P2Y1 receptor antagonists mitigate oxygen and glucose deprivation-induced astrocyte injury

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Abstract. The aim of the present study was to elucidate the effects of blocking the calcium signaling pathway of astrocytes (ASs) on oxygen and glucose deprivation (OGD)-induced AS injury. The association between the changes in the concentrations of AS-derived transmitter ATP and glutamic acid, and the changes in calcium signaling under the challenge of OGD were investigated. The cortical ASs of Sprague Dawley rats were cultured to establish the OGD models of ASs. The extracellular concentrations of ATP and glutamic acid in the normal group and the OGD group were detected, and the intracellular concentration of calcium ions (Ca^{2+}) was detected. The effects of 2'-deoxy-N6-methyl adenosine 3', 5'-diphosphate diammonium salt (MRS2179), a P2Y1 receptor antagonist, on the release of calcium and glutamic acid of ASs under the condition of OGD were observed. The OGD challenge induced the release of glutamic acid and ATP by ASs in a time-dependent manner, whereas elevation in the concentration of glutamic acid lagged behind that of the ATP and Ca^{2+}. The concentration of Ca^{2+} inside ASs peaked 16 h after OGD, following which the concentration of Ca^{2+} was decreased. The effects of elevated release of glutamic acid by ASs when challenged by OGD may be blocked by MRS2179, a P2Y1 receptor antagonist. Furthermore, MRS2179 may significantly mitigate OGD-induced AS injury and increase cell survival. The ASs of rats cultured *in vitro* expressed P2Y1 receptors, which may inhibit excessive elevation in the concentration of intracellular Ca^{2+}. Avoidance of intracellular calcium overload and the excessive release of glutamic acid may be an important reason why MRS2179 mitigates OGD-induced AS injury.

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Introduction

Previous studies on hypoxic brain injury centered on neurons while ignoring the role of astrocytes (ASs) in hypoxic injury. With the performance of in-depth studies on ASs in recent years, ASs have been identified as potentially crucial in numerous central nervous system diseases, and regulation of these processes by ASs is achieved by regulating the release of ATP and glutamic acid of calcium-dependent gliotransmitters (1). Studies have indicated that ATP and glutamic acid may be key molecules in the transmission of AS-derived signals; they were closely associated with the changes in calcium signaling, and regulation of the AS signaling network may be achieved by regulating the above-mentioned two key molecules (2,3), thereby providing a novel perspective for studies on neurological diseases.

Calcium overload is an important factor in oxygen and glucose deprivation (OGD)-induced nerve cell damage. As an energy molecule that is widely present in organisms and is an important neurotransmitter, ATP regulates the physiological function of nerve cells via P2 purine receptors (4). P2 receptors are divided into two types: P2X and P2Y. P2Y is a class of G protein-coupled receptors, which activate coupled G proteins and regulate the release of calcium ions (Ca^{2+}) from the intracellular calcium library following activation (5). It was demonstrated that AS expressed P2Y1 receptors in the cortex and hippocampus of rats, and P2Y1 receptors were associated with ATP-induced calcium mobilization (6,7). Glutamic acid is an important excitatory neurotransmitter, while its excessive extracellular accumulation leads to neuronal and glial cell death. It was found that the release of AS calcium-dependent glutamic acid was involved in the regulation of neuronal activities (8,9).

ATP is the most responsive signal molecule in hypoxic injury, and the concentration of extracellular ATP is significantly elevated during the first minute of hypoxia. The release of ATP by injured or dead/apoptotic cells is the primary source of elevation in extracellular ATP. Current studies indicate that the regulation of ATP release may intervene in OGD-induced brain injury. Therefore, ASs of rats were purified and cultured

to gain an insight into the association between changes in the concentration of ATP and glutamic acid, and changes in the concentration of calcium within ASs. In addition, changes in the expression of P2Y receptor proteins under the challenge of OGD were observed, as well as the effects of blocking the calcium signaling pathway of AS using the specific antagonist of P2Y1 receptors, 2'-deoxy-N6-methyl adenosine 3',5'-diphosphate diammonium salt (MRS2179), on OGD-induced injury of ASs, providing a theoretical basis for the involvement of P2Y1 receptors in the regulation of AS activities.

