Inhibition of inflammatory mediator release from microglia can treat ischemic/hypoxic brain injury

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Abstract
Interleukin-1α and interleukin-1β aggravate neuronal injury by mediating the inflammatory reaction following ischemic/hypoxic brain injury. It remains unclear whether interleukin-1α and interleukin-1β are released by microglia or astrocytes. This study prepared hippocampal slices that were subsequently subjected to oxygen and glucose deprivation. Hematoxylin-eosin staining verified that neurons exhibited hypoxic changes. Results of enzyme-linked immunosorbent assay found that interleukin-1α and interleukin-1β participated in this hypoxic process. Moreover, when hypoxic injury occurred in the hippocampus, the release of interleukin-1α and interleukin-1β was mediated by the P2X4 receptor and P2X7 receptor. Immunofluorescence staining revealed that during ischemia/hypoxia, the P2X4 receptor, P2X7 receptor, interleukin-1α and interleukin-1β expression was detectable in rat hippocampal microglia, but only P2X4 receptor and P2X7 receptor expression was detected in astrocytes. Results suggested that the P2X4 receptor and P2X7 receptor, respectively, mediated interleukin-1α and interleukin-1β released by microglia, resulting in hippocampal ischemic/hypoxic injury. Astrocytes were activated, but did not synthesize or release interleukin-1α and interleukin-1β.

Key Words
neural regeneration; brain injury; inflammatory; P2X4 receptor; P2X7 receptor; interleukin-1α; interleukin-1β; microglia; astrocytes; oxygen-glucose deprivation; hippocampal slices; grants-supported paper; neuroregeneration

Research Highlights
(1) Purinergic receptors are associated with vascular responses, cell apoptosis and cytokine secretion.
(2) P2X4 and P2X7 receptors participated in ischemic/hypoxic injury, but the precise mechanisms of this kind of injury remain poorly understood.
(3) Interleukin-1 plays a key role in ischemic/hypoxic brain injury. This study investigated whether microglia induce interleukin-1 release during ischemia/hypoxia.
(4) P2X4 receptor and P2X7 receptor mediated interleukin-1α and interleukin-1β release from microglia resulted in hippocampal ischemic/hypoxic injury.
(5) Astrocytes were activated, but did not synthesize or release interleukin-1α and interleukin-1β.
INTRODUCTION

The inflammatory response plays an important role in ischemic/hypoxic brain injury\(^1\)–\(^2\). Following ischemic/hypoxic injury, increased release of adenosine triphosphate leads to microglial activation. Activated microglia release interleukin, interferon, tumor necrosis factor, prostaglandin and reactive oxygen species\(^3\). The above factors, especially interleukin, have strong toxic effects on neuronal cells. They mediate the inflammatory reaction and aggravate ischemic/hypoxic injury which results in cell death. Interleukin-1 is an important inflammatory cytokine and exerts a crucial effect on the initial stage of the inflammatory reaction\(^4\)–\(^8\). Interleukin-1 is composed of interleukin-1α and interleukin-1β and exerts biological effects via interleukin-1 receptors. Interleukin-1α is mostly membrane-associated and exerts a biological activity, but interleukin-1β mainly exists in the circulation. Interleukin-1 receptor antagonists\(^9\) are specific inhibitors of interleukin-1, and can suppress the activities of interleukin-1α and interleukin-1β, thus having a protective effect against ischemic/hypoxic injury\(^9\)–\(^10\).

