ATP metabolism in astrocytes.

In this study, a rat spinal astrocyte culture system was used to observe the effect of P2Y(1) activation on extra- or intracellular ATP contents, mitochondrial membrane potential (MMP) and probe into the relationship of these changes with AMPK expression.

Primary dissociated culture of the dorsal spinal cord was prepared from the postnatal Sprague-Dawley rats (1–3 days old) in a manner consistent with the National Institutes of Health guidelines that stressed minimizing animal pain and suffering and limiting to the smallest number of the animals used. All experiments were

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carried out in accordance with the China animal welfare legislation and were approved by the Third Military Medical University Committee on Ethics in the Care and Use of Laboratory Animals. Primary dissociated culture of the dorsal spinal cord astrocytes was prepared and maintained as described previously [19]. After confirmation of the purification by anti-glial fibrillary acidic protein (GFAP, Sigma), the cells were incubated and used in the subsequent procedures.

Cellular ATP levels were measured using a firefly luciferase ATP assay kit (Beyotime, China) according to the manufacturer's instructions. Briefly, the astrocytes were incubated with PBS, ADP, GDPbetaS + ADP (or ADPbetaS) and MRS2179 + ADP (or ADPbetaS) in a mixed medium for 24 h. Next, a 0.5 ml culture medium was transported to 1.5 ml EP tube and was kept on ice to precisely determine the extracellular ATP concentration. Then, the cells were schizolysised and centrifuged at 12,000 × *g* for 5 min. In 1.5 ml EP tubes, 100 μl of each supernatant was mixed with 100 μl of ATP detection working dilution. Luminance (RLU) was measured by a GloMax 20/20 luminometer (Promega, USA). Standard curves were also generated, and the protein concentration of each treatment group was determined using the Bradford protein assay. The intracellular ATP level was normalized by protein content in each sample (unit: nmol/mg protein).

MMP was assessed by using 5,5′,6,6′-tetrachloro-1,1′3,3′-tetraethylbenzamidazol-carboncyanine (JC-1; Genmed Scientifics Inc., USA) in both a direct and an indirect manner [8]. JC-1 is a cationic dye whose mitochondrial uptake was related directly to the magnitude of the mitochondrial membrane potential. Direct measurement of MMP was taken by adding 100 μl of 2 mmol/l JC-1 to the astrocytes after treating in the manner above mentioned. After 20 min incubation at 37 °C in the dark, a positive result was detected by an Olympus BX60 microphotography system (Olympus, USA) under a rhodamine (red) or fluorescein (green) spectral filter. For indirect verification, the astrocytes were collected by scraping after being trypsinized. The normalized isolated cells (concentration 10^5 cells/ml) for 1 ml was incubated with 10 ml of 200 mmol/l JC-1 (final JC-1 concentration of 2 mmol/l) for 20 min at 37 °C with 5% CO_2. The cells were subsequently pelleted by centrifugation (5000 × *g* for 5 min at 4 °C) and resuspended in PBS. The JC-1 fluorescence for the cell suspensions and PBS controls were measured in triplicate in the black costar 96-well plates (Corning Inc., NY) using a microplate reader (Ex/Em(green)/Em(red): 485/538/590 nm) (SpectraMAX Gemini EM, USA). A higher red:green ratio indicated a greater polarization, that is, more negative and hyperpolarized MMP.

Astrocyte proteins were extracted using a T-PER protein extraction reagent (Pierce, USA) and separated on 10% SDS polyacrylamide gels. The proteins were electrophoretically transferred onto the polyvinylidene fluoride membranes. The membranes were shaken in a blocking buffer (TBST with 5% fat free dry milk) for 6 h and then incubated with rabbit anti-AMPK (1:200, Millipore) overnight at 4 °C. The membranes were then incubated with a donkey anti-rabbit IgG antibody fragment (1:1000) for 2 h and with the horseradish peroxidase-conjugated streptavidin (1:1000) for 1 h at 37 °C, and then the proteins were visualized using the chemiluminescence method. The immune-reactive density was analyzed by Quantity One software.