Materials and methods

Experimental animals. Healthy male and female Sprague Dawley (SD) rats of clean grade (n=48 rats; weight, 9.26±0.22 g; age, 3 days) were provided by Huaxi Experimental Animal Center, Sichuan University (license no. 2004A067; Chengdu, China). The rats were housed with free access to food and water in a controlled environment (temperature, 21˚C and humidity 35%) with a 12-h light/dark cycle. All the animal experiments were conducted according to the Animal Research Reporting in *In Vivo* Experiments guidelines. Animals were sacrificed under anesthesia and every effort was made to minimize their suffering.

Main reagents. The basic medium, Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and trypsin, 10% fetal bovine serum (FBS) and polylysine were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Rabbit anti-glial fibrillary acidic protein (GFAP) was purchased from EMD Millipore (Billerica, MA, USA; cat. no. AB5804), and fluorescent tetramethylrhodamine (TRITC)-labeled secondary antibody (cat. no. 76925) was from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd. (Beijing, China). In addition, Fura-2-AM (F-1201) and Pluronic F-127 were purchased from Invitrogen (Thermo Fisher Scientific, Inc.), the ATP bioluminescence assay kits from Promega Corporation (cat. no. 259885), and the P2Y1 receptor antagonist, MRS2179, Hanks' balanced salt solution (HBSS), hydroxyethyl piperazineethanesulfonic acid, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), dimethyl sulfoxide (DMSO; AR) and glutamic acid standard products were purchased from Sigma-Aldrich (Merck KGaA). The apoptosis detection kit of terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) was purchased from Roche Diagnostics (Basel, Switzerland; cat. no. 11684817910). The nuclear content was stained with 1 mg/ml 4',6-diamidino-2-phenylindole (DAPI; cat. no. TB267; Sigma; Merck KGaA) in PBS in all experiments.

Main devices. A laminar flow bench, dissecting microscope (Olympus), CO_2 incubator (Thermo Fisher Scientific, Inc.), thri-gas incubator (Thermo Fisher Scientific, Inc.), electrophoresis and membrane transfer device (Bio-Rad Laboratories, Inc., Hercules, CA, USA), inverted fluorescence microscope (Zeiss Axiovert 200), ultra-high performance liquid chromatography-mass spectrometry instrument (UPLC/MS; Waters Corporation, Milford, MA, USA) and multi-mode full wavelength microplate reader (Thermo Fisher Scientific, Inc.) were all used during the present study.

Culture of AS. SD rats (age, <3 days) were subjected to isolation of their cerebral cortices under sterile conditions, and removal of their soft meninges and blood vessels. After being sliced into sections (1-2 mm), the cerebral cortex samples were digested with 0.125% trypsin at 37˚C for 10 min, combined with DMEM containing 10% FBS to terminate digestion, and dissociated into a single cell suspension. Subsequently, the cells were seeded into polylysine-coated 6-well plates (cell density, $1x10^6$/ml), and cultured in an incubator at 37˚C with 5% CO_2 for 3 weeks, with the medium replaced every 3 days. The cells were cultured at 37˚C for 10-14 days, and underwent passage subsequent to reaching confluence. Prior to passaging, the culture flask was vigorously shaken. The medium was aspirated, washed with phosphate-buffered saline (PBS) three times, digested with 0.125% trypsin for 3-5 min and combined with fresh DMEM containing 10% FBS to terminate digestion. The cells were seeded at a concentration of $1x10^4$/ml, and identified after undergoing the second passage. The above-mentioned cells were cultured in 24-well plates for the Ca^{2+} experiment, and culture flasks (25 cm^2) were used to extract the whole cell lysates for western blotting. The cells cultured on coverslips were employed for immunofluorescence assays.

Identification of cortical ASs in rats. Indirect immunofluorescence was adopted to monitor GFAP, the specific marker of AS, with positive cells identified as ASs. ASs seeded on coverslips were fixed with 4% paraformaldehyde for 20 min at room temperature, and washed with PBS three times, for 5 min each time. ASs were treated with 0.3% Triton-X-100 for 5 min at room temperature, and then incubated for 30 min with PBS containing 1% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA) at room temperature. ASs were combined with primary antibodies (rabbit anti-rat GFAP; 1:1,000) and placed in a refrigerator at 4˚C overnight. The ASs were washed with PBS three times, for 5 min each time. Subsequently, the ASs were added to TRITC-labeled goat anti-rabbit secondary antibodies (1:200) and incubated in the dark at room temperature for 1 h. After washing with PBS 3 times, the ASs were mounted with anti-fluorescent quencher, observed under an Olympus fluorescence microscope and photographed.