Normal brain tissue expresses interleukin-1, but this low level of expression cannot induce neuronal death. Neurons, microglia and vascular endothelial cells can synthesize interleukin-1. A large number of interleukin-1β can be detected during ischemic/hypoxic injury. Interleukin-1β mediated cytokine release from microglia, promoted adhesion molecule expression in endothelial cells, stimulated the production of interleukin-6, interleukin-8 and tumor necrosis factor in monocytes and macrophages, strengthened leukocyte infiltration, released inflammatory mediators, and aggravated local inflammatory reaction. In addition, following brain ischemia, increased exogenous interleukin-1β obviously aggravated brain injury\(^10\). Touzani et al\(^11\) injected interleukin-1β into the cerebral ventricles of rats with middle cerebral artery occlusion and found that the injury was aggravated, and a remarkable increase in infarct volume was observed. Following cerebral ischemia, interleukin-1 receptor expression in the cerebral cortex was significantly increased at 6 hours, which coincided with interleukin-1β levels. An interleukin-1 receptor antagonist used as a preventative factor can inhibit interleukin-1β activity, and lessen neuronal injury in the ischemic region\(^12\)–\(^13\). Caso et al\(^14\) reported that following ischemic/hypoxic injury, an interleukin-1 receptor antagonist inhibited interleukin-1β expression. The interleukin-1 receptor antagonist lessened cerebral edema, reduced infarct area, decreased leukocyte infiltration, and blocked interleukin-1β-induced neural cell injury\(^15\). To further confirm this observation, interleukin-1β gene knock-out mice display significantly reduced infarct area following cerebral ischemia\(^16\)–\(^19\).

Our previous studies confirmed that interleukin-1β regulated the opening and closing of L-type Ca\(^{2+}\) channels, decreased L-type Ca\(^{2+}\) current, suppressed Ca\(^{2+}\) overload, reversed glutamate cytotoxicity, and exerted a neuroprotective effect. Nesic et al\(^20\) and Weng et al\(^21\) confirmed that interleukin-1β can protect injured spinal nerves. These studies demonstrated that interleukin-1β expression increased during ischemic/hypoxic injury, but whether interleukin-1β contributed to or relieved brain injury required further elucidation. Moreover, few studies addressing the effects of interleukin-1α in ischemic brain injury have been published, with the results remaining controversial.

Interleukin-1α and interleukin-1β have been shown to participate in ischemic/hypoxic brain injury, but their manner of release remains unclear\(^22\)–\(^25\). Under the stimulation of lipopolysaccharide, the P2X7 receptor induced interleukin-1β release in macrophages or microglia\(^23\)–\(^25\). Therefore, during ischemic/hypoxic injury, P2X7 receptor mediated interleukin-1β release from microglia or astrocytes is involved in neuronal injury. The concentration of adenosine triphosphate, an endogenous ligand of the P2X7 receptor, has been shown to increase in extracellular fluid during cerebral ischemia\(^26\). Seven subtypes of adenosine receptors for adenosine triphosphate, P2X1–7 receptors, have been identified. Of these, P2X4 and P2X7 expression is abundant in the central nervous system\(^27\)–\(^29\). Previous studies have demonstrated that the P2X4 receptor participated in the inflammatory reaction after traumatic brain injury, and P2X4 receptor expression was upregulated during brain ischemia\(^30\)–\(^31\). Thus, the P2X4 receptor possibly mediates interleukin-1 release, together with the P2X7 receptor. Furthermore, these receptors may participate in the starting and development of ischemic/hypoxic brain injury.

In the central nervous system, microglia are strongly associated with interleukin-1 during ischemic/hypoxic brain injury\(^32\)–\(^33\). Recent studies showed that activated astrocytes take on an active role in the inflammatory reaction following traumatic brain injury\(^34\)–\(^35\). Whether microglia or astrocytes induce interleukin-1 release requires further investigations. This study investigated
the effects of interleukin-1α and interleukin-1β after hypoxic brain injury in hippocampal slices subjected to oxygen and glucose deprivation. The source of interleukin-1α and 1β during hypoxia, and the precise mechanisms of their release were also investigated, so as to provide firm experimental evidence for future treatment of ischemic brain injury.

RESULTS

Effect of interleukin-1 on neuronal injury in hippocampal slice cultures

At 75 minutes following oxygen-glucose deprivation, the morphology of nerve cells in the control group was normal. Lightly stained basophilic granules were visible in the cytoplasm. The boundary between nerve cell membranes and surrounding regions was unclear. In the oxygen-glucose deprivation group, nerve cells displayed hypoxic injury, cell atrophy, acidophilic granules in the cytoplasm, and a clear boundary between nerve cell membranes and surrounding regions was observed. Some nerve cells exhibited vacuole-like changes. In the oxygen-glucose deprivation + interleukin-1 receptor antagonist group, nerve cells displayed normal morphology, lightly stained basophilic granules were observed in the cytoplasm and an unclear boundary between nerve cell membranes and surrounding regions was evident. No significant difference between this group and controls was seen (Figure 1).

