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Original Article

Neuroprotective effect of A2AR knockout on cerebral ischemia/reperfusion injury via the inhibition of microglia activation

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Abstract: The present study was designed to investigate the neuroprotective effects of A2AR inhibition in stroke and to elucidate the mechanisms. Focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO) for 30 min in mild mice and A2AR knockout mice followed by 1 d, 3 d, and 7 d reperfusion. The neurobehavioral scores and the activities of microglia were evaluated after MCAO. We found that A2AR knockout inhibiting microglial activation. But there was no significantly difference of neurobehavioral scores between wild type and A2AR knockout mice in every time point. Perhaps it related with few sample size. To further seek the mechanism, we checked the effects of A2AR blockade on the TNF-a expression in the primary microglia in an oxygen-glucose deprivation/reperfusion (OGD/R) condition. In vitro data showed that A2AR blockade decreased the TNF-a expression. A2AR knockout might improve neurological functional deficits in mice with cerebral ischemia/reperfusion (I/R) injury in which may relate with inhibition of microglia activation and with reducing inflammatory cytokines expression in brain.

Keywords: A2AR knock out, A2AR antagonist, microglia, cerebral ischemia-reperfusion injury, oxygen-glucose deprivation/reperfusion

Introduction

Ischemic stroke is one of the leading causes of death and disability globally [1, 2] and represents a major public health problem. In recent years, a growing numbers of studies had found that it is difficult to recover the neurological function after brain ischemic injury. One reason may be the activation of microglia (MG). After ischemic stroke, an inflammatory response that involves the activation of the resident brain microglia is initiated within a few hours [3] and persists for months. Microglia settled in the brain as the macrophage is considered the key to cause inflammation [4]. Recent studies have revealed that post-stroke neuroinflammation is an important determinant of acute outcomes and long-term prognoses [5, 6]. The suppression of microglial activation following stroke can reduce brain injury and therefore represents an attractive therapeutic strategy for stroke [7, 8]. Understanding the mechanisms underlying the activation of microglia will help to minimize ischemic brain injury.

Adenosine is a potent biological mediator whose concentration dramatically increases during brain ischemia [9, 10]. It is a neuromodulator with receptor-mediated effects and has dual role. Adenosine receptors are important targets for therapeutic implementation in the treatment of stroke. A2AR is one of adenosine receptors, widely exists in glial cells. There are obvious neuroprotective effects in adult cerebral ischemia/reperfusion (I/R) mice with A2AR knock out or using selective antagonist SCH5826l [11, 12]. And the specific mechanism is not yet clear. In recent years, vitro experiments have been show that A2AR receptor antagonists can inhibit the activation of microglia and astrocyte [13, 14]. Other evidences have begun to emerge indicating that brain adenosine or its simulation agent role on microglia A2AR can promote the proliferation and activation of microglia, prompt release

inflammatory factor, thus enhancing local inflammation, increasing tissue damage [15]. Given the A2AR correlation with the activity of glial cells, when it is necessary to study the A2AR regulation function of glial cells in cerebral I/R injury.

In the present study, we investigated whether A2AR blockade affects ischemic brain injury by altering microglia-mediated post-stroke neuroinflammation.

Experimental animals and grouping

A2AR knockout (A2AR (-/-), KO) and A2AR wild (A2AR (+/+), WT) C57/BL6 mice (25±2 g) were used for this experiment, the KO breeders were provided by the Boston University Professor Chen Jiangfan. Mice were housed in SPF Laboratory Animal Center of Wenzhou Medical University Laboratory (SYXK, Zhejiang-2005-0061) during a 12 h reverse light/dark cycle. Mice were dieting and activity in free. Gene was identified before the experiment. KO and WT mice randomly assigned into eight groups: KO sham-operated (SKO, n=7), WT sham-operated (SWT, n=7), KO 1d model group (MKO1d, n=7), KO 3d model group (MKO3d, n=7), KO 7d model group (MKO7d, n=7), WT 1d model group (MWT1d, n=7), WT 3d model group (MWT3d, n=7), WT 7d model group (MWT7d, n=7). All animal experiments were approved by the guidelines of Institute for Laboratory Animal Research of Wenzhou Medical University and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals.