Grouping. ASs on the seventh day of culture after passaging were randomly assigned to three groups as follows: A, control group; B, OGD group and C, MRS2179 intervention group. The OGD time was determined according to pre-experiment results, the hypoxia time was set at 0, 2, 4, 8, 16 and 24 h, where 0 h served as the baseline control.

At total of 1 h prior to OGD, astrocytes were combined with MRS2179 blockers to a final concentration of 20 μmol/l. When the blockers took effect, the cells were added to OGD solution, and placed in the tri-gas incubators at 37˚C with 1% O_2, 94% N_2 and 5% CO_2 for OGD at 0, 2, 4, 8, 16 and 24 h. The culture medium was collected at the different time points of OGD, and the level of ATP was detected using the bioluminescent assay kit. The content of glutamic acid in

Table I. Concentrations of extracellular ATP in the OGD and control groups (n=3 per group) at different time points of OGD (values presented as means ± standard deviation).

	Time points of OGD (h)				
Group	2	4	8	16	24
OGD (mmol/l)	8.16±0.56[a]	11.13±0.85[a]	26.82±1.92[a]	38.06±2.18[a]	40.78±1.04[a]
Control (mmol/l)	5.11±1.07	5.41±0.89	6.32±1.12	5.95±1.34	6.57±1.53

[a]P<0.05 vs. control group. OGD, oxygen and glucose deprivation.

the culture medium was detected using UPLC/MS, and the medium was quickly discarded following OGD. After the 6-well plates were washed with HBSS-BSA solution three times, the Fura-2/AM/Pluronic F-127 load solution (1 ml) was added in the presence of light, and the control group was combined with 1 ml DMSO and maintained in an incubator at 37˚C with CO_2 for 45 min. The calcium probe load solution was discarded and the cells were washed with PBS three times, after which 0.25% trypsin (1 ml) was added to uniformly cover the 6-well plates. Subsequently, the trypsin was discarded and the remaining contents were placed in incubators at 37˚C and 5% CO_2 for digestion for 3-5 min. After the majority of cells became round, HBSS-BSA was added to suspend the cells and the number of cells was adjusted to $1.0x10^5$/ml. The cell suspension (200 μl) was added to 96-well plates. A multi-mode full wavelength microplate reader was adopted to detect the concentration of intracellular Ca^{2+}. The maximum fluorescence (F_{max}) under excited wavelength was detected by 0.2% Triton X-100 and the minimum fluorescence (F_{min}) was detected by adding EGTA (pH 9) with a final concentration of 3 mmol/l. The concentration of intracellular free Ca^{2+} was calculated according to the Grynkiewicz formula: $[Ca^{2+}]$ $i=Kd[(F-F_{min})/(F_{max}-F)]$. To quantify AS apoptosis with different treatments, ASs were processed for TUNEL staining using the *in situ* cell death detection kit (cat. no. 11684817910; Roche Diagnostics). Briefly, following fixation in 4% paraformaldehyde at room temperature for 15 min and incubation in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) at 4˚C for 2 min, coverslips were incubated with the TUNEL reaction mixture at 37˚C for 1 h. Subsequently, the coverslips were stained with DAPI for 5 min at room temperature to evaluate the surviving neurons. The cells were collected at various time points of OGD, and lysed according to previous literature (10). The modified BCA was employed for protein quantification, with total proteins per well injected. Equal amounts (30 μg) of the proteins were subjected to 10% SDS-PAGE, which were then transferred to nitrocellulose membranes (Pharmacia Biotech; GE Healthcare, Chicago, IL, USA). Blots were blocked with 5% fat-free milk in Tris-buffered saline (pH 8.0) for 30 min at 4˚C, and incubated with the P2Y1 receptor antibody (1:200; cat. no. orb11205; Biorbyt Ltd., Cambridge, UK) for 1 h at room temperature, and then the goat anti-rabbit immunoglobulin G peroxidase-labeled secondary antibody (1:2,000; cat. no. A27036; Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h at room temperature, immunoreactivity was detected by Pierce™ enhanced chemiluminescence western blotting substrate). GAPDH was used as the reference protein (1:1,000; cat. no. ab37168; Abcam). The western blot image was analyzed by Bio-Rad Image Lab 4.1 (Bio-Rad Laboratories, Inc.). Experiments were performed for three times.