At 75 minutes after OGD, the release of lactate dehydrogenase in hippocampal slices subjected to oxygen-glucose deprivation (OGD).

Release of interleukin-1α and interleukin-1β in hippocampal slices subjected to oxygen-glucose deprivation

At 75 minutes after oxygen-glucose deprivation, interleukin-1α or interleukin-1β concentration significantly increased in artificial cerebrospinal fluid (P < 0.01). The P2X7 receptor antagonist, A438079, did not affect interleukin-1α concentration. However, oxygen-glucose deprivation-induced interleukin-1α release was completely inhibited after P2X4 receptor antibody was added to artificial cerebrospinal fluid (P < 0.01; Figure 3). A438079 could completely suppress oxygen-glucose deprivation-induced interleukin-1β release (P < 0.01), but P2X4 receptor antibody did not affect interleukin-1β release (Figure 4).

Effects of exogenous interleukin-1α and interleukin-1β in hippocampal slices

A significant increase in lactate dehydrogenase release was observed at 75 minutes following incubation in artificial cerebrospinal fluid with the addition of exogenous interleukin-1α or interleukin-1β (P < 0.01). A further increase in lactate dehydrogenase release was observed following the combined addition of interleukin-1α and interleukin-1β compared with the addition of interleukin-1α alone or interleukin-1β alone (P < 0.01; Figure 5).
In double immunofluorescence labeling, hippocampal slices subjected to oxygen-glucose deprivation were assigned to eight groups. Using double labeling immunofluorescence, monoclonal antibodies of OX42 and glial fibrillary acidic protein were used to label microglia and astrocytes, respectively. At 75 minutes after oxygen-glucose deprivation, staining for these two markers was visible. P2X4 receptor and P2X7 receptor were successfully stained in astrocytes and microglia, suggesting that microglia and astrocytes were activated. Interleukin-1α and interleukin-1β were seen in OX42-positive cells, suggesting that microglia could synthesize interleukin-1α and interleukin-1β. These interleukins could not be immunostained in glial fibrillary acidic protein-positive cells, suggesting that astrocytes were activated during oxygen-glucose deprivation, but could not synthesize or secrete interleukin-1α and interleukin-1β (Figure 6).

**DISCUSSION**

During cerebral ischemia and hypoxia, necrosis and apoptosis, two types of neuronal death, can occur[36-40]. Whether it is necrosis or apoptosis depends on the character and intensity of various nociceptive stimuli, and also ischemic time. Apoptosis frequently occurs after slight ischemia or postischemic reperfusion[31-43]. Apoptosis is a naturally occurring programmed and targeted cause of cellular death, showing cell shrinkage, with the presence of intact cell membrane and apoptotic bodies, which are phagocytized. Cellular contents are contained within the cell membrane, and do not induce an inflammatory reaction. Apoptotic cells are often scattered throughout a sample[40]. Necrosis is a form of cell injury that results in the premature death of cells in living tissue, displaying cell swelling, broken cell membranes, leakage of cellular contents, slow changes in nuclei, insufficient DNA degradation, and local severe inflammatory response. Necrotic cells are widely distributed throughout ischemic tissue. Hematoxylin-eosin staining in the oxygen-glucose deprivation group showed nerve cell atrophy and a clear boundary between cell membranes.
Double fluorescence staining for the P2X4 receptor, P2X7 receptor, interleukin (IL)-1α and IL-1β expression in astrocytes and microglia in hippocampal slices subjected to oxygen-glucose deprivation (confocal laser scanning microscope, × 100).

