·Original Article·

Effects of $P2Y_1$ receptor on glial fibrillary acidic protein and glial cell line-derived neurotrophic factor production of astrocytes under ischemic condition and the related signaling pathways

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Abstract Objective The present study aimed to explore the role of $P2Y_1$ receptor in glial fibrillary acidic protein (GFAP) production and glial cell line-derived neurotrophic factor (GDNF) secretion of astrocytes under ischemic insult and the related signaling pathways. **Methods** Using transient right middle cerebral artery occlusion (tMCAO) and oxygen-glucose-serum deprivation for 2 h as the model of ischemic injury *in vivo* and *in vitro*, immunofluorescence, quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR), Western blotting, enzyme linked immunosorbent assay (ELISA) were used to investigate location of $P2Y_1$ receptor and GDNF, the expression of GFAP and GDNF, and the changes of signaling molecules. **Results** Blockage of $P2Y_1$ receptor with the selective antagonist N^6-methyl-2'-deoxyadenosine 3',5'-bisphosphate diammonium (MRS2179) reduced GFAP production and increased GDNF production in the antagonist group as compared with simple ischemic group both *in vivo* and *in vitro*. Oxygen-glucose-serum deprivation and blockage of $P2Y_1$ receptor caused elevation of phosphorylated Akt and cAMP response element binding protein (CREB), and reduction of phosphorylated Janus kinase2 (JAK2) and signal transducer and activator of transcription3 (STAT3, Ser727). After blockage of $P2Y_1$ receptor and deprivation of oxygen-glucose-serum, AG490 (inhibitor of JAK2) reduced phosphorylation of STAT3 (Ser727) as well as expression of GFAP; LY294002, an inhibitor of phosphatidylinositol 3-kinase (PI3-K), decreased phosphorylation of Akt and CREB; the inhibitor of mitogen-activated protein kinase kinase1/2 (MEK1/2) U0126, an important molecule of Ras/extracellular signal-regulated kinase (ERK) signaling pathway, decreased the phosphorylation of JAK2, STAT3 (Ser727), Akt and CREB. **Conclusion** These results suggest that $P2Y_1$ receptor plays a role in the production of GFAP and GDNF in astrocytes under transient ischemic condition and the related signaling pathways may be JAK2/STAT3 and PI3-K/Akt/CREB, respectively, and that crosstalk probably exists between them.

Keywords: $P2Y_1$ receptor; gliosis; glial fibrillary acidic protein; glial cell line-derived neurotrophic factor; PI3-K/Akt/CREB; JAK2/STAT3; Ras/ERK

1 Introduction

Reactive astrogliosis is a repair process caused by various insults to the central nervous system (CNS). Astrogliosis manifests hypertrophy with increase of its glial fibrillary acidic protein (GFAP) contents as well as hyperplasia of astrocyte. The relevance of astrogliosis remains controversial, especially with respect to the beneficial or detrimental influence of reactive astrocytes on CNS recovery[1]. The reactive astrocytes can secret neurotrophic factors including GDNF and confine the spread of inflammation and toxic chemicals[2,3]. At the same time, the formation of a glial scar may interfere with neuronal repair or axonal regeneration in the CNS. Therefore, controllable astrogliosis might be an admirable goal: to enhance its beneficial effects of gliosis and to avoid its detrimental effects.

Extracellular ATP and its derivants have been considered as transmitters for years[4,5]. "Purinergic" receptors were first formally recognized and classified as P1 and P2 sub-

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Article ID: 1673-7067(2008)04-0231-13
CLC number: Q42
Document code: A
Received date: 2008-04-30

types by Burnstock in 1978[6]. The widespread and abundant distribution of the $P2Y_1$ receptor within the brain arouses many interests.

P2 receptors can activate STAT3 serine 727 phosphorylation in astrocytes and activation of STAT3 maybe play a key role in astrogliosis[7]. ERK attributes to CREB phosphorylation that leads to GDNF production in cultured astrocytes[8]. The roles of $P2Y_1$ receptor in astrogliosis, STAT3 phosphorylation and GDNF secretion as well as the related signal pathways under ischemic brain injury remain to be elucidated.

Our study is targeting on the roles of $P2Y_1$ receptor during the process of astrogliosis and GDNF secretion with the transient right middle cerebral artery occlusion (tMCAO) *in vivo*, and oxygen-glucose-serum deprivation of cultured astrocytes *in vitro* as the model of transient ischemic injury. The related signaling pathways would be investigated. We hope our work will help us to deepen our understanding of related pathophysiological processes and might arouse new therapeutic approaches.

2 Material and methods

2.1 Reagents and antibodies Mouse anti-GFAP monoclonal antibody, mouse anti-β-actin monoclonal antibody, MRS2179, and proteinase inhibitor kit set [aprotinin, leupeptin, pepstatin, and 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF)] were purchased from Sigma (Saint Louis, Missouri, USA). Mouse anti-NeuN monoclonal antibody, rabbit anti-$P2Y_1$ receptor polyclonal antibody and rabbit anti-JAK2 polyclonal antibody were purchased from Chemicon international (Temecula, CA, USA). Monoclonal anti-GDNF antibody was purchased from R&D system, Inc. Phospho-state-specific and non-phospho (pan) antibodies to Akt (Ser473), CREB (Ser133), STAT3 (Ser727), phospho-state-specific antibody to JAK2(Tyr1007/1008) and LY294002, U0126 were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). AG490 and phosphatase inhibitor cocktail set III (sodium fluoride, sodium orthovanadate, sodium pyrophosphate decahydrate, β-glycerophosphate) were purchased from Calbiochem. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG were purchased from Proteintech Group, Inc. (Chicago, IL, USA). Fluorescein (FITC)-conjugated affinipure rabbit anti-mouse IgG and rhodamine (TRITC)-conjugated AffiniPure goat anti-mouse IgG were purchased from Jackson Immuno Research Laboratories Inc. (Baltimore, PA, USA). M-MLV reverse transcriptase and GDNF enzyme linked immunosorbent assay (ELISA) kit were purchased from Promega (Madison, WI, USA). Enhanced chemiluminescence (ECL) immunoblotting substrate was purchased from Pierce. Genbox anaer and Genbox jar were purchased from bioMériux (Marcy-I'Etoile, France).

2.2 Cell culture Pregnant Sprague-Dawley (SD) rats were bought from Shanghai Laboratory Center of Chinese Academy Sciences. All procedures were conducted in accordance with NIH guidelines for the care and use of laboratory animals. Heterogeneous primary cells were cultured from embryonic day 16 (E16) SD rat embryos[9]. Pregnant SD rat was anesthetized with 10% chloral hydrate (0.4 g/kg, i.p.). Pups were taken out, embryo cerebral cortex was dissected free of adherent meninges, and the tissue was minced and dissociated into single-cell suspension after incubation in D-Hanks containing 0.12% trypsin for 10 min at room temperature. And placed in cell culture dishes containing DMEM/F-12 (1:1) with 15% fetal calf serum and penicillin-streptomycin (100 U/mL and 100 μg/mL, respectively) at the density of 6×10^6 per 10-mm dish. Cultures were maintained at 37 ºC in a humidified 5% CO_2-containing atmosphere until they reached 100% confluence. Cells were fed twice a week, and at DIV (days *in vitro*) 5 they were treated with cytosine arabinoside (10 μmol/L) to eliminate the dividing microglia from the cultures. The cultures were used for experiments at 13-15 d in culture.

2.3 Ischemia model *in vitro* An *in vitro* ischemia model (oxygen-glucose-serum deprivation) was used to investigate the possible cellular and molecular mechanisms responsible for cerebral ischemia[10]. One day before experiment, cells were cultivated in a glucose- and serum- free DMEM-F12. The culture plates were put into an anoxic chamber which could consume all oxygen in 15 min and keep deprivation status for 2 h. During this incubation, cells grew in an environment similar to the ischemic condition *in vivo*. After 2 h, cultured cells returned to normal cultivation condition.