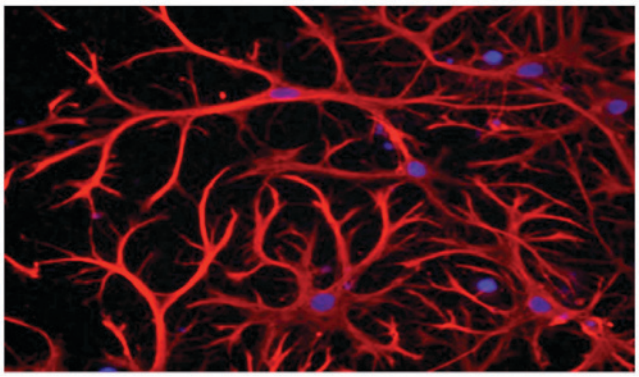

Figure 1. Hippocampal astrocytes of rats 14 days after primary culture *in vitro* (magnification, x400).

Statistical analysis. All measurement data were expressed as means ± standard deviation. SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for analysis. The independent-samples t-test was employed for comparison between two groups, and analysis of variance and post-hoc Newman-Keuls analysis were performed for comparisons among multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of cortical ASs in rats. GFAP, the cytoskeletal proteins of ASs, are located in the cytoplasm and projections, and are recognized as specific markers of ASs. Therefore, amongst cultured cells, those with positive GFAP expression are ASs. Under a fluorescence microscope, GFAPs are expressed in the cytoplasm and projections of ASs (Fig. 1). Within ten visual fields, 100 cells were counted, and the count ratio of cells was >98% in purified ASs subsequent to passaging.

OGD induced the release of ATP by ASs. Table I and Fig. 2 demonstrates that the challenge of OGD induced the release of ATP by ASs in a time-dependent manner. Specifically,

Table II. Concentrations of calcium ions (nmol/l) in the astrocytes of the various blocker intervention groups (n=3) at different time points of OGD (values presented as means ± standard deviation).

	Concentration of calcium ions (nmol/l)				
Group	2 h	4 h	8 h	16 h	24 h
OGD	104.2±4.6[a]	162.2±11.8[a]	340.1±7.6[a]	638.6±32.1[a]	250.2±10.6[a]
Control	81.5±1.9	79.2±4.7	78.4±2.5	85.6±4.8	83.8±2.6
MRS2179	83.2±4.3[b]	101.5±5.8[b]	186.3±6.8[b]	350.9±16.7[b]	107.3±8.8[b]

[a]P<0.01 vs. control; [b]P<0.01 vs. OGD. OGD, oxygen and glucose deprivation.

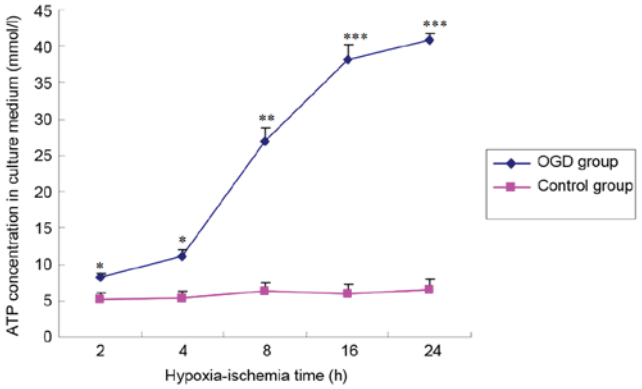

Figure 2. OGD (induced the release of ATP by astrocytes. *P<0.05 vs. control. **P<0.01 vs. control. ***P=0.000 vs. control. OGD, oxygen and glucose deprivation.