At 75 minutes after oxygen-glucose deprivation, in green astrocytes (Cy2-stained; glial fibrillary acidic protein, GFAP), P2X4 and P2X7 receptors were stained red with Cy3. After merging the two images, yellow staining appeared (arrows; A) indicating colocalization. IL-1α and IL-1β were not stained with Cy3. After merging of these images, no colocalization was observed (arrows; B). In red microglia (Cy3-stained; OX42), P2X4 receptor, P2X7 receptor, IL-1α and IL-1β all stained green with Cy2, and after merging of the images yellow double staining appeared (arrows; C, D), indicating colocalization.
Interleukin-1 receptor antagonist could block this change in the oxygen-glucose deprivation group, suggesting that apoptotic hippocampal nerve cells were found after ischemia/hypoxia, and the occurrence of apoptosis was mediated by interleukin-1, which was consistent with previously published results\[^{[40]}\]. Lactate dehydrogenase is known to catalyze the conversion of pyruvic acid to lactic acid\[^{[41]}\]. In the oxygen-glucose deprivation group, expression of lactate dehydrogenase significantly increased, and the addition of interleukin-1 receptor antagonist could partially inhibit the increased expression of oxygen-glucose deprivation-mediated lactate dehydrogenase, which was consistent with the results from the hematoxylin-eosin staining studies. These findings indicated that this study successfully established a hippocampal slice model of hypoxia, and the hippocampal nerve cells experienced anaerobic glycolysis, showing hypoxic injury. Simultaneously, these findings suggested that interleukin-1 may promote the effects on hypoxic injury in rat hippocampal neurons. Following oxygen-glucose deprivation, even with the addition of interleukin-1 receptor antagonist, the concentrations of lactate dehydrogenase remain higher than the control group. The above-mentioned results suggested that during cerebral ischemia/hypoxia, other factors, besides the interleukin-1-mediated inflammatory reaction, also participated in neuronal injury.

This study verified that the addition of exogenous interleukin-1α and interleukin-1β could directly induce nerve injury, which confirmed that interleukin-1α and interleukin-1β exert a crucial effect on ischemia/hypoxia-induced nerve injury. Furthermore, the augmentation of the release of lactate dehydrogenase seen following the combined addition of interleukin-1α and interleukin-1β was greater than that using interleukin-1α or interleukin-1β alone. This finding suggests that the mechanisms of action of interleukin-1α and interleukin-1β are different, and their combination shows a synergistic effect on lactate dehydrogenase release. The precise mechanism remains unclear and requires further investigations.

During cerebral ischemia/hypoxia, damaged or necrotic nerve cells significantly release adenosine triphosphate, so that the intracellular and extracellular concentrations of adenosine triphosphate are altered\[^{[29]}\]. Elevated adenosine triphosphate can lead to cerebral injury\[^{[45-46]}\]. Thus, during hypoxia in hippocampal slices, interleukin-1α- and interleukin-1β-mediated nerve injury may possibly be associated with adenosine triphosphate. Adenosine triphosphate is an endogenous ligand of P2X4 and P2X7.

Adenosine triphosphate receptors have seven subtypes, of these, P2X4 and P2X7 receptors are highly expressed in the central nervous system\[^{[28-29]}\]. A selective antagonist of the P2X7 receptor is A438079\[^{[47]}\]. The P2X4 receptor does not have a specific antagonist. A previous study showed that P2X4 receptor antibody could effectively suppress the P2X4-gating current\[^{[49]}\]. Therefore, P2X4 receptor antibody and A438079, respectively, blocked the P2X4 and P2X7 receptors to observe their effects in oxygen-glucose deprivation-mediated interleukin-1α and interleukin-1β expression. Results revealed that oxygen-glucose deprivation-mediated release of interleukin-1α and interleukin-1β can only be completely suppressed by P2X4 receptor antibody and A438079. These findings suggested that during ischemia/hypoxia, interleukin-1α is mediated by P2X4, but interleukin-1β is mediated by P2X7. The pathway and mechanism of high expression of interleukin-1α and interleukin-1β are not identical.

After P2X4 and P2X7 receptors were blocked, the hypoxia-mediated release of interleukin-1α and interleukin-1β was inhibited, but their physiological release was not affected (as shown in the control group), indicating that the mechanisms of physiological release and hypoxia-induced release are different. Physiological release of interleukin-1α and interleukin-1β was not mediated by the interaction of adenosine triphosphate and its P2X receptor. On the basis of previous studies, this study further confirmed that interleukin-1α and interleukin-1β were involved in hippocampal neuronal injury following oxygen-glucose deprivation via P2X4 and P2X7 receptors, respectively.