2.4 Microinjection into cerebro-ventricular system Adult male Wistar rats weighing 250-300 g, which were bought from Shanghai Laboratory Center of Chinese Academy Sciences, were used for the experiments .

Rats were anesthetized with 10% chloral hydrate (0.4 g/kg, i.p.). The head was immobilized in a small animal stereotaxic device[11]. A scalpel was used to expose the skull, and a hole

was drilled with a needle at 1.6 mm rostrally, 2.0 mm laterally, and 0-0.5 mm ventrally to bregma. The needle was inserted vertically 3.5 mm into the predrilled hole. MRS2179 or vehicle was infused into the cerebro-ventricular system to the final concentration of 10 μmol/L in the cerebrospinal fluid. After injection, the needle was left in the place for 5 min before removed, and then the skin was sutured.

2.5 Ischemia model *in vivo* Thirty minutes after injection of MRS2179 or vehicle, the animals in the experimental groups were subjected to tMCAO as described by Longa with minor modifications[12]. In brief, the right cervical carotid bifurcation was exposed, and both the proximal common carotid artery (CCA) and the external carotid artery (ECA) were ligated. A 4-0 nylon suture with a thermically rounded tip was introduced into the distal CCA through ECA and advanced into the internal carotid artery between 18 and 20 mm until a faint resistance was felt. The suture was secured in this position using a tight ligature around the ECA. After occlusion for 2 h, animals were re-anesthetized and the occluding filament was withdrawn to achieve a reperfusion condition. Sham surgeries were performed in the same way except that the filament was advanced only a few millimeters inside the internal carotid artery.

For immunofluorescence staining, animals were anesthetized with chloral hydrate and transcardially perfused with ice-cold 0.9% NaCl and then followed by 4% paraformaldehyde until the livers turned yellow and hardened, removed the brains and fixed in 4% paraformaldehyde for 24 h and cryoprotected in 30% sucrose at 4 ºC for 24 h, then embedded in optimal cutting temperature compound (OCT) and placed in –70 ºC immediately for further frozen slide making. For Western blotting, ELISA and reverse transcription-polymerase chain reaction (RT-PCR) experiments, animals were sacrificed by decaptation under anesthesia at 0, 2, 6, 24, 48, or 72 h after reperfusion and the brains were removed. Two coronal sections of 5-mm thick were taken at the optic chiasm and placed under -70 ºC condition immediately.

2.6 Immunofluorescence Immunofluorescence labeling of tissue. The sections were permeabilized and blocked with 0.3% Triton X-100 and 10% normal goat serum in 0.01 mol/L PBS (pH 7.4) for 30 min, and primary antibodies (1:200) were then applied to the sections overnight at room temperature. On the following day, sections were incubated with either fluorescein-conjugated goat anti-rabbit or rhodamine-conjugated goat anti-mouse antibodies (1:200). Slides were washed, mounted, and examined using a Nikon fluorescence microscope.

Cells grown on coverslips were washed briefly with PBS and fixed with 4% paraformaldehyde for 20 min, blocked with 10% normal goat serum at room temperature for 20 min and then incubated with primary antibodies (1:200) at 4 ºC overnight followed by fluorescent secondary antibodies (1:200) at room temperature for 45 min. Coverslips were mounted with 90% glycerol in PBS and examined by fluorescence microscope.

2.7 Quantitative real-time RT-PCR for GFAP mRNA Cells or tissue were lysed in TRIzol, total RNA was extracted from primary cultures in 100-mm dishes or tissues with TRIzol reagent according to the manufacturer's instruction and the RNA concentration was measured photometrically. First strand cDNA was synthesized from 1 μg of total RNA using M-MLV reverse transcriptase and $(dT)_{16\text{-}18}$. Total cDNA was amplified in a 25-μL reaction mixture using forward and reverse primers. Real-time PCR was performed using the TaKaRa PCR premix on a machine (Corbett research) according to the manufacturer's protocol. The sequence of GFAP fragments (171 bp) (GenBank: U03700) was: forward 5'- GTT GTG AAG GT C TAT CCT GGT TG-3', reverse 5'- GTC TA G GCG ATA CTC CGT ACA TG-3'. As an internal control for the correct PCR conditions and RNA amount in each sample, cDNA fragments (220 bp) of the constitutively expressed β-actin (GenBank: NM031144.2) were amplified using the following primers: forward 5'-AGG ATG CAG AAG GA G ATT ACT GC-3'; reverse 5'-AAA ACG CAG CTC A GT AAC AGT GC-3'. Samples were cycled by an initial denaturation at 95 ºC for 15 s, 35 cycles of denaturation at 95 ºC for 10 s, annealing, elongation and acquiring the fluorescence signal at 60 ºC for 40 s, 72 ºC for 20 s. PCR fragments were separated by agarose gel electrophoresis and identified by ethidium bromide staining. The data were dealt with the Rotor Gene 6 software and the results were expressed as fold change relative to β-actin.

2.8 Western blotting Tissue or serum-starved cells was incubated with MRS2179 and inhibitors (DMSO solution) or only DMSO in the glucose- and serum-free DMEM/F-12 for 45 min. After treatment, tissue or cells was lysed in 100 μL of ice-cold homogenate buffer [50 mmol/L Tris-HCL(pH 8.0), 150 mmol/L NaCl, 0.02% NaN_3, 0.1% SDS, 1% NP-40, 0.5% hyodeoxycholic acid sodium] at 4 ºC for 25 min. The lysate was centrifuged at 12 000×*g* for 15 min at 4 ºC, and then aliquots were taken for protein determination using a BCA

protein assay kit. The cell lysate was boiled in loading buffer containing 150 mmol/L Tris (pH 6.8), 3 mmol/L DTT,10% SDS, 0.3% bromophenol blue and 30% glycerol. After adding protease inhibitor and/or phosphatase inhibitor, aliquots containing 20 μg total protein were applied to SDS-PAGE and blotted to PVDF membranes. The blotted membranes were incubated with 5% non-fat dry milk in PBS containing 0.1% Tween 20 for 45 min followed by incubation with primary antibodies. The membranes were subsequently incubated with a peroxidase-conjugated secondary antibody and the immunoreactive bands were detected with an enhanced chemiluminescence detection kit. After detection of GFAP or the phosphor-proteins, the antibodies were stripped from the membrane by incubation with a solution for 5 min. Then β-actin or total proteins were measured with related antibodies. The integrated optical density (IOD) of lanes was measured with Gel-Pro 4.0 software.

2.9 ELISA ELISA on the cell-free culture supernatants and the tissue extracts was carried out with commercially available ELISA kits for GDNF according to manufacture's instruction.

2.10 Statistics Mean values for each group were statistically compared using one-way ANOVA and significant results ($P < 0.05$) were followed by Tukey's *post hoc* test. Paired-samples were assessed using *t*-test.

3 Results

3.1 $P2Y_1$ receptor and GDNF colocalized in astrocytes after tMCAO *in vivo* and neurons died after oxygen-glucose-serum deprivation *in vitro* After tMCAO for 2 h *in vivo*, double labeling immunofluorescence studies revealed that $P2Y_1$ receptor was expressed by astrocytes (Fig. 1). Double labeling immunofluorescence studies also showed that GDNF and $P2Y_1$ receptor colocalized in the same kind of cells at 2 h after reperfusion (Fig. 2). After oxygen-glucose-serum deprivation for 2 h *in vitro*, neurons died with Hoechst33258 staining (Fig. 3). Therefore, this heterogeneous culture mimicked the physiological condition *in vivo* and met our requirement of investigating astrocytes after deprivation of oxygen-glucose-serum.