In muti-labeling immunofluorescence experiments, the sections were pre-treated as described above and then were incubated with 10% normal goat serum. Subsequently, the sections were incubated with AMPK rabbit monoclonal (1:250, Millipore) and GFAP (1:300, Sigma) mouse polyclonal antibodies at 37 °C for 1 h, followed by incubation at 4 °C overnight. The antibody binding to the tissue sections was visualized with a Cy3 labeling goat anti-rabbit (1:300 in PBS, Jackson Immuno-Research, West Grove, PA, USA) IgG and FITC labeling goat anti-mouse IgG antibody fragments (1:300 in PBS, Jackson Immuno-Research) at 37 °C for 1.5 h. Finally, the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, 1:1000, Roche). For immunofluorescence preparations that required labeling of only one protein, the sections were incubated with AMPK rabbit polyclonal antibody and then with Cy3-conjugated goat anti-rabbit IgG as a secondary antibody. The positive results were detected with an Olympus BX60 microscope (Olympus, USA).

The number of the primary cultured astrocytes was determined by application of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (Beyotime, China), which provided an index of cell proliferation [1]. Astrocytes were plated onto the 96-well plates (5 × 104/0.1 ml/well) for 48 h and then incubated in regents mixed serum-free medium for 24 h. The cells were washed with serum-free medium 3 times, and then 10 μl of MTT solution was added to each well. After incubation for 4 h at 37 °C, 100 μl of solubilizing buffer was added to each well and incubated for 3 h to stop the reaction. The 96-well plates were read by an iMark microplate reader (Bio-RAD, Japan) at 570 nm for optical density values to determine the cell proliferation.

All data were presented as mean ± SEM. Statistical analysis was performed with one-way analysis of variance (ANOVA) followed by least significant difference (LSD) post-hoc tests when multiple comparisons were made (SPSS16.0, SPSS Inc., Chicago, IL, USA). A p-value of 0.05 or less was considered statistically significant.

Our results suggested that ADP and ADPbetaS induced a significant increase of both the intracellular (Fig. 1A) and extracellular (Fig. 1B) ATP concentration, and which could be fully blocked by GDPbetaS. Pretreatment with MRS2179 did not inhibit ATP content increase, but augmented it to a much higher level.

To investigate the mechanism that caused ATP accumulation in the astrocytes, we detected the MMP of the astrocytes to determine whether ATP biosynthesis was increased. The results suggested that ADP or ADPbetaS significantly improved MMP after 24 h, for which MRS2179 had a significant enhancing effect (Fig. 2A1–F3). The results showed that the red fluorescence in the astrocytes turned stronger than normal. In the presence of 100 μM MRS2179, red fluorescence in the astrocytes was strengthened further and green fluorescence turned weaker. The red:green ratio indicated the degree of MMP. It was found that 100 μM MRS2179 significantly enhanced the MMP increase induced by ADP or ADPbetaS (Fig. 2G).

The AMPK expression in the astrocytes was initially detected with multi-channel immunofluorescence, which indicated AMPK (green) was in a good concordance with the astrocyte specific glial fibrillary acidic protein (GFAP) (red) and DAPI (blue, stained for nuclei) in the normal cultured spinal astrocytes (Fig. 3).

To define the influence of P2Y(1) receptor activation on AMPK expression, the immunofluoresence was used to detect the effect of ADP on AMPK expression. After ADP treatment, the expression of AMPK was much stronger than that in the untreated cells. On co-incubation with MRS2179 and ADP, the immunodetection of AMPK in the astrocytes was significantly increased (Fig. 4A–C). Similar changes were also found following ADPbetaS treatment (Fig. 4D and E). The amount of AMPK protein in the astrocytes was also measured by Western blotting and analyzed with Quantity One software. The results revealed that 100 μM MRS2179 increased the AMPK protein expression induced by ADP and ADPbetaS (Fig. 4G1 and G2).

To determine the direct effect of AMPK, 20 μM Compound C was used to directly block the AMPK activation and investigated its effect on the ADP-induced ATP accumulation and astrocyte proliferation. The results revealed that the extra- and intracellular ATP concentrations were decreased significantly regardless of the presence of MRS2179 (Fig. 5A and B). JC-1 assay suggested that MMP