The hippocampal cell type that releases interleukin-1α and interleukin-1β remains unclear. A previous study reported that P2X7 receptor mediated interleukin-1β release occurs in macrophages or microglia after stimulation with lipopolysaccharide\[^{[49]}\]. It remains unclear whether the mechanism of interleukin-1β synthesis and release during cerebral ischemia/hypoxia is similar to the mechanism of interleukin-1β synthesis and release in response to stimulation with lipopolysaccharide. Microglia and astrocytes are activated and involved in neuronal injury during ischemic/hypoxic brain injury\[^{[34-35]}\]. Microglia are considered as “macrophages of the central nervous system”, and play a key role in the innate immune\[^{[30-51]}\] and adaptive immune response\[^{[82]}\]. Normally, microglia are in the resting state, and mainly nourish and support neurons. Simultaneously, microglia are the principal immune cells in
the brain and function in immune defense. During cerebral ischemia/hypoxia, microglia are activated and proliferate, with the presence of reduced number of shortened processes, resulting in microglial pinocytosis and phagocytosis. Thus, microglia can secrete various cytokines, and express various specific proteins, such as CD68 and CD116. Appropriate microglial activation contributes to injury repair, but excessive activation will release interleukin, interferon and tumor necrosis factor, thereby aggravating injury[53-55].

To explore which kind of cells released interleukin-1α and interleukin-1β and mediated neuronal injury during ischemia/hypoxia, this study investigated the expression of P2X4 receptor, P2X7 receptor, interleukin-1α and interleukin-1β in astrocytes and microglia using double immunofluorescence labeling. Results demonstrated that at 75 minutes following oxygen-glucose deprivation, astrocytes and microglia were activated, and P2X4 and P2X7 receptors were expressed in both astrocytes and microglia. Interleukin-1α and interleukin-1β, however, were only expressed in microglia. These results verified that interleukin-1α and interleukin-1β were synthesized and released by microglia during cerebral hypoxia, and discovered the source of significant increase in interleukin-1α and interleukin-1β expression under hypoxia.

Following hypoxia, astrocytes were activated, but there was no immunostaining for interleukin-1α and interleukin-1β under these conditions, even though staining for the P2X receptor subtypes was present. These observations may be the case because (1) astrocytes did not synthesize interleukin-1α and interleukin-1β during hypoxia, but mediated the inflammatory reaction of ischemic/hypoxic brain injury by synthesizing other inflammatory factors. (2) Astrocytes synthesized interleukin-1α and interleukin-1β during hypoxia, but interleukin-1α and interleukin-1β were fully released from astrocytes at 75 minutes following hypoxia. Thus, expression of interleukin-1α and interleukin-1β was not detectable in astrocytes. (3) Astrocytes were activated in an early stage of cerebral hypoxia, but astrocytes did not synthesize interleukin-1α and interleukin-1β. The synthesis of interleukin-1α and interleukin-1β began at 75 minutes following oxygen-glucose deprivation, so the expression of interleukin-1α and interleukin-1β was not detected at the sampling time points. Nevertheless, results from previous studies demonstrated that interleukin-1β expression peaked at 60 minutes following cerebral ischemia/hypoxia, which differs from the latter two presumptions[56].

The inflammatory reaction is a major characteristic of continuous damage following cerebral ischemia/hypoxia, and is involved in the whole process of ischemic/hypoxic brain injury, especially in ischemia/reperfusion brain injury. Therefore, studying the pathogenesis of the inflammatory reaction and pathways used by inflammatory molecules allows further understanding for future lessening and prevention of ischemic/hypoxic brain injury.

Studies confirmed that P2X4 and P2X7 receptors, respectively, mediated the release of interleukin-1α and interleukin-1β from microglia during hypoxia. Astrocytes were activated during cerebral hypoxia, but did not express interleukin-1α and interleukin-1β, which further revealed the mechanisms of action of interleukin-1α and interleukin-1β in ischemic/hypoxic brain injury. This observation provides important theoretical evidence for the treatment of ischemic cerebrovascular disease, such as stroke. However, the way that the P2X4 and P2X7 receptors control the release of interleukin-1α and interleukin-1β from microglia and how P2X4 and P2X7 activation mediates neuronal injury and death remain poorly understood and require further investigations.