3.2 The expression level of GFAP mRNA increased after tMCAO *in vivo* and oxygen-glucose-serum deprivation *in vitro*, but reduced after adding MRS2179 In order to investigate the influence of $P2Y_1$ receptor on GFAP expression under the ischemic condition, quantitative real-time RT-PCR was conducted to measure the expression level of GFAP

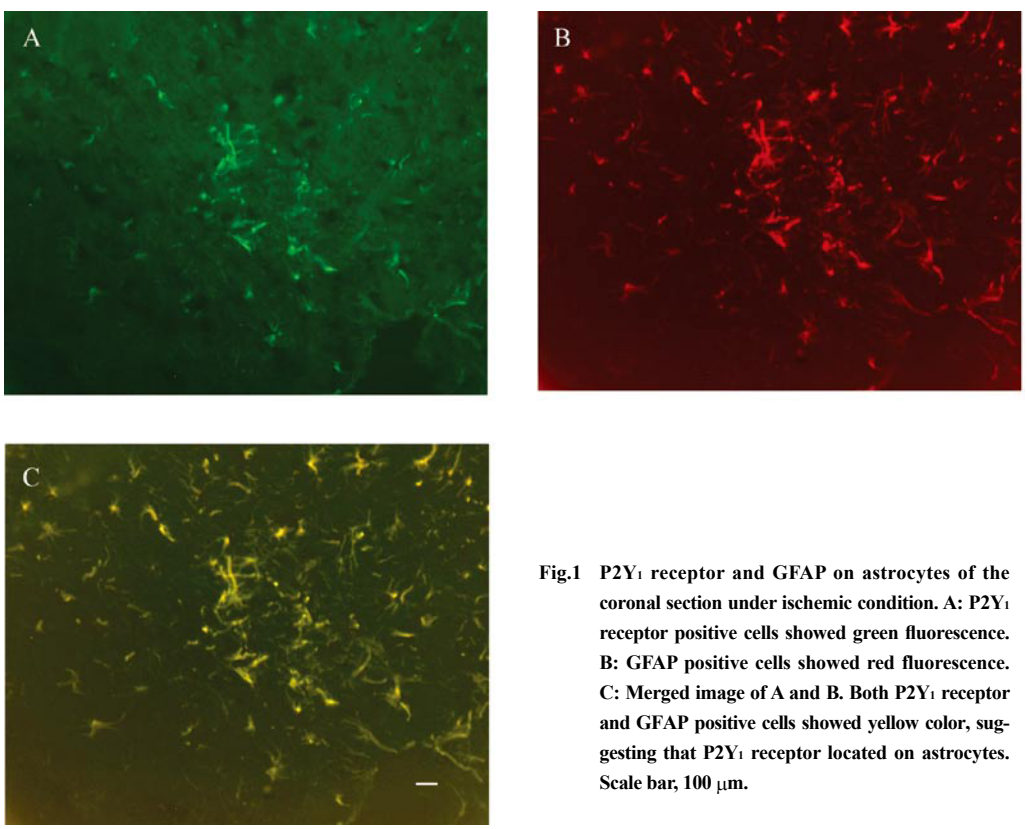

Fig.1 $P2Y_1$ receptor and GFAP on astrocytes of the coronal section under ischemic condition. A: $P2Y_1$ receptor positive cells showed green fluorescence. B: GFAP positive cells showed red fluorescence. C: Merged image of A and B. Both $P2Y_1$ receptor and GFAP positive cells showed yellow color, suggesting that $P2Y_1$ receptor located on astrocytes. Scale bar, 100 μm.

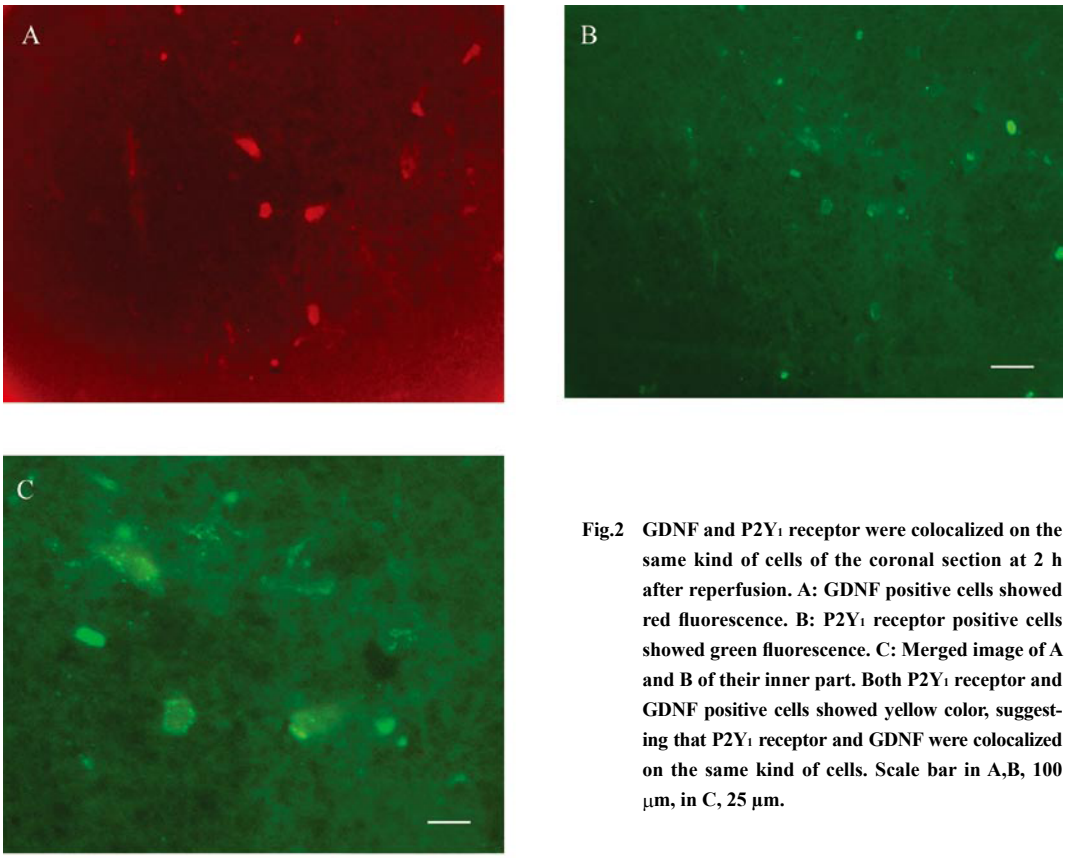

Fig.2 **GDNF and $P2Y_1$ receptor were colocalized on the same kind of cells of the coronal section at 2 h after reperfusion. A: GDNF positive cells showed red fluorescence. B: $P2Y_1$ receptor positive cells showed green fluorescence. C: Merged image of A and B of their inner part. Both $P2Y_1$ receptor and GDNF positive cells showed yellow color, suggesting that $P2Y_1$ receptor and GDNF were colocalized on the same kind of cells. Scale bar in A,B, 100 μm, in C, 25 μm.**