Animal model

Mice were subjected to transient middle cerebral artery occlusion (MCAO) as mentioned earlier [16]. Briefly, mice were anaesthetized with intra-peritoneal injection of 10% chloral hydrate 350 mg/kg, i.p. Rectal temperature was monitored throughout the operation and maintained at 37°C with a circulating heating pad. The left common carotid artery (CCA), internal carotid artery (ICA), and external carotid artery (ECA) were surgically exposed. The CCA was ligated distally and the ECA was ligated proximally to the bifurcation of the ICA and the ECA. A microvascular clip was placed distally across the ICA. A filament (2636-A3, Beijing ShaDong biological technology co.) was inserted into the ICA through the CCA and gently advanced from the common carotid artery bifurcation to block the middle cerebral artery (MCA) at its origin (9 ± 0.5 mm). The suture around the ICA was tightened, and the microvasculature clip was removed. The suture was pulled back until the tip reached the suture around the ICA to restore blood flow (reperfusion) after 30 min of MCAO. The mice were killed 1, 3, and 7 days after MCAO, and the impaired cortical areas were extracted for subsequent biochemical analysis. Sham control animals were subjected to cut the skin, and sew up immediately after separating the left carotid artery. After recovery from anesthesia, all mice were returned to their cages with free access to food and water.

Behavioral neurological assessment

The functional outcome of the animals was assessed at 1, 3 and 7 days after MACO. The Zea Longa's [17] score was determined according to the following scoring system: no deficits score, 0; unable to extend the right forelimb score, 1; decreased grip of the right forelimb score, 2; mild circling to the right score, 3; spontaneous right circling score, 4; falling to the contralateral side score, 5. Score 1 to 4 was the efficacy model.

TTC staining

TTC (2,3,5-triphenyl-2h-tetrazolium chloride, 2%, Sigma) was used to estimate the extent of brain infarct. One animal of each group was deeply anaesthetized with chloral hydrate (350 mg/kg, i.p.). After intracardiac perfusion with 0.9% NaCl, the brains were quickly removed coronally sectioned into 2-mm-thick slices using a brain slicer. The slicers were immersed in TTC at 37°C for 30 min, and then fixed in 4% paraformaldehyde for 24 h at 4°C. The infarct volumn in the ischemic mice were observed and photographed.

Iba1 Immunofluorescence staining

Mice were sacrificed after the completion of Behavioral Neurological evaluation at 1, 3, and 7 days after MCAO (6 per group at each time point) with 10% chloral hydrate and perfused transcardially with 0.9% saline at 4°C followed by 4% paraformaldehype in phosphate buffer. The brains were removed, fixed in the 4% para-