In summary, interleukin-1α and interleukin-1β played a key role in hypoxic injury in hippocampal slices. The P2X4 receptor and P2X7 receptor, respectively, mediated the release of interleukin-1α and interleukin-1β from microglia. In hypoxic hippocampal slices, astrocytes were activated, but did not synthesize or release interleukin-1α or interleukin-1β. The above mentioned results provide further evidence for developing and applying targeted drugs for the prevention and treatment of neuronal injury following ischemia/hypoxia.

MATERIALS AND METHODS

Design
A randomized, controlled in vitro study.

Time and setting
Experiments were performed at the Clinical Skill Center, Affiliated Hospital of Guangdong Medical College, China from June 2011 to March 2012.

Materials
A total of 15 healthy, clean, male, Sprague-Dawley rats aged 7 or 8 weeks old and weighing 150–200 g were
provided by the Experimental Animal Center, Guangdong Medical College, China with license No. A20100067, SCXX (Yue) 2008-0008. Rats were housed in a quiet room with ventilation and air filtration system at 20°C and 50% humidity, and allowed free access to food and water. Bedding and drinking water were replaced daily.

All protocols were in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China[57].

**Methods**

**Preparation of rat hippocampal slices**

Rats were decapitated under anesthesia. Hippocampi were separated and washed with artificial cerebrospinal fluid (Shanghai Chemical Agent Company, Shanghai, China) at 0–4°C. Two minutes later, hippocampi were sliced into 400-μm thick coronal sections on a vibratome (Leica, Wetzlar, Germany) and incubated at 35°C in artificial cerebrospinal fluid supplemented with NaCl 126.5 mmol/L, KCl 2.4 mmol/L, KH₂PO₄ 0.5 mmol/L, CaCl₂ 1.1 mmol/L, MgCl₂ 0.83 mmol/L, NaHCO₃ 27.5 mmol/L, Na₂SO₄ and glucose 11.8 mmol/L, pH 7.3, and maintained in 95% O₂ + 5% CO₂. In glucose-free artificial cerebrospinal fluid, glucose was replaced by 10 mmol/L sucrose (Shanghai Chemical Agent Company).

**Oxygen-glucose deprivation tests**

Hippocampal slices were randomly assigned into three groups. In the control group, slices were incubated in artificial cerebrospinal fluid in 95% O₂ + 5% CO₂. In the oxygen-glucose deprivation group[58-59], slices were incubated in artificial cerebrospinal fluid in 95% O₂ + 5% CO₂ for 20 minutes, and then in glucose-free artificial cerebrospinal fluid in 95% N₂ + 5% CO₂ for 75 minutes. In the oxygen-glucose deprivation + interleukin-1 receptor antagonist group, slices were incubated in artificial cerebrospinal fluid containing interleukin-1 receptor antagonist (1 mg/mL; R&D, Minneapolis, MN, USA) in 95% O₂ + 5% CO₂, and then in glucose-free artificial cerebrospinal fluid containing interleukin-1 receptor antagonist (1 mg/mL) in 95% N₂ + 5% CO₂ for 75 minutes.

Lactate dehydrogenase detection kit (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) was used to measure the concentration of lactate dehydrogenase in artificial cerebrospinal fluid, which determined whether hippocampal slices subjected to oxygen-glucose deprivation successfully displayed hypoxic injury. Hematoxylin-eosin staining was utilized to observe morphological changes in neurons. Lactate dehydrogenase detection (lactate dehydrogenase colorimetry) occurred as follows. Standards (10 μL) and samples of artificial cerebrospinal fluid (10 μL) were added in microwell plates. Substrate buffer (reagent A) was mixed with beta-nicotinamide adenine dinucleotide (reagent B) at a ratio of 5:1, and 60 μL of the mixture was added in each well. This mixture was placed in a water bath at 37°C for 15 minutes. 2,4-dinitrophenylhydrazine (reagent C; 50 μL) was mixed with standard preparations or specimens in a water bath at 37°C for 15 minutes. Stop solution (150 μL) was added at room temperature for 3 minutes. Specimens were measured at 440 nm. All experiments were performed at 35°C and repeated six times in six separate cultures.