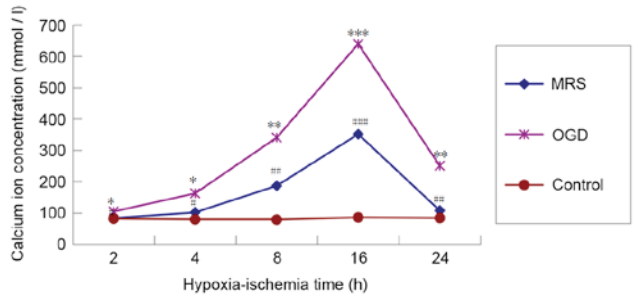

Figure 3. OGD challenge induced an elevation in the concentration of calcium ions in astrocytes. *P<0.01 vs. control. **P<0.01 vs. control. ***P=0.001 vs. control. #P<0.05 vs. OGD. ##P<0.01 vs. OGD. ###P<0.001 vs. OGD. OGD, oxygen and glucose deprivation; MRS, MRS2179 intervention group.

the concentration of ATP was marginally increased in the early stage of OGD (2-4 h), sharply increased in the middle stage (4-16 h), and did not significantly fluctuate in the late stage (16-24 h).

Changes in concentrations of Ca^{2+} in ASs in the P2Y1 receptor antagonist intervention group at different time points of OGD. Table II and Fig. 3 demonstrate that the concentration of free Ca^{2+} within ASs during OGD was not increased linearly, but fluctuated with the extension of OGD duration. The concentration of Ca^{2+} in ASs peaked 16 h after OGD, following which the concentration of Ca^{2+} was decreased

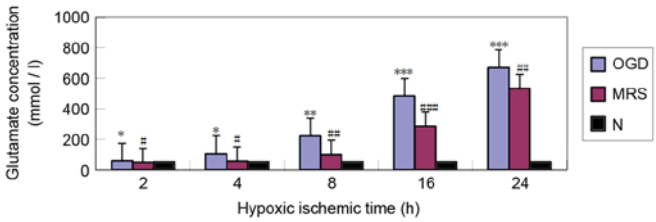

Figure 4. Comparison of glutamic acid release between the MRS2179 treatment group and single hypoxia-ischemia deprivation group. *P<0.01 vs. N. **P<0.01 vs. N. ***P=0.001 vs. N. and #P<0.05 vs. OGD. ##P<0.01 vs. OGD. ###P<0.001 vs. OGD. OGD, oxygen and glucose deprivation; MRS, MRS2179 intervention group; N, normal control.

(P<0.01). The addition of MRS2179, a P2Y1 receptor-specific antagonist, significantly reduced the concentration of Ca^{2+} in the ASs under OGD.

Effects of MRS2179 on the release of glutamic acid by ASs. UPLC/MS was adopted to assess the contents of glutamic acid in the culture medium at various time points of OGD, and the results demonstrated that the content of glutamic acid 2 h after OGD was not significantly different from that in the normal culture group (P>0.05), and from 4 h after OGD, the concentration of extracellular glutamic acid began to increase in a time-dependent manner, with the peak reached at 24 h. The release of glial cell-derived neurotransmitter, glutamic acid was increased, however following the MRS2179 intervention, the OGD-induced release of glutamic acid was significantly reduced (P<0.05; Table III and Fig. 4).

Immunofluorescence observation of GFAP within ASs at different time points of OGD following the addition of MRS2179. Fluorescence microscopy using GFAP staining demonstrated that a short-term challenge of OGD (2-4 h) induced activation of ASs (Fig. 5A). The ASs exhibited increased volume, numerous and bulky projections and the fluorescence intensity of GFAP increased. Sustained challenge with OGD (8-24 h) induced AS death (Fig. 5B and C). Compared with the pure OGD control group, the MRS2179 intervention did not affect the glial reaction of ASs, while it significantly improved the OGD-induced injury of ASs, which manifested as an increased number of viable cells and relatively intact cell morphology (Fig. 5D-F).

Table III. Comparison of contents of glutamic acid at different time points of OGD between the MRS2179 intervention group and the control group (n=3; values are presented as means ± standard deviation).

	Time points of ischemia-hypoxia (h)				
Group	2	4	8	16	24
OGD	56.12±6.90[a]	104.33±10.69[a]	219.65±10.33[a]	481.82±8.67[a]	668.37±15.49[a]
MRS2179	46.31±5.83[b]	54.63±6.98[b]	99.857±7.36[b]	284.052±19.64[b]	531.805±25.33[b]
Control	49.65±3.87	46.87±2.12	51.93±6.16	48.12±3.47	53.16±4.93

[a]P<0.01 vs. control; [b]P<0.05 vs. OGD. OGD, oxygen and glucose deprivation.