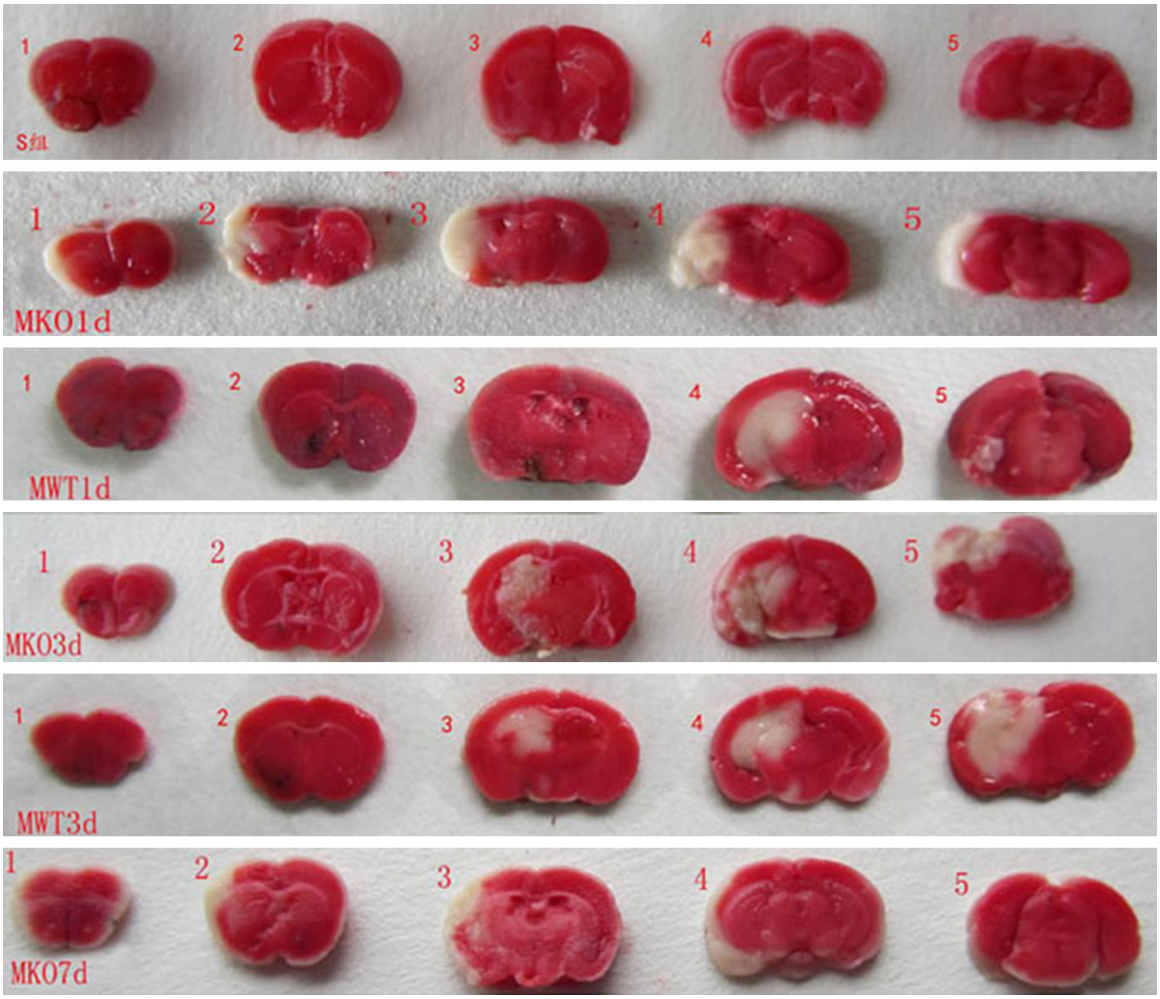

Figure 1. TTC staining of brain slices. Infarct images obtained by TTC staining at 1 d, 3 d, and 7 d after MCAO. The normal tissue was stained deep red and the infarct was stained milky. The sham group had no cerebral infarcts. In the M groups, however, the ischemic zone was identified as a distinct pale-stained area in the right cortex and striatum in the ipsilateral ischemic hemisphere.

formaldehyde for 6 h at 4°C, and then immersed sequentially in 10%, 20% and 30% sucrose until sinking occurred. 10-μm frozen brain sections were used for immunofluorescence staining of Iba1. Sections were washed 3 times for 5 minutes each with PBS for antigen retrieval, followed by 5% normal donkey serum for 30 minutes at room temperature. Next, sections were incubated with rabbit anti-Iba1 (1:200, Wako, Japan) for 24 h at 4°C. After rinsing in PBS 3 times for 5 minutes each, sections were incubated with TRITC-marked secondary antibody (1:100, Jackson, USA.) for 12 h at 4°C. Fluorescence signals were detected with a microscope (CLSM, Texas instruments co.). Negative control sections were incubated with PBS instead of primary antibodies and showed no positive signals. All histological images were captured at the same exposure and analyzed with Image-Pro Plus image 6.0 analysis software (The American Media Cybernetics co.) by one author who was not aware of the animals' group assignment. For cell counting of Iba1 immune positive cells, four non-overlapping fields were analyzed and presented as the average cell number per field on each section. The final cell number per mice was the average cell number of all the sections.