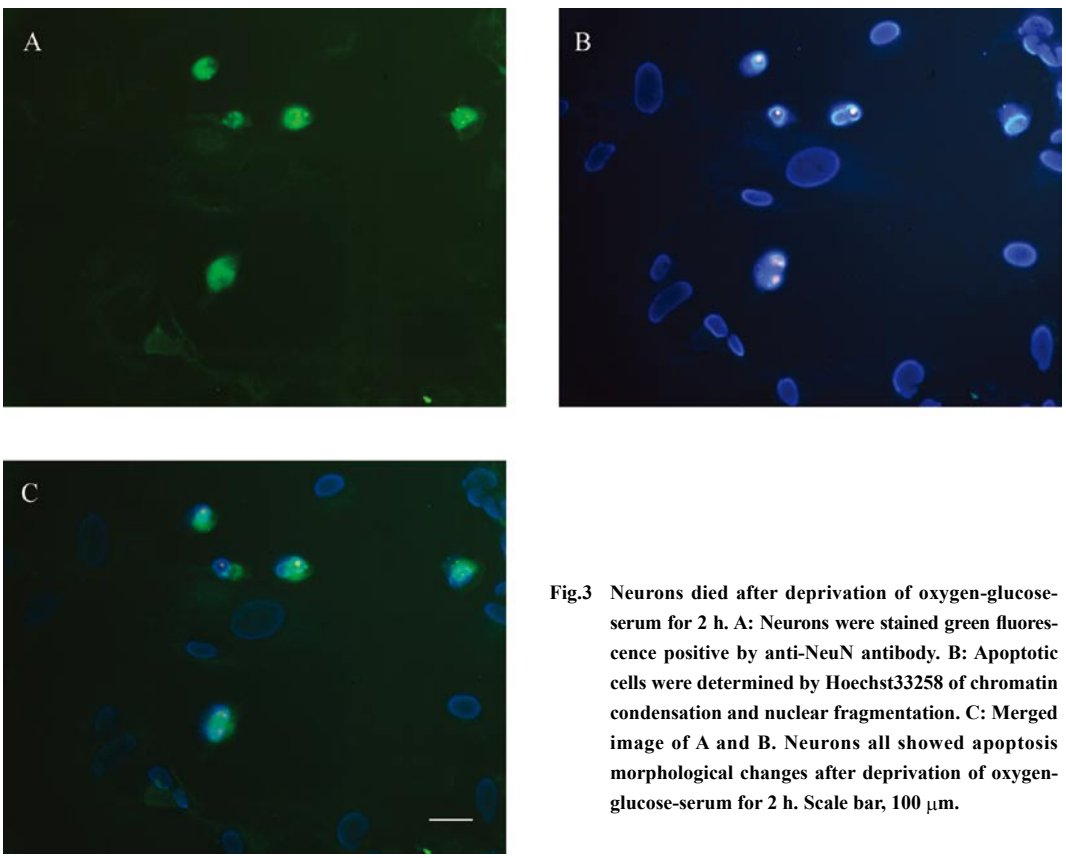

Fig.3 **Neurons died after deprivation of oxygen-glucose-serum for 2 h. A: Neurons were stained green fluorescence positive by anti-NeuN antibody. B: Apoptotic cells were determined by Hoechst33258 of chromatin condensation and nuclear fragmentation. C: Merged image of A and B. Neurons all showed apoptosis morphological changes after deprivation of oxygen-glucose-serum for 2 h. Scale bar, 100 μm.**

mRNA after tMCAO *in vivo* and oxygen-glucose-serum deprivation *in vitro*.

According to the *in vivo* study, GFAP mRNA in the simple tMCAO group increased at 6 h, 24 h, and 72 h after reperfusion as compared with the control (sham operation, Fig. 4A). In the antagonist group (adding MRS2179), GFAP mRNA increased at 24 h and 72 h after reperfusion as compared with the control (sham operation, Fig. 4B). Compared with simple tMCAO group, GFAP mRNA of the antagonist group reduced at 6 h, 24 h, and 72 h after reperfusion (Fig. 4C).

In vitro, the simple oxygen-glucose-serum deprivation group showed that GFAP mRNA increased at 2 h after deprivation as compared with the cells with normal cultivation (Fig. 4D). There was no difference between the cells in the antagonist group and those in normal cultivation (Fig. 4E). Compared with the simple oxygen-glucose-serum deprivation group, the GFAP mRNA level in the antagonist group showed reduction at control, 0 h, 2 h, 6 h, and 72 h after returning of normal cultivation (Fig. 4F).

The results showed that blockage of $P2Y_1$ can inhibit

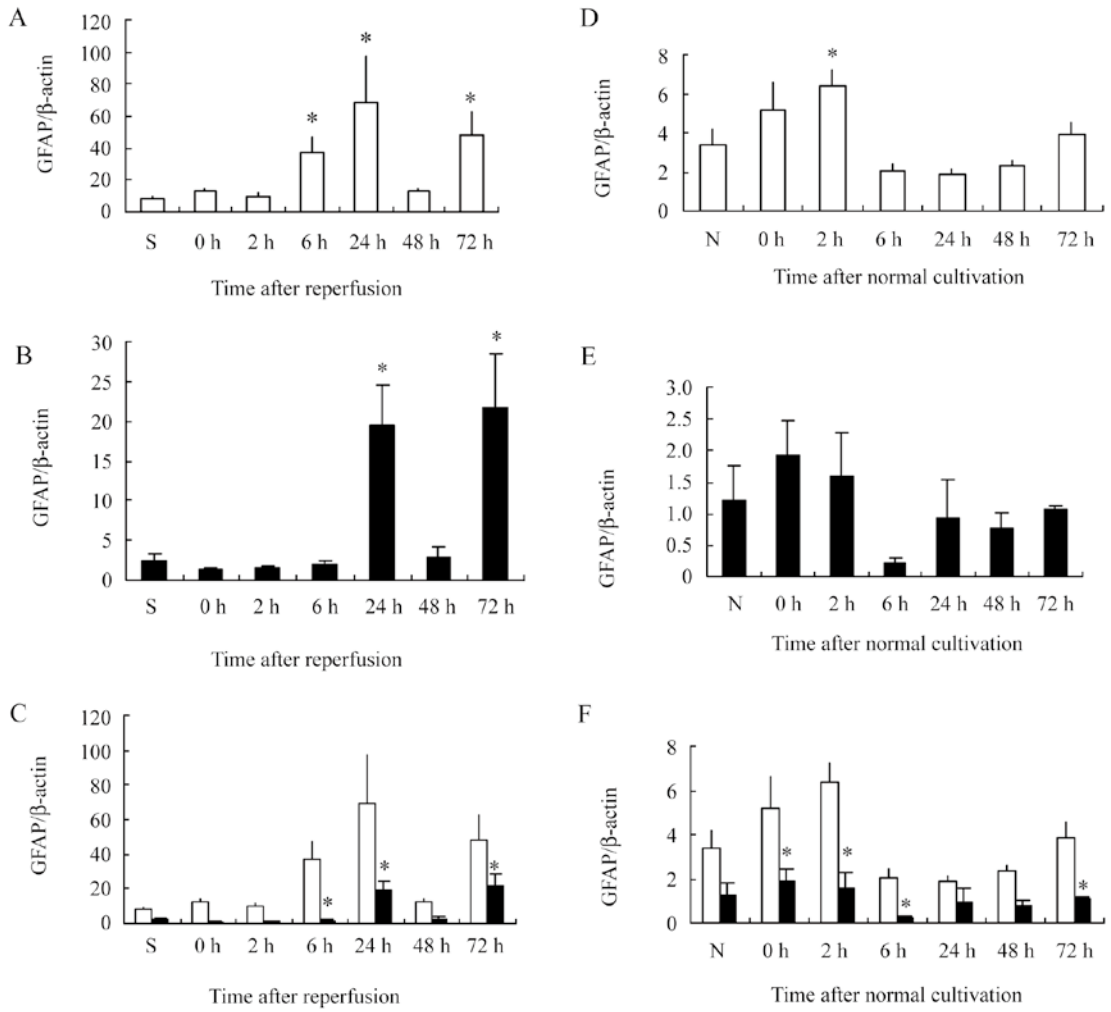

Fig.4 Expression of GFAP mRNA in the injuried region and cultured cells induced by temporal ischemia with or without blocking of $P2Y_1$ receptor with MRS2179 (10 μmol/L) as determined by quantitative real time RT-PCR. A: tMCAO group *in vivo*. B: The antagonist group *in vivo*. C: Compared A with B. D: Oxygen-glucose-serum deprivation *in vitro*. E: The antagonist group *in vitro*. F: Compared D with E. cDNA samples obtained from the injured region or cultured cells at various times (0, 2, 6, 24, 48, 72 h) after reperfusion were amplified by real-time PCR. Sample from a sham-operated rat (lane S) 2h after the sham operation without occlusion or cultured cells without deprivation of oxygen-glucose-serum (lane N). Quantification of the RT-PCR data for GFAP was performed by Rotor Gene 6 software. Data are shown as mean±SEM in three independent experiments. White columns stand for simple tMCAO group *in vivo* or deprivation of oxygen-glucose-serum group *in vitro*. Black columns stand for the antagonist groups *in vivo* and *in vitro*. A, B, D: *$P < 0.05$ compared with S or N; C, F: *$P < 0.05$ compared with simple tMCAO group *in vivo* or deprivation of oxygen-glucose-serum group *in vitro* at the same time point.

the expression of GFAP mRNA under the ischemic model both *in vivo* and *in vitro*.