**Detection of interleukin-1α and interleukin-1β release in hippocampal slices subjected to oxygen-glucose deprivation**

In the oxygen-glucose deprivation + A438079 group, slices were incubated in artificial cerebrospinal fluid containing A438079 (10 μmol/L) in 95% O₂ + 5% CO₂ for 20 minutes, and then in glucose-free artificial cerebrospinal fluid containing A438079 (10 μmol/L) in 95% N₂ + 5% CO₂ for 75 minutes. In the oxygen-glucose deprivation + P2X4 receptor antibody group, slices were incubated in artificial cerebrospinal fluid containing rabbit P2X4 receptor monoclonal antibody (1 μg/mL; Guangzhou Qiyun Biotechnology Co., Ltd., Guangzhou, Guangdong Province, China) in 95% O₂ + 5% CO₂ for 20 minutes, and then in glucose-free artificial cerebrospinal fluid containing rabbit P2X4 receptor monoclonal antibody (1 μg/mL) in 95% N₂ + 5% CO₂ for 75 minutes.

Interleukin-1α and interleukin-1β concentration were measured using enzyme-linked immunosorbent assay[60]. Artificial cerebrospinal fluid was centrifuged at 1 000 r/min at room temperature for 20 minutes, and the supernatant was obtained. A 96-well assay plate was divided into blank wells, standard wells and pending test sample wells. Sample diluent (100 μL) was added in the blank wells. Standard preparations (100 μL) and pending test samples (100 μL) were separately added to other wells. Samples were covered with a film and maintained at 37°C for 120 minutes. After removal of the liquid,
specimens were dried in air. Detection solution A working fluid (100 μL) was added in each well and the sample was covered with a film for incubation at 37°C for 60 minutes. After removal of the liquid, specimens were dried in air. Liquid A (400 μL/well) was added for 2 minutes. The plate was washed three times with PBS pH 7.4. Detection solution B working fluid (100 μL) was added and the sample was covered with a film for incubation at 37°C for 60 minutes. After removal of the liquid, the specimens were dried in air. Liquid B (350 μL/well) was added for 2 minutes, followed by drying in air. Substrate solution (90 μL) was added to each well in order. The assay plate was covered with a film and maintained at 37°C in the dark for coloration. Stop solution (50 μL) was then added in each well in order to terminate the reaction.

Absorbance values of each well were measured at 450 nm using a microplate reader (Shanghai Kang Medical Technology Development Co., Ltd., Shanghai, China). A standard curve was drawn using curve expert 1.3 curve software (Daniel G. Hyams, Hixson, TN, USA) where standard substance concentration was on the Y-axis and absorbance was on the X-axis. Thus, interleukin-1β concentrations were obtained according to the standard curve. The detection procedure of interleukin-1α was identical to that of interleukin-1β. Experiments were repeated six times.

**Determination of the effects of exogenous interleukin-1α and interleukin-1β in hippocampal slices subjected to nerve injury**

Hippocampal slices were assigned to four groups. In the control group, slices were incubated in artificial cerebrospinal fluid in 95% O₂ + 5% CO₂ for 75 minutes. In the exogenous interleukin-1α group, exogenous interleukin-1β group and exogenous interleukin-1α + interleukin-1β group, slices were incubated in artificial cerebrospinal fluid containing exogenous interleukin-1α (100 ng/mL) and/or interleukin-1β (100 ng/mL) in 95% O₂ + 5% CO₂ for 75 minutes. The concentrations of lactate dehydrogenase were measured.

The measurement method was identical to that used in oxygen-glucose deprivation studies. Experiments were repeated six times.