Oxygen and glucose deprivation and reperfusion (OGD/R) protocol

Primary mouse microglia was purchased from Shanghai Yaji Biological Technology (Co., LTD). The cell was produced according to the manu-

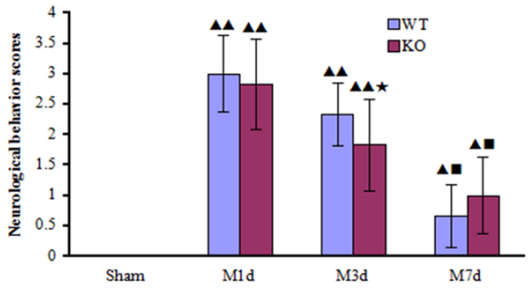

Figure 2. The effect of A2AR knockout on the changes of neurological behavior scores in focal cerebral ischemia and reperfusion mice ($\bar{x}$ ± s) (n=7). The neurological function was assessed with a five-point neurological scale. ▲▲P<0.01: Model vs Sham group; ▲P<0.05: Model vs Sham group; ★P<0.05: vs MKO1d group; ■P<0.01: vs other groups in same genotype.

facturer's protocol. For the oxygen glucose deprivation (OGD), we put the plates into an incubator under 94% N_2, 1% O_2 and 5% CO_2 adding with 100 nM SCH58261 (Sigma.) or DMSO at 37°C for 4 h, 8 h, or 12 h. Then, the cells were cultured at 37°C in a 5% CO_2/air environment for 12 h of reperfusion. Control cell cultures not deprived of oxygen and glucose were incubated under normal conditions. The group M was microglia of OGD/R, and classified into SCH58261 intervention group (MS) and DMSO control group (MC). According to the different time of OGD, MS and MC classified into three groups respectively: MS_{4h}, MS_{8h}, MS_{12h}, MC_{4h}, MC_{8h}, MC_{12h}. The group O was microglia of OGD but not reperfusion, and then it classified into O_{4h}, O_{8h}, O_{12h}. The group C was microglia without OGD/R. The group CS was incubated with SCH58261, group CD was incubated with DMSO. The group CB was control group.

Microglia viability after OGD/R

Primary cells were seeded in 96-well plate. Cells were treated with OGD or OGD/R or incubated under normal conditions. Examination of cell damage was quantitatively assessed by measuring the reduction of 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT Sigma.) after 4 h, 8 h, 12 h, or 24 h after OGD or OGD/R. Absorbance values at the test wavelength of 490 nm were determined with an automatic microplate reader (Bio-Tek, USA). The absorbance that was revealed directly correlated with the metabolically active number of microglia.

Quantitative real-time PCR

RT-PCR was performed to detect the A2AR and TNF-a levels in the primary microglia according to the protocol. RT-PCR was performed with light cycler and the fast start DNA master SYBR green I kit (Roche). The mouse specific primer (Shanghai Jikang biotechnoloty co.) are as follows. TNF-α, forward primer: 5'-CACCACGCTCTTCTGTCTACTGA-3'; reverse primer: 5'-AAGGTACAACCCATCGGCTG-3'; A2AR, forward primer: 5'-TCACGCAGAGTTCCATCTTCAG-3'; reverse primer: 5'-CAGTTGTTCCAGCCCAGCAT-3'.

The $2^{\Delta\Delta CT}$ formula was used for the calculations of the relative quantifications.