3.3 The expression level of GFAP protein increased after tMCAO *in vivo* and oxygen-glucose-serum deprivation *in vitro*, but reduced after adding MRS2179 In order to investigate the effects of $P2Y_1$ receptor on GFAP expression under ischemic condition, Western blotting was conducted to measure the expression level of GFAP protein after tMCAO *in vivo* and oxygen-glucose-serum deprivation *in vitro*.

According to the *in vivo* study, GFAP increased at 2 h after reperfusion in the simple tMCAO group compared with the control (Fig. 5A). In the antagonist group (adding MRS2179), GFAP expression reduced at 2 h and 24 h after reperfusion compared with the control (Fig. 5B). Compared with simple tMCAO, the antagonist group showed reduction of GFAP except at 48 h time point after reperfusion (Fig. 5C).

In vitro, the simple oxygen-glucose-serum deprivation group showed that the expression of GFAP increased at 72 h after returning to the normal cultivation condition as compared with the control (Fig. 5D). The antagonist group showed no significant increase compared with the control (Fig. 5E). Compared with the simple oxygen-glucose-serum deprivation group, the GFAP expression of the antagonist group reduced at 48 h and 72 h after returning to the normal cultivation (Fig. 5F).

The results showed that blockage of $P2Y_1$ could inhibit the expression of GFAP protein under the ischemic model *in vivo* and *in vitro*.

3.4 GDNF production increased under ischemic condition after inhibiting the $P2Y_1$ receptor both *in vivo* and *in vitro* ELISA results showed that GDNF production increased at 2 h after reperfusion in the antagonist group *in vivo* as compared with other time points in the simple tMCAO group and the antagonist group, except at 0 h in the antagonist group (Fig. 6A). *In vitro* study, GDNF could be detected only in the supernate of antagonist group immediately after oxygen-glucose-serum deprivation (0 h). Supernate of other time points in antagonist group and all simple oxygen-

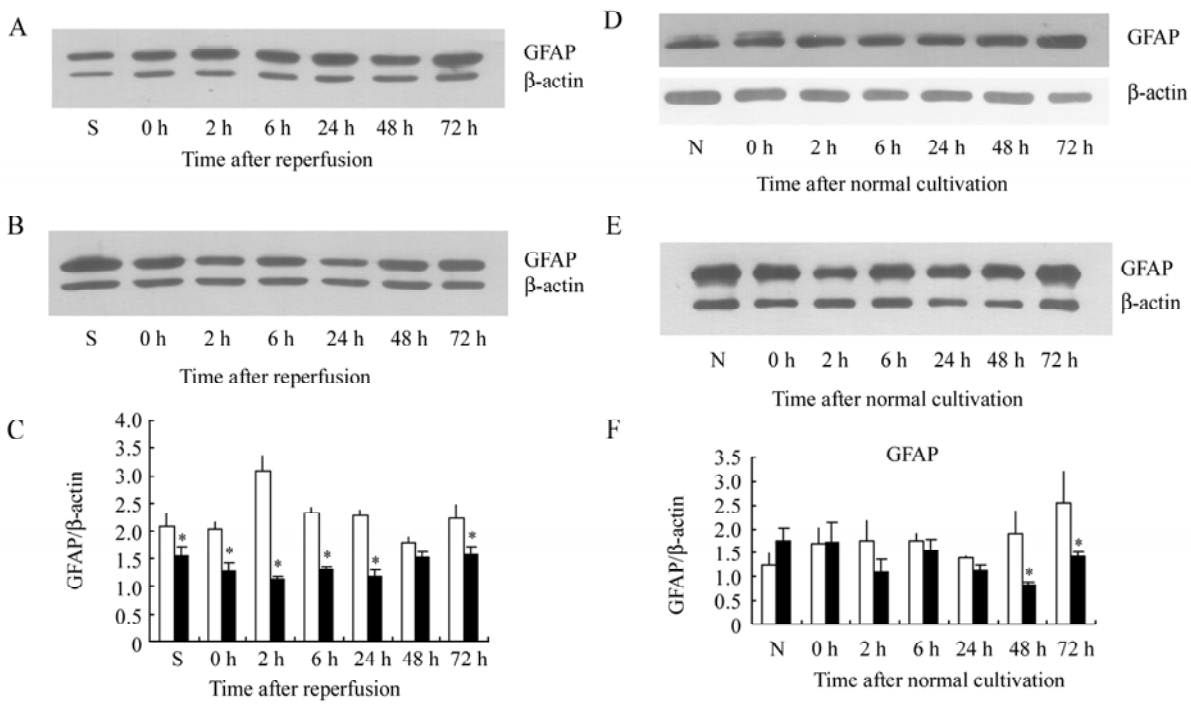

Fig.5 Transient ischemia-induced GFAP production in the injuried region and cultured cells with or without blocking of $P2Y_1$ receptor. A: tMCAO group *in vivo*. B: The antagonist group *in vivo*. C: Compared A with B. D: Oxygen-glucose-serum deprivation *in vitro*. E: The antagonist group *in vitro*. F: Compared D with E. Total homogenates of the injured regions or dishes at various times (0, 2, 6, 24, 48, 72 h) after reperfusion and sham-operated rats or control cells were analyzed with Western blotting. Data are shown as mean±SEM in three independent experiments. S: Sham-operated sample *in vivo*. N: Sample without deprivation of oxygen-glucose-serum *in vitro*. White columns stand for simple tMCAO group *in vivo* or deprivation of oxygen-glucose-serum group *in vitro*. Black columns stand for the antagonist groups *in vivo* and *in vitro*. *$P < 0.05$ compared with the simple tMCAO group *in vivo* or the deprivation of oxygen-glucose-serum group *in vitro* at the same time point.

glucose-serum deprivation could not be measured (Fig. 6B).

3.5 JAK2/STAT3 signaling pathway took part in GFAP production after blockage of $P2Y_1$ receptor and deprivation of oxygen-glucose-serum and Ras/ERK pathway was also involved in this process Given the fact that JAK2/STAT3 signaling pathway takes part in astrogliosis under some conditions[7,13], we investigated the possible involvement of this signaling pathway with GFAP production under ischemic

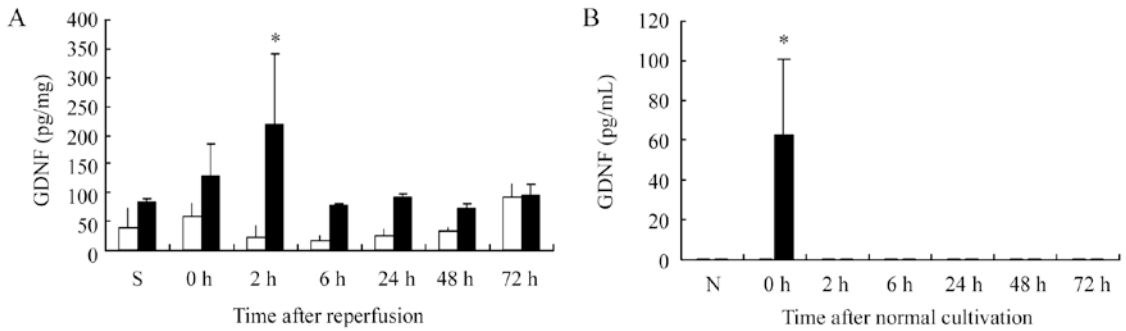

Fig. 6 Temporal ischemia-induced GDNF production in the total homogenates of the injuried regions and supernates of cultured cells with or without blocking of $P2Y_1$ receptor. A: GDNF production (pg/mg tissue extract) *in vivo*. B: GDNF production (pg/mL) *in vitro*. The total homogenates of the injured regions and supernates of cultured cells at various time points (0, 2, 6, 24, 48, 72 h) after reperfusion and sham-operated rats or control cells were analyzed with ELISA. Data are shown as mean±SEM in three independent experiments. White columns stand for simple tMCAO group *in vivo* or deprivation of oxygen-glucose-serum group *in vitro*. Black columns stand for the antagonist groups *in vivo* and *in vitro*. *$P < 0.05$ compared with simple tMCAO group *in vivo* or deprivation of oxygen-glucose-serum group *in vitro* at the same time point.

