Microglial and Neuronal Cell Pyroptosis Induced by Oxygen–Glucose Deprivation/Reoxygenation Aggravates Cell Injury via Activation of the Caspase-1/GSDMD Signaling Pathway

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Abstract
Pyroptosis is a new type of programmed cell death, which induces a strong pro-inflammatory reaction. However, the mechanism of pyroptosis after brain ischemia/reperfusion (I/R) and the interaction between different neural cell types are still unclear. This study comprehensively explored the mechanisms and interactions of microglial and neuronal pyroptosis in the simulated I/R environment \textit{in vitro}. The BV2 (as microglial) and HT22 (as neuronal) cells were treated by oxygen–glucose deprivation/reoxygenation (OGD/R). Both BV2 and HT22 cells underwent pyroptosis after OGD/R, and the pyroptosis occurred at an earlier time point in HT22 than that of BV2. Caspase-11 and Gasdermin E expression in BV2 and HT22 cells did not change significantly after OGD/R. Inhibition of caspase-1 or GSDMD activity, or down-regulation of GSDMD expression, alleviated pyroptosis in both BV2 and HT22 cells after OGD/R. Transwell studies further showed that OGD/R-treated HT22 or BV2 cells aggravated pyroptosis of adjacent non-OGD/R-treated cells, which could be relieved by inhibition of caspase-1 or GSDMD. These results suggested that OGD/R induces pyroptosis of microglia and neuronal cells and aggravates cell injury via activation of caspase-1/GSDMD signaling pathway. Our findings indicated that caspase-1 and GSDMD may be therapeutic targets after cerebral I/R.

Keywords Pyroptosis · Neuron · Microglia · Inflammation · GasderminD (GSDMD) · Necrosulfonamide

Introduction
Stroke is a disease with high morbidity and mortality worldwide. Ischemic stroke accounts for about 80% of neurovascular injury [1]. At present, the treatment strategy of acute ischemic stroke is to restore blood flow as soon as possible, by using drug thrombolysis and mechanical thrombolysis [2, 3]. However, the time frame for safe intervention is still limited for these treatments. It is estimated that less than 10% of patients with acute stroke benefit from reperfusion treatments, because delayed treatment can lead to worsened outcome [4], caused by reperfusion injury. An important mechanism of reperfusion injury is the inflammatory response following cerebral ischemia/reperfusion (I/R) [5]. However, the inflammatory response after cerebral I/R is a very complex process, which still needs to be explored.

Recent studies have shown that the inflammasomes formed within brain cells (including neurons, microglia and astroglia) play an important role in inflammatory response to brain I/R injury [6–9]. Activation of caspase-1, a component of inflammasomes, induces the processing of
pro-inflammatory cytokines pro-IL-1β and pro-IL-18 into their matured forms. Activated caspase-1 is also known to induce a lytic type of cell death called pyroptosis. When cells undergo pyroptosis, membrane pores with diameters of 10–20 nm are formed and water influx is driven by intracellular non-ionic osmolytes, which causes cells swelling, membrane rupture, the release of cellular contents, and finally cell death. Pyroptosis can induce a strong pro-inflammatory reaction [10–14]. Activation of Gasdermin D (GSDMD), one of the gasdermin proteins family, is necessary for activated caspase-1 to induce cell pyroptosis. The activated caspase-1 cleaves GSDMD between the N-terminal and C-terminal domains. The N-terminal domain of GSDMD (GSDMD-N) has pyroptosis-inducing activity and plays a decisive role in pro-IL-1β and pro-IL-18 processing and secretion [15–17]. The inflammasome/caspase-1/GSDMD pathway is called the canonical inflammasome pathway. GSDMD can also be activated by active caspase-11 (rodent-derived) or caspase-4/5 (human-derived) in the case of lipopolysaccharide action, known as the non-canonical inflammasome pathway [11]. In addition, recent studies showed that caspase-3, a well-known indicator of apoptosis all the time, also induces secondary necrosis (pyroptosis) through activation of Gasdermin E (GSDME) [18–20].

Although some studies have suggested the activation of GSDMD and pyroptosis in microglia and neurons in simulated ischemic conditions in vitro [21–23], however, it is still unclear whether there is interplay between microglial and neuronal pyroptosis after I/R, which results in inflammatory propagation. Moreover, there are no reports about activation of GSDME following cerebral I/R. This study comprehensively explores the mechanisms and interactions of microglial and neuronal pyroptosis during mimic I/R injury in vitro.

Materials and Methods

Experimental Design

This study consisted of three parts: In part 1 experiment, proportion of pyroptic BV2 (as microglial) and HT22 (as neuronal) cells, expression of pyroptosis-related protein and cell viability were observed during OGD/R (supplemental Fig. 4A). Part 2 experiment explored the effect of inhibition of caspase-1 or GSDMD on BV2 and HT22 cell pyroptosis following OGD/R (supplemental Fig. 4B). Part 3 experiment demonstrated the interaction of BV2 and HT22 cell pyroptosis after OGD/R by the transwell co-culture system (supplemental Fig. 4C, D).