Statistical analysis

Statistical analysis was performed using SPSS 19.0. The data are presented as mean ± SD. Multiple comparisons were evaluated by one-way ANOVA. Two-group comparisons were analyzed by the two-tailed Student t test. For all analyses, A *P*-value <0.05 was considered statistically significant; A *P*-value <0.01 was considered obvious statistically significant.

Results

TTC staining of brain sections

The brain infarct size was assessed with TTC staining 1 d, 3 d, and 7 d after MCAO. None of the sham mice had cerebral infarcts. The infarct volume in M3d groups was bigger than other groups. There were hardly any white infarct lesions in MWT7d group (**Figure 1**).

A2AR knockout improved functional recovery

We examined the effects of A2AR knockout on neurological statuses. Neurological function assessment was performed 1 d, 3 d, and 7 d after MCAO. The results revealed that mice exposed to ischemia followed by 7 days demonstrated significantly better neurological statuses compared with the mice exposed to ischemia by 1 or 3 days either in MKO groups or in MWT groups (P<0.05 or P<0.01). The neurological scores in group MKO3d decreased significantly compared with MKO1d (P<0.05). There was no significantly difference of the neurological scores in group MWT3d compared with group MWT1d (P>0.05). There was no sig-

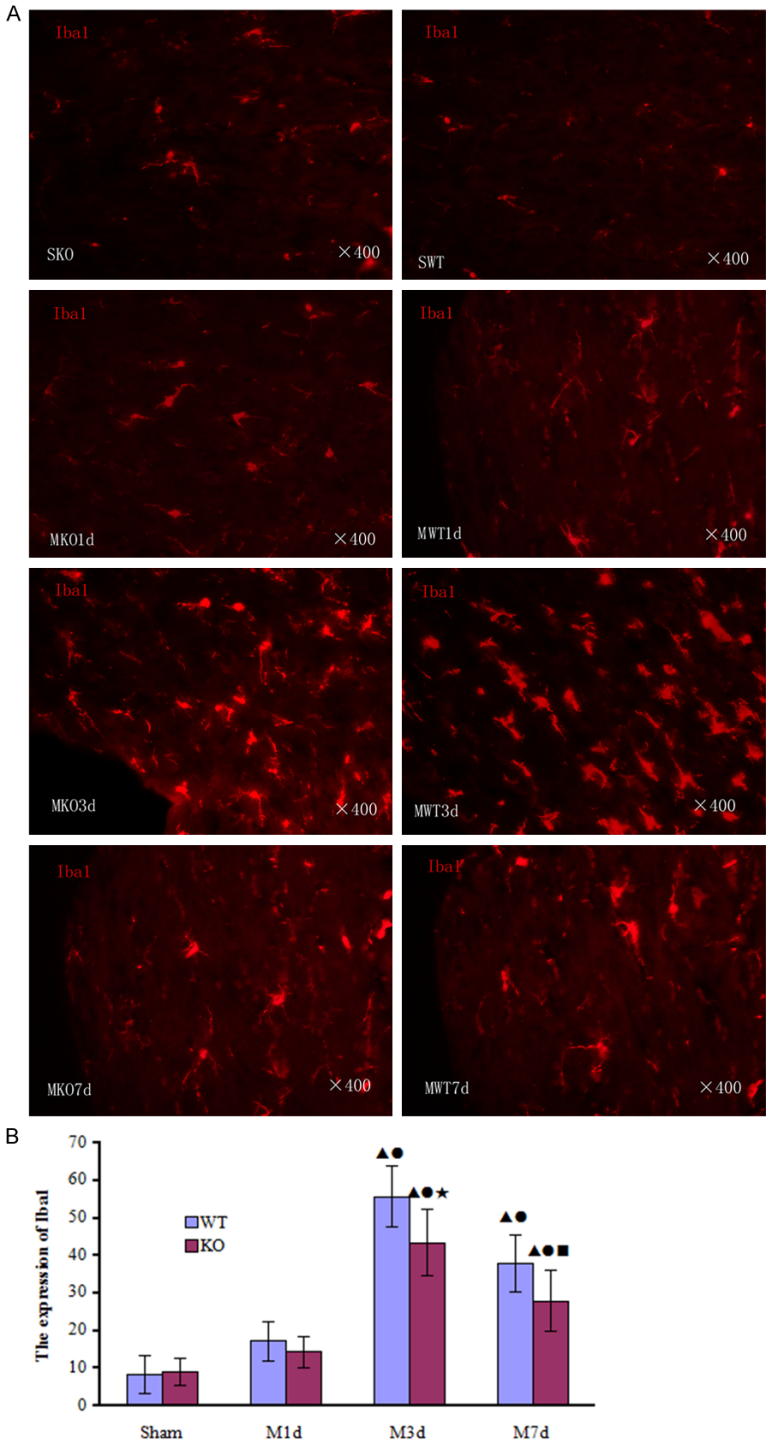

Figure 3. Immunofluorescence staining of Iba1 of each group in focal cerebral ischemia and reperfusion mice. ($\bar{x}$ ± s) (n=6). A: Immunohistochemistry with Iba1 antibodies revealed reduced microglial activation (red) in the A2AR knockout group compared to the wild type. B: Statistical analyze of microglial mean fluorescent intensity. ▲P<0.01: vs S groups; ★P<0.01: vs MWT3d group; ■P<0.05: vs MWT7d group; ●P<0.01: vs other groups in same genotype.