**Immunofluorescence for the expression of the P2X4 receptor, P2X7 receptor, interleukin-1α and interleukin-1β in astrocytes and microglia**

Hippocampal slices were incubated in glucose-free artificial cerebrospinal fluid in 95% N₂ + 5% CO₂ for 75 minutes. The expression of the P2X4 and P2X7 receptor and interleukin-1α and interleukin-1β in astrocytes and microglia were determined using immunofluorescence. Following oxygen-glucose deprivation, hippocampal slices were divided into eight groups. Precise fluorescent labeling is as follows:

| Group | Marker                   |
|-------|--------------------------|
| 1     | GFAP/P2X4                |
| 2     | GFAP/P2X7                |
| 3     | GFAP/IL-1α               |
| 4     | GFAP/IL-1β               |
| 5     | OX42/P2X4                |
| 6     | OX42/P2X7                |
| 7     | OX42/IL-1β               |
| 8     | OX42/IL-1α               |

GFAP: Glial fibrillary acidic protein; IL: interleukin.

Hippocampal slices in groups 1–4 were washed with Tris buffered saline (0.05 mol/L, pH 7.6), blocked with 5% fetal calf serum, incubated with rat glial fibrillary acidic protein monoclonal antibody (1:1 000; Guangzhou Qiyun Biotechnology Co., Ltd.), and then with secondary antibody targeted against Cy2-labeled goat anti-rat IgG (1:400; Guangzhou Qiyun Biotechnology Co., Ltd.) for fluorescence staining. Subsequently, slices in groups 1 and 2 were incubated in primary antibody rabbit P2X4 and P2X7 receptor monoclonal antibody (P2X4: 1:500, P2X7: 1:400; Guangzhou Qiyun Biotechnology Co., Ltd.). Slices in groups 3 and 4 were incubated with primary antibody hamster interleukin-1 monoclonal antibody (interleukin-1α: 1:200, interleukin-1β: 1:200; Guangzhou Qiyun Biotechnology Co., Ltd.). Subsequently, slices were treated with secondary antibody Cy3-labeled goat anti-rabbit IgG (1:400; Guangzhou Qiyun Biotechnology Co., Ltd.) or secondary antibody Cy3-labeled goat anti-hamster IgG (1:200) for fluorescence staining.

Hippocampal slices in groups 5–8 were incubated in rat OX42 monoclonal antibody (1:2 000; Guangzhou Qiyun Biotechnology Co., Ltd.), and then in the secondary antibody Cy3-labeled goat anti-rat IgG (1:500; Guangzhou Qiyun Biotechnology Co., Ltd.) for fluorescence staining. Subsequently, hippocampal slices in groups 5 and 6 were incubated with primary antibody targeted towards the rabbit P2X4 receptor and P2X7 receptor monoclonal antibody (P2X4: 1:500, P2X7: 1:500). Hippocampal slices in groups 7 and 8 were incubated with primary antibody hamster interleukin-1 monoclonal antibody (interleukin-1α: 1:200; interleukin-1β: 1:200). Secondary antibody Cy2-labeled goat anti-rabbit (1:400; Guangzhou Qiyun Biotechnology Co., Ltd.) or secondary antibody Cy2-labeled goat anti-hamster IgG (1:200; Guangzhou Qiyun
Biotechnology Co., Ltd.) were used for fluorescence staining.

Double fluorescence detection was performed using confocal laser scanning microscope (Olympus, Tokyo, Japan). A red signal was revealed by immunofluorescence for Cy3 at an excitation wavelength of 543 nm, and a green signal was revealed by immunofluorescence for Cy2 at an excitation wavelength of 488 nm.

**Statistical analysis**

Data were expressed as mean ± SD. Data were analyzed using SPSS 16.0 software (SPSS, Chicago, IL, USA). Intergroup differences were compared by one-way analysis of variance. Intergroup paired comparison was performed using least significant difference t-test. A value of \( P < 0.05 \) was considered statistically significant.

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**Author contributions:** Huaibo Wang designed the experiments, wrote and revised the manuscript. Weitao Guo was in charge of funding and conducted the experiments. Hongliang Liu conducted the experiments. Rong Zeng analyzed the data. Mingnan Lu performed the experiments and revised the article. Ziqiu Chen and Qixian Xiao conducted the experiments and revised the article. All authors approved the final version of the paper.

**Conflicts of interest:** None declared.

**Ethical approval:** The study gained full ethical approval from the Animal Ethics Committee of Guangdong Medical College in China.

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