Cell Culture

BV2 microglia and HT22 neurons were purchased from American Type Culture Collection (ATCC). BV2 cells and HT22 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) High Glucose (Gibco, Invitrogen, USA) with 10% fetal bovine serum (Gibco, Invitrogen, USA) and 1% penicillin/streptomycin (Gibco, Invitrogen, USA) at 37 °C in a 5% CO₂ atmosphere.

Oxygen–Glucose Deprivation/Reoxygenation (OGD/R) Model

In order to mimic cerebral I/R an OGD/R in vitro model was used, the OGD/R model was constructed by replacing normal culture medium with glucose-free DMEM medium (Gibco, Invitrogen, USA), and then the cells was placed in a hypoxic incubator (Anoxomat Mark II, Mart Microbiology B.V, Netherlands) that contained a gas mixture of 1% O₂, 5% CO₂ and 94% N₂ for 4 h (BV2 cells) or 2 h (HT22 cells) at 37 °C and then recovering normal gas and culture medium at the optimal time. Samples collected at various time points after OGD/R for subsequent experiments. Normal cultured cells were used as the control group.

Detection of Cell Pyroptosis by Dye Uptake

The dye uptake method was carried as previously reported [12]. The formation of discrete pores in the plasma membrane of cells underwent pyroptosis resulted in increased uptake of YO-PRO-1 iodide, a small (629 Da) membrane impermeable dye, with exclusion of a larger (1293 Da) membrane impermeable dye, ethidium homodimer-2 (Eth-D2). The cells at various time points after OGD/R in the culture dish of 10 cm in diameter was added to 1 µM YO-PRO-1 iodide (Invitrogen, Shanghai, China), 1 µM Eth-D2 (Invitrogen, Shanghai, China) and 2 µM Hoechst 33,342 (Invitrogen, Shanghai, China) for 10 min. Then wash the cells with PBS twice, 5 min each. Triton X-100 detergent (0.1%) was used as a positive control for dye uptake. Normally cultured cells are treated as controls. Images were captured using a ZEISS A2 inverted microscope (ZEISS, Germany). Dye-positive cells were counted in a total of 18 fields per group (6 wells per group, 3 fields per well).

Western Blot Analysis

The cells were lysed using ice-cold Radio-Immunoprecipitation Assay (RIPA) buffer (Beyotime, P0013B). The extracts were separated on 12% SDS-PAGE, transferred to nitrocellulose membrane. The membrane was incubated with
primary antibodies against GSDMD (Abcam, ab209845, 1:2000), GSDME (Abcam, ab215191, 1:2000), caspase-1 (Abcam, ab179515, 1:1000), caspase-11 (Abcam, ab22684, 1:1000), β-actin (Abcam, ab115777, 1:3000) at 4 °C overnight. The membranes were then incubated with an HRP-conjugated secondary antibody for 1 h at room temperature. The epitopes were visualized using an ECL western blot detection kit (Millipore, USA).

**Immunofluorescence (IF) Analysis**

The cells were cultured in confocal dish. Then, the cells were fixed with 4% paraformaldehyde for 30 min, permeabilized for 10 min in 0.3% Triton X-100 and blocked using 5% BSA for 1 h. The cells were incubated with primary antibodies against caspase-1 (Abcam, ab179515, 1:200) or GSDMD (Abcam, ab209845, 1:200) at 4 °C overnight. The cells were washed three times for 10 min each with PBST (PBS tween-20, 0.05%), and then the secondary anti-Rabbit Alexa Fluor-488-conjugated antibody (Zsbio, 1:300) was added for 1 h at room temperature on the dark. Nuclear were stained with 4′,6-diamidino-2-phenylindole (DAPI). The images were taken with ZEISS confocal microscopy system and fluorescence intensity analysis was performed with ImageJ software.

**Enzyme Linked Immunosorbent Assay (ELISA)**

The levels of the inflammatory cytokines IL-1β, IL-18 and the Lactate dehydrogenase (LDH), an indicator for cell injury/death, were measured in the cell supernatants by ELISA according to the manufacturer’s instructions (eBioscience, Thermo Fisher).

**Cell Viability**

The cell viability was assessed using MTT assay Kit (Pro-mega, USA) according to manufacturer’s instructions. Approximate 4.0 × 10^4 cells were plated in 96-well plates overnight. The cells were collected at various time points after exposing OGD/R. Ten microlitre MTT solution was added to 100 µL of culture medium in each well and incubated for 4 h at 37 °C. Then the solution was removed and 100 µL of DMSO was added to each well. After mixed by oscillation 10 min, the optical density was measured at 570 nm by using the enzyme standard instrument. The normal cultured cells were as the control.

**Inhibition of Caspase-1 and GSDMD**

According to the results of our preliminary experiments, caspase-1 inhibitor AC-YVAD-CMK at 10 µM (supplemental Fig. 1), chemical inhibitor of GSDMD Necrosulfonamide (