nificantly difference between MWT and MKO groups in each time point (P>0.05) (**Figure 2**).

A2AR knockout inhibits microglia activated in the ischemic brain following MCAO

Following cerebral ischemia and reperfusion, resident microglia are reapidly activeted and mobilized to the injury site to mediate neuroinflammation. To investigate the effects of A2AR knockout on microglia activation. We used immunofluorescence analysis the activated microglia, the result showed that MCAO robustly activated the microglia in the ipsilateral cortex, as evidenced by the remarkably enhanced immunofluorescence intensity of Ibal staining, which was significantly attenuated in the ipsilateral cortices of the A2AR knockout mice. The immunofluorescence intensity of Iba1 staining in group MKO3d and group MKO7d were decreased significantly compared with group MWT3d and group MWT7d respectively. The results revealed that the immunofluorescence intensity of Iba1 staining of injury brain after exposing to ischemia by 7 days demonstrated significantly reduced compared with the mice exposed to ischemia by 1 or 3 days either in MKO groups or in MWT groups (**Figure 3**).

Activity of microglia after OGD/R

Cell viability assays revealed that it was reduced after OGD (O) by 4 h, 8 h, 12 h and 24 h (P<0.01, or P<0.05) and had a significant difference compared with group C (P<0.01). The microglia vitality of OGD/R (M) 4 h, 8 h, 12 h and 24 h had no significant difference compared with group C (P>0.05), except M_{24h} group (P<0.01). It showed that the activity of microglia recovery after reperfusion. The microglia vitali-

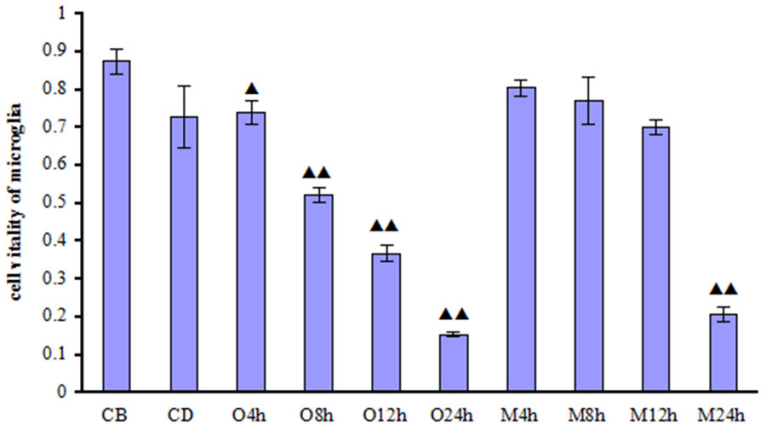

Figure 4. The cell vitality of microglia.($\bar{x} \pm s$) (n=5). Cell viability assays by MTT, the result revealed that the treatment of OGD resulted in cell death, and reperfusion increased the resistance to OGD-induced cell damage. ▲P<0.01, ▲▲P<0.05, vs control group.