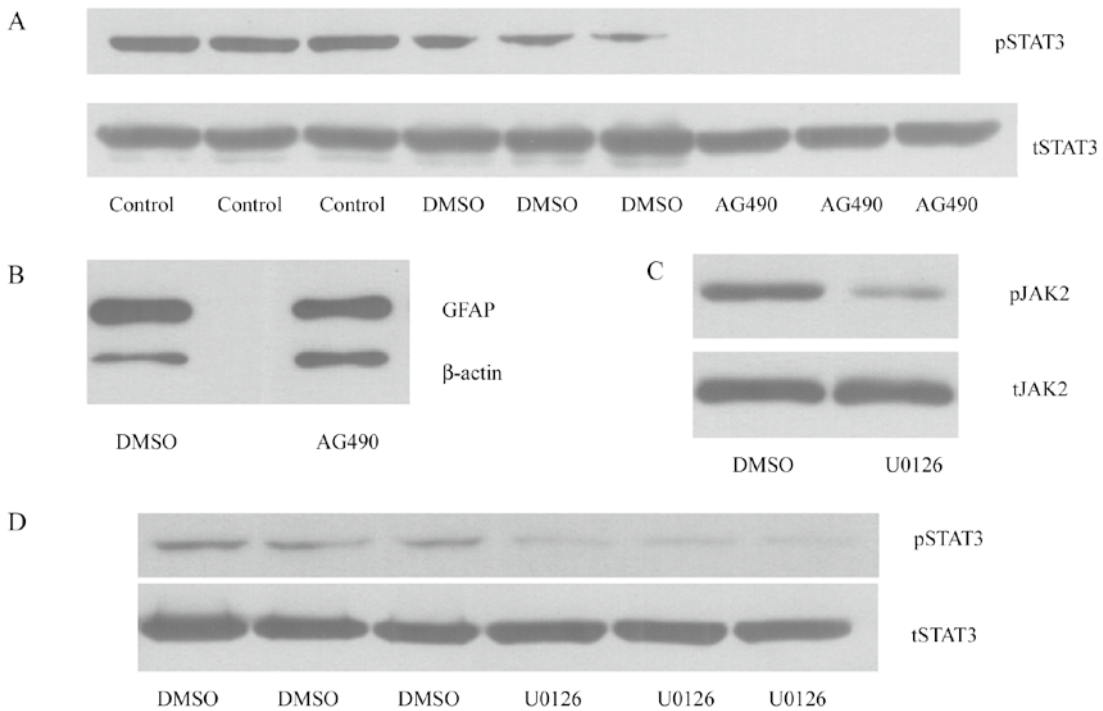

Fig. 7 Activation of STAT3 after deprivation of oxygen-glucose-serum and blocking of $P2Y_1$ receptor and variation of GFAP after inhibiting JAK2 and the effects on phosphorylated JAK2 and STAT3 of U0126. A: Expression of phosphorylated STAT3. B: Expression of GFAP. C: Expression of phosphorylated JAK2. D: Expression of phosphorylated STAT3. All of the samples were treated with MRS2179 (10 μmol/L) 30 min prior to experiment. Control: Normal cells were treated with DMSO (25 μL) 30 min prior to experiment without deprivation of oxygen-glucose-serum. DMSO: Cells were treated with DMSO 30 min prior to experiment with deprivation of oxygen-glucose-serum for 2 hours. AG490 or U0126: Cells were treated with AG490 (50 μmol/L, DMSO solution) or U0126 (10 μmol/L, DMSO solution) 30 min prior to experiment with deprivation of oxygen-glucose-serum for 2 h. Data are shown as mean±SEM in three independent experiments. A: *$P < 0.05$ compared with control; B, C, D: *$P < 0.05$ compared with DMSO.

condition and blockage of $P2Y_1$ receptor. The results showed that after deprivation of oxygen-glucose-serum, phosphorylated STAT3 (Ser727) reduced compared with the control. After adding AG490 (inhibitor of JAK2), phosphorylated STAT3 (Ser727) almost disappeared and GFAP expression reduced compared with the oxygen-glucose-serum deprivation group at the same time point (Fig. 7A, B).

Since ERK is involves in STAT3 (Ser727) phosphorylation[7], we were interested if some molecules of Ras/ERK were involved in JAK2/STAT3 pathway of GFAP production in our system. With U0126 as the MEK1/2 inhibitor, we found that the phosphorylations of JAK2 (Tyr1007/1008) and STAT3 (Ser727) were both lower than that in DMSO group (Fig. 7 C, D).

The results revealed that JAK2/STAT3 signaling pathway took part in GFAP production under ischemic condition and blockage of $P2Y_1$ receptor, and some molecules of Ras/ERK pathway were the upstream molecules of JAK2/ STAT3 signaling pathway under this condition.

3.6 GDNF production was mediated by PI3-K/Akt/CREB pathway and had relations with Ras/ERK pathway After blockage of $P2Y_1$ receptor with MRS2179, Western blotting displayed that phosphorylated Akt and CREB increased significantly in the oxygen-glucose-serum deprivation group compared with the control group. After blockage of $P2Y_1$ receptor with MRS2179 and inhibition of PI3-K with LY294002, phosphorylated Akt and CREB reduced significantly compared with the oxygen-glucose-serum deprivation group that was treated with MRS2179 and DMSO (Fig. 8A, B). Blockage of $P2Y_1$ receptor with MRS2179, GDNF variation was parallel to that of phosphorylated Akt and CREB under oxygen-glucose-serum deprivation condition. Inhibition of PI3-K led to reduction of phosphorylated Akt and