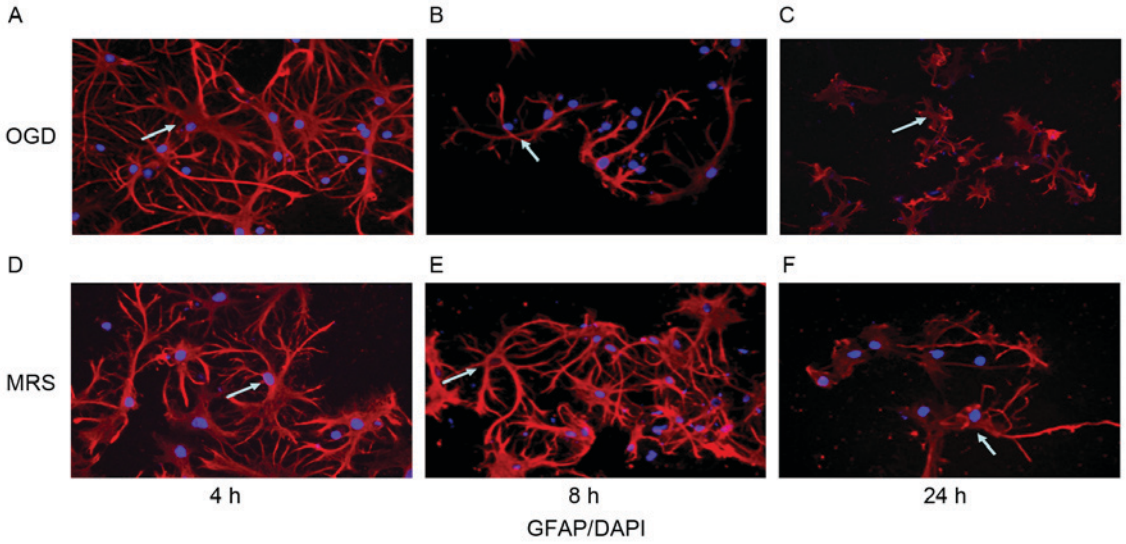

Figure 5. Morphological changes of astrocytes at different time points of OGD subsequent to MRS2179 intervention. Morphological changes of astrocytes (A) 4, (B) 8 and (C) 24 h after OGD. Morphological changes of astrocytes (D) 4, (E) 8 and (F) 24 h after OGD and MRS2179 intervention. OGD, oxygen and glucose deprivation; MRS, MRS2179 intervention group; GFAP, glial fibrillary acidic protein; DAPI, 4',6-diamidino-2-phenylindole.

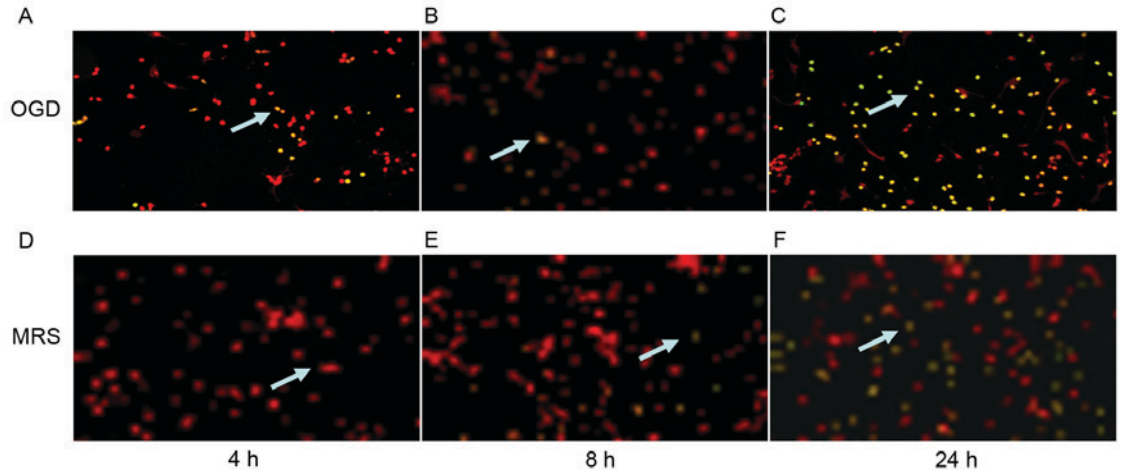

Figure 6. Apoptosis of astrocytes at different time points of OGD subsequent to MRS2179 intervention. Apoptosis of astrocytes at (A) 4, (B) 8 and (C) 24 h of OGD. Apoptosis of astrocytes treated with MRS2179 at (D) 4, (E) 8 and (F) 24 h. OGD, oxygen and glucose deprivation; MRS, MRS2179 intervention group.