ty in OGD 24 h and OGD/R 24 h was very lower, and it showed that cells may not recover to survival after long time oxygen. Therefore, this study chose 4 h, 8 h and 12 h time point for following study (**Figure 4**).

Expression of A2AR was increased in the microglia after OGD/R

To better mimic in vivo ischemia, we further examined A2AR expression in activated microglia in an OGD/R condition, which is a well-established cellular ischemia model. After 4 h and 8 h of OGD and reperfusion, the expression of A2AR was remarkly increased in the microglia (P<0.01). After 12 h of OGD and reperfusion, A2AR expression was significantly reduced compared to control group, especially group MS_{12h} (P<0.05). But, there was no obvious difference between MC and MS groups in every time point respectively. The results imply that the increasing of A2AR expression was likely a universal aspect of microglial activation (**Figure 5**).

Inhibition of A2AR suppresses the expression of TNF-α in activated microglia

To investigate whether the reduced expression of A2AR following microglial activation plays an important role in microglia-mediated neuroinflammation, so we used cultured microglia to investigate the effects of A2AR antagonist SCH58261 on microglia-mediated neuroinflammation in vitro. The results showed that the TNF-α level was significantly elevated in OGD/R groups compared to that of the control group, especially at 8 h after OGD (P<0.01). The TNF-α was obviously increased at 8h after OGD in SCH58261-treated compared to the control group (P<0.01). There were significant difference in the TNF-α expression between the SCH58261-treated and control groups (DMSO-treated) in every time point (P<0.01, P<0.05) (**Figure 6**).

Discussion

The modified MCAO model is in line with the pathophysiological changes of stroke used in this experiment [18]. Cerebral ischemia and reperfusion trigger microglial activation, which leads to proinflammatory responses and thus essentially contributes to secondary brain ischemic injury. In the current study, we show that the microglia was activated in the brains of mice following experimental findings. Although microglial activation has been linked to neuroinflammation and neurotoxicity conventionally, recent reports have shown that microglial activation leads to neurodegeneration and microglia can generate neuronal growth factors and anti-inflammatory cytokines contributing to neuroprotection [19-21]. Therefore, post-ischemic neuroinflammation is thought to be a good target for stroke therapies [22, 23].

Many studies have showed that A2AR have the ability to suppress the activity of microglia and play anti-inflammatory and neuroprotective effects in ischemic brain injury. Adenosine is an important transmitter in the central nervous system [11, 24]. Its tissue levels increase dramatically during ischemia as a consequence of energy metabolism failure [25]. Of the four so far identified receptors, evidence indicates that the A2A receptor plays an important role in ischemia; most evidence indicates that A2A antagonists are protective in different animal models of cerebral ischemia. Indeed, A2A knockout mice are protected against cerebral infarction and against the neurological outcome of focal ischemia. We investigated the effect of A2AR knockout on microglia activation after cerebral ischemia/reperfusion. The