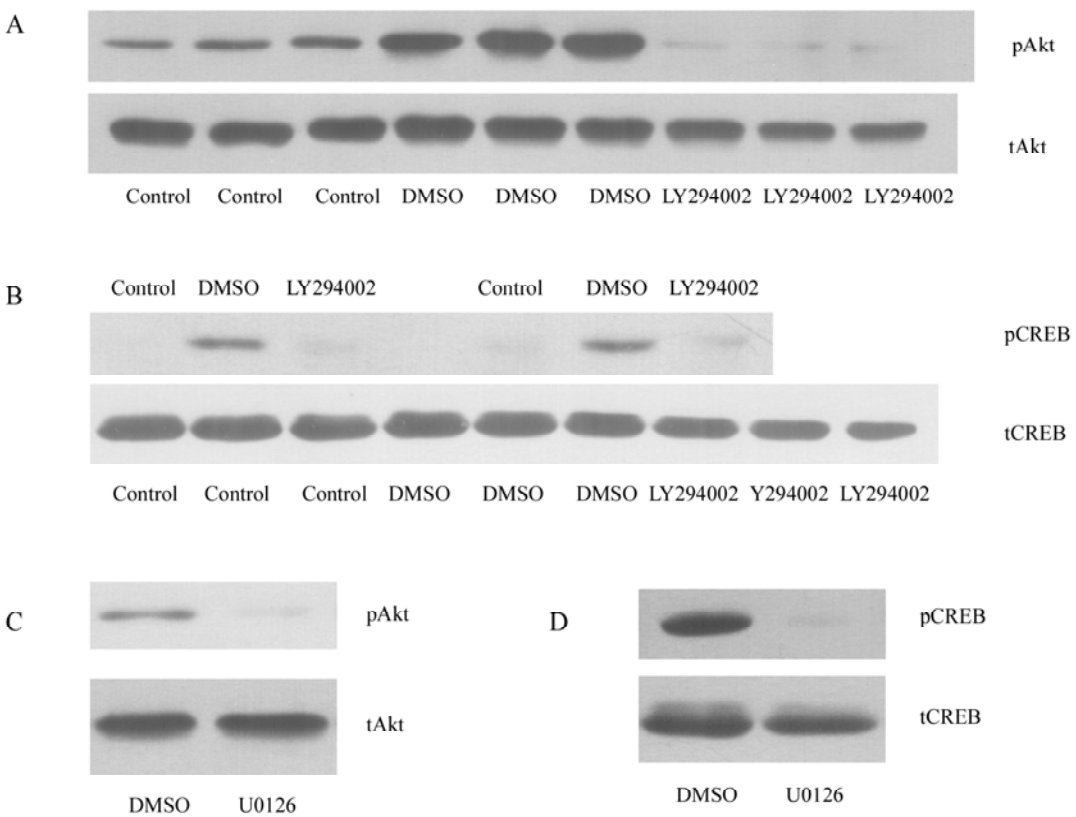

Fig. 8 Activation of Akt and CREB after deprivation of oxygen-glucose-serum and blocking of $P2Y_1$ receptor and the effects on phosphorylated Akt and CREB of U0126. A: Expression of phosphorylated Akt after adding LY294002. B: Expression of phosphorylated CREB after adding LY294002. C: Expression of phosphorylated Akt after adding U0126. D: Expression of phosphorylated CREB after adding U0126. All of the samples were treated with MRS2179 (10 μmol/L) 30 min prior to experiment. Control: Normal cells were treated with DMSO (25 μL) 30 min prior to experiment without deprivation of oxygen-glucose-serum. DMSO: Cells were treated with DMSO (25 μL) 30 min prior to experiment with deprivation of oxygen-glucose-serum for 2 h. LY294002 or U0126: Cells were treated with LY294002 (50 μmol/L, DMSO solution) or U0126 (10 μmol/L) 30 min prior to experiment with deprivation of oxygen-glucose-serum for 2 h. Data are shown as mean±SEM in three independent experiments.

CREB. Therefore, we concluded that PI3-K was the upstream molecule of Akt/CREB, and GDNF production might be mediated by PI3-K/Akt/CREB signaling pathway under oxygen-glucose-serum deprivation and blockage of $P2Y_1$ receptor.

It is reported that ERK attributes to CREB phosphorylation, which can induce GDNF production in cultured astrocytes[8]. We would investigate if some molecules of Ras/ERK signaling pathway was involved in GDNF production in our system. After blockage of $P2Y_1$ receptor with MRS2179 and inhibition of MEK1/2 with U0126, we found that the expressions of phosphorylated Akt and CREB reduced significantly (Fig. 8C, D). It meant that some molecules of Ras/ERK were involved in PI3-K/Akt/CREB signaling pathway under the conditions of oxygen-glucose-serum deprivation and blockage of $P2Y_1$ receptor.

4 Discussion

Nucleotides, the substrates for nucleic acid synthesis, are also ubiquitous extracellular mediators. High concentrations of cytosolic nucleotides are released into the extracellular space in response to cell death and hypoxia[14]. Extracellular nucleotides can interact with cell surface P2 receptors both in the CNS and in the peripheral tissues to produce a broad range of physiological effects. The P2 family is divided into two main types as follows: the P2X receptors are ligand-gated ion channels, and the P2Y receptors are G protein-coupled[15,16]. $P2Y_1$ is the first member of the P2Y family to be identified and is widely distributed in various tissues[17-19]. Astrocytes have multiple functions in the brain and are the predominant neuroglial cells of the CNS. They are integral part of synapses and provide physical support to neighboring neurons, meninges, and vasculature. Reactive astrogliosis is characterized by hypertrophic and hyperplastic changes of astrocytes and is a hallmark of disease-, trauma-, and chemical-induced damage to the central nervous system. A biomarker for astrogliosis is GFAP, a major intermediate structural filament protein that is expressed predominantly in mature astrocytes of the CNS[20]. Neurons in the core lesion die immediately after ischemia. But some neurons in the penumbra are still viable for several hours. To rescue neurons at penumbra is critical in considering strategies for ischemia treatment. Under the ischemia condition, the role of astrocytes is controversial. Activated astrocytes are thought to provide support for damaged neural tissues through several mechanisms, including release of neurotrophic factors[21]. Meanwhile the formation of a glial scar may interfere with neuronal repair or axonal regeneration in the CNS[1, 22]. Therefore how to control the astrogliosis and the release of neurotrophic factors attracts our attention.

P2Y ($P2Y_1$ or $P2Y_{12}$) receptor subtypes involve in the generation of astrogliosis in the NAc of rats. P2 receptor agonists can induce astrogliosis changes including hypertrophy of astrocytes, elongation of astrocytic processes and up-regulation of GFAP. P2 receptor antagonist PPADS decreases the injury-induced proliferation[23]. $P2Y_1$ receptor expression on astrocytes can be raised by stab wound injury and the $P2Y_1$, $P2Y_{12}$, $P2Y_{13}$ receptor agonist adenosine 5'-O-(2-thiodiphosphate) (ADPβS) can also raise the expression of $P2Y_1$ receptor and GFAP. $P2Y_1$ receptor antagonist MRS2179 and the $P2Y_1$ receptor-antibody itself inhibit the agonist-induced proliferation effects[24]. We were interested if $P2Y_1$ receptor was involved in astrogliosis under ischemic condition.

In the present study, we found that both the expression of GFAP mRNA and protein raised under ischemic condition. After blockage of $P2Y_1$ receptor, both the expressions of GFAP mRNA and protein decreased to some degree under ischemic condition. Although the reason why the levels of GFAP mRNA and protein of 48 h after reperfusion or normal cultivation were not coincident with the variation of 24 h and 72 h remains unclear, we concluded that $P2Y_1$ receptor interfered in astrogliosis under ischemic condition. It is known that the concentration of agonists of $P2Y_1$ receptor raise rapidly after ischemic injury[14]. Blockage of $P2Y_1$ receptor could inhibit the astrogliosis under ischemic injury. It provided us some new therapeutic implications to deal with the astrogliosis or glial scar after ischemic injury.

ATP can increase Ser727 phosphorylation of STAT3 in primary cultures of rat cortical astrocytes and the effect is mediated by P2 receptors. Inhibition of ERK signaling blocks phosphorylation of Ser727-STAT3, and inhibition of STAT3 activation prevents ATP-stimulated mitogenesis[7]. ATP released by injuried astrocytes activates ERK through $P2X_2$ and $P2Y_1$[25]. ADP can activate ERK1/ERK2 through $P2Y_1$ and $P2Y_{12}$ which leads to cell proliferation of cultured glioma C6 cells[26]. Activation of the JAK/STAT pathway following transient focal cerebral ischemia is mediated through JAK1 and STAT3 in astrocytes, and this signaling pathway involves in the astroglial response to focal cerebral is-

chemia[27]. It has been observed that rapid tyrosine (Tyr-705) phosphorylation and nuclear translocation of STAT3 in astrocytes, prior to the induction of GFAP mRNA and protein during a glial response elicited by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)[13]. Inhibition of JAK2 with AG490 attenuates both the phosphorylation of STAT3 and induction of GFAP. These results suggest that the glial response is mediated by JAK2/STAT3 (Tyr705)[13]. Ciliary neurotrophic factor induces glial fibrillary acidic protein in retinal Müller cells through the JAK/STAT (Tyr705) signal pathway[28]. Astrogliosis in brains of scrapie-infected mice is mediated by JAK2/STAT1 (Ser727 and Tyr701) signaling pathway[29].

Taken above reports together, different injury-induced astrogliosis is mediated by different signaling pathways which involve in JAK1 or JAK2 and phosphorylation of Tyr705 or Ser727 of STAT3 respectively. Which signaling pathway does involve in inhibiting astrogliosis after blockage of $P2Y_1$ receptor under ischemic injury?

In the current study, we found that MRS2179, the selective antagonist of $P2Y_1$ receptor, could reduce the production of GFAP under ischemic condition. Inhibition of JAK2 with AG490 could decrease the expression of activated STAT3 (Ser727) as well as GFAP protein. Inhibition of MEK1/2 with U0126 reduced the phosphorylation of both JAK2 and STAT3 (Ser727). We concluded that blockage of $P2Y_1$ receptor led to reduction of GFAP through JAK2/STAT3 signaling pathway and which was downstream event of Ras/ERK signaling pathway.

In recent years, neurotrophic factors have become a therapeutic promise for brain repair in neurodegenerative diseases or after ischemia and trauma. Among these factors, GDNF, a distant member of the transforming growth factor-β (TGF-β) superfamily, has potent neuroprotective and neurotrophic effects on several neuronal cell types in both the central and peripheral nervous systems. GDNF has been originally isolated based on its ability to promote the survival and differentiation of dopaminergic neurons in primary cultures of embryonic ventral midbrain[30,31]. GDNF regulates the development, migration, and survival of neurons, and has therapeutic implications for neurodegenerative disorders[3,32]. The GDNF and PI3-K immunoreactivity in astrocytes achieves highest at the same time after ischemic insult[2]. GDNF immunoreactivity appears greatest in cells with glial morphology in the cortex and greatest in neuronal-like cells in the hippocampus after traumatic brain injury[33]. Ischemia-induced reactive astrocytes, as well as surviving neurons, can produce GDNF after the ischemic injury[34]. The effect of $P2Y_1$ on the GDNF expression of astrocytes under focal brain ischemia remains currently unknown.

Measuring GDNF in the brain tissue extract of Wistar rats after ischemic injury, we found that GDNF increased significantly at 2 h after reperfusion and blockage of $P2Y_1$ receptor. In the supernate of heterogeneous brain primary cells culture, GDNF increased significantly when measured immediately after oxygen-glucose-serum deprivation and blockage of $P2Y_1$ receptor. This meant that blockage of $P2Y_1$ receptor could increase GDNF production after oxygen-glucose-serum deprivation. Interestingly, we found a negative correlation between the variation of GDNF and GFAP under ischemic condition and blockage of $P2Y_1$ receptor *in vivo* and *in vitro*. This provide a possibility that an antagonist can hamper the formation of glia scar and stimulate GDNF production in astrocytes at the same time. Administration of this kind of agents at the later stage of ischemic brain injury will benefit the recovery of patients.

ERK attributes to CREB phosphorylation that leads to GDNF production in cultured astrocytes[8]. GDNF stimulation can induce the activation of signaling molecules PI3-K/Akt, Ras/ERK, and CREB, *etc*.[35-37]. CREB plays a key role in transcriptional regulation of GDNF expression[38]. Astrocytes can secret GDNF after transient ischemia and PI3-K maybe have relations with GDNF secretion[2]. Rehmannia glutinosa, a Chinese traditional herb, induces GDNF gene expression in astroglial cells via cPKC and ERK1/2 pathways independently[39]. ADP evokes an increase in the PI3-K activity and blocking of the $P2Y_1$ receptor by MRS2179 additionally increases this ADP response. These results suggest that the $P2Y_1$ receptor has an inhibitory effect on PI3-K signaling pathway[25]. In our study, we found that blockage of $P2Y_1$ receptor could increase the production of GDNF. We want to further explore if GDNF production of astrocytes under ischemic condition is mediated by PI3-K/Akt/CREB signaling pathway and its relations with Ras/ERK pathway.

In the present study, we found that phosphorylated Akt and CREB increased significantly after deprivation of oxygen-glucose-serum and blockage of $P2Y_1$ receptor. This variation was parallel to that of GDNF. Addition of U0126 reduced the expression of phosphorylated Akt and CREB

significantly. This indicated that some molecules in Ras/ERK signaling pathway were upstreams of PI3-K/Akt/CREB signaling pathway. It will be of interest to get a causal relationship between the variation of PI3-K/Akt/CREB and that of GDNF and to investigate whether there is an autocrine loop of GDNF or not under this system.

In conclusion, the present study showed that blockage of $P2Y_1$ receptor might decrease the GFAP production, while increase the GDNF production of astrocytes under ischemic condition. It indicated that the synthesis of GFAP and GDNF, which are both influenced by $P2Y_1$ receptor, had negative correlation. $P2Y_1$ receptor on astrocytes membrane affected GFAP synthesis through JAK2/STAT3 pathway, and might influence GDNF production via PI3-K/Akt/CREB pathway. Some molecules such as MEK1/2, or its downstream molecules such as ERK1/2, in Ras/ERK pathway might be the upstream signaling molecules of JAK2/STAT3 and PI3-K/Akt/CREB. Crosstalk exists between the signaling pathways of JAK/STAT3 and PI3-K/Akt/CREB.

Acknowledgments: This work was supported by the National Natural Science Foundation of China (No. 30500189).

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$P2Y_1$ 受体对缺血状态下星形胶质细胞产生胶质原纤维酸性蛋白与胶质细胞源性神经营养因子的影响及相关信号通路

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摘要：目的 研究$P2Y_1$受体对缺血时星形胶质细胞产生胶质原纤维酸性蛋白(glial fibrillary acidic protein, GFAP)及胶质细胞源性神经营养因子(glial cell line-derived neurotrophic factor, GDNF)的影响及其相关信号通路。**方法** 分别利用右侧大脑中动脉线拴阻塞及培养细胞缺氧无营养后恢复正常培养，造成体内、外缺血再灌注模型。用免疫荧光标记、实时定量 RT-PCR、Western blotting、酶联免疫吸附试验观察 $P2Y_1$ 受体、GDNF 定位，检测 GFAP、GDNF 及信号分子的表达变化。**结果** 与单纯性缺血组比较，用选择性拮抗剂 MRS2179 阻断 $P2Y_1$ 受体后，可使体内、外星形胶质细胞产生的 GFAP 减少，同时使其产生 GDNF 增加。体外缺氧无营养并阻断 $P2Y_1$ 受体后：可使磷酸化蛋白激酶B(Akt)及cAMP反应元件结合蛋白(cAMP response element binding protein, CREB)升高，而使磷酸化JAK2及STAT3 (Ser727)降低；JAK2的抑制剂AG490在降低磷酸化STAT3(Ser727)的同时也降低GFAP表达水平；PI3-K的抑制剂LY294002可降低磷酸化的Akt及CREB；MEK1/2抑制剂U0126可同时降低磷酸化的JAK2、STAT3 (Ser727)、Akt及CREB。**结论** $P2Y_1$ 受体参与短时性缺血时星形胶质细胞GFAP及GDNF的产生过程，相关信号途径分别为JAK2/STAT3 和 PI3-K/AKT/CREB，并且两条途径存在串话。

关键词： $P2Y_1$ 受体；胶质化；胶质原纤维酸性蛋白；胶质细胞源性神经营养因子；PI3-K/Akt/CREB；JAK2/STAT3；Ras/ERK