Detection of AS apoptosis in the OGD group and the MRS2179 intervention group using TUNEL assay. The TUNEL method was applied to detect AS apoptosis. It was observed under a florescence microscope using propidium iodide staining that, subsequent to OGD challenge, the number of apoptotic ASs was increased with the extension of OGD duration (Fig. 6A-C),

and the addition of MRS2179 may significantly decrease AS apoptosis at various time points (Fig. 6D-F; P<0.01).

Discussion

With more in-depth studies on AS in recent years, it has been identified that, in addition to supporting nutrition, AS is also important in neuronal excitability, inhibition of heterologous synapsis and cerebral angiogenesis (11-13). The regulation of these processes by AS was achieved by regulating the release of ATP and glutamic acid of calcium-dependent gliotransmitters (1). ATP and glutamic acid may be key molecules in the transmission of AS-derived signals and they were closely associated with the changes in calcium signaling, the regulation of AS signaling may be achieved by regulating the above-mentioned two key molecules (2,3), thereby providing a novel perspective for studies on neurological diseases.

The results of the present study indicated that 2 h following OGD, the concentration of ATP released by ASs to outer regions began to increase, and at 4 h, the concentration of ATP began to increase significantly, peaking at 16 h. The concentration of Ca^{2+} inside ASs was increased with the extension of OGD time at 0-16 h, following which the concentration began to decrease gradually with the extension of OGD duration, which was consistent with the changes of ATP. While the concentration of glutamic acid released by ASs to outer regions at 2 h was not significantly changed when compared with the control group, it began to increase at 4 h and peaked at 24 h. Based on the current results, it was concluded that the challenge of OGD may activate the calcium signaling system. At 4 h after hypoxia, the concentration of intracellular Ca^{2+} was significantly increased and it peaked at 16 h after hypoxia, indicating that 4 h after hypoxia, the calcium signaling system had been fully activated, and the time from 4 to 16 h after hypoxia may be a crucial period for the calcium signaling to initiate AS injury. The elevation in the concentration of glutamic acid lagged behind that of Ca^{2+}. Therefore, it was speculated that glutamic acid may not be the initiating factor for calcium signaling of ASs. In addition, the level of extracellular glutamic acid was not significantly changed in the early stage of ischemia (2-4 h), which may be associated with the enhanced capacities of ASs in glutamic acid uptake as a result of elevation in the glutamine synthetase synthesized by ASs in early ischemia (14,15).

To further define the chemical neurotransmitter that initiated the elevation in the concentration of Ca^{2+} in ASs, MRS2179, a P2Y1 receptor-specific antagonist, was used to block the transmission of P2Y1R. The concentration of Ca^{2+} in the ASs was measured and then it was determined which (ATP or glutamic acid) was the calcium signaling pathway in the ASs. The results indicated that MRS2179, a P2Y1 receptor-specific antagonist, significantly decreased the concentration of intracellular Ca^{2+} of astrocytes under OGD. In addition, the release of glial cell-derived neurotransmitter, glutamic acid was significantly reduced following the addition of MRS2179, which further confirmed that it was ATP that activated the calcium signaling of microglia. In addition to suppressing the accumulation of glutamic acid outside ASs, MRS2179 significantly reduced OGD-induced AS injury and improved cell viability, indicating that inhibition of the excessive elevation in the concentration of intracellular Ca^{2+} and avoidance of intracellular calcium overload and the excessive release of glutamic acid may be an important reason as to why MRS2179 mitigates OGD-induced AS injury.

The calcium signaling system is considered a key molecule in AS network signaling. The surface receptors of ASs sense the changes of glutamic acid, ATP, norepinephrine and 5-hydroxytryptamine, amongst others, in the local microenvironment, and increase the concentration of intracellular Ca^{2+} and generate calcium oscillation, thereby causing the release of gliotransmitters (including glutamic acid and ATP). These gliotransmitters may bind with the corresponding receptors on neurons, whereby ASs may regulate neuronal activities (4,8,9).

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