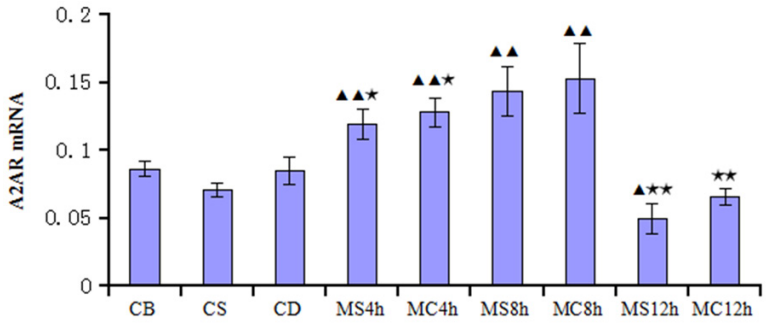

Figure 5. SCH58261 affects adenosine A2AR mRNA expressing after microglia OGD/R ($\bar{x} \pm s$) (n=3). Activated microglia expressed increased A2AR mRNA. ▲P<0.01, ▲▲P<0.05, vs control group; ★P<0.01, ★★P<0.05, vs 8 h of each group.

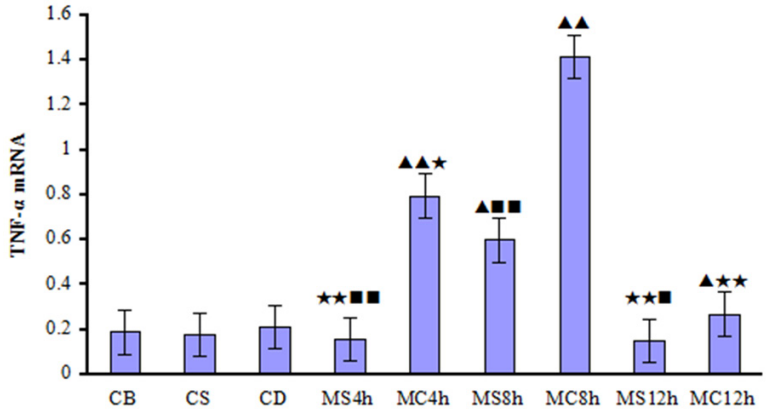

Figure 6. SCH58261 affects TNF-α mRNA expressing after microglia OGD/R ($\bar{x} \pm s$) (n=3). A2AR antagonist suppressed the expression of TNF-α mRNA level in activated microglia. ▲P<0.01, ▲▲P<0.05, vs control group; ★P<0.01, ★★P<0.05, vs 8 h of each group; ■P<0.01, ■■P<0.05, vs the same time points of group M.

results of study presented two important findings. First, cerebral ischemia/reperfusion trigger microglial activation and A2AR knockout were inhibited the activity of microglia as evidenced by decreasing microglial Iba1 staining. In addition, we observed that brain infarct volumes after stroke were alleviated in A2AR knockout mice. The neurological functions were recovered quickly in A2AR knockout mice campared with wild type mice, whereas there was not significantly difference between wild type and A2AR knockout mice in every time point. Perhaps it related with few sample size. Moreover, by using primarily cultured microglia, we further investigated whether A2A receptor expression was decreased in activated microglia and whether the A2AR overexpression alleviated microglia-mediated neuroinflammation. OGD/R has been used to mimic the in vivo ischemia/reperfusion brain injury. In accordance with the in vivo results, A2AR levels were remarkably increased in the activated microglia.

It has been well established that proinflammatory gene expression leads to stroke damage [26, 27]. During brain ischemia, proinflammatory cytokines such as TNF-α, IL-1β, IL-6 are produced by a variety of activated cell types, including endothelial cells, microglia, astrocytes, and neurons [27]. Blocking the production of these proinflammatory cytokines will be an important strategy to protect against ischemia brain injury. In vitro, we present that blockaded the A2AR, would blunt the activation-induced production of TNF-α. Taken together, our results from both in vivo and in vitro experimental stroke models suggest that the inhibition of microglial activation and the related inflammatory response is likely a mechanism that underlies the protective effect of blocking A2AR following cerebral ischemia and reperfusion.

All these findings indicate that the A2AR blockade protected the brain from ischemic reperfusion injury by inhibiting post-stroke microglial activation, so as to reduce the inflammatory responses caused by stroke. Moreover, our study suggests that A2AR blockade might provide a promising therapeutic approach for ischemic stroke.

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Disclosure of conflict of interest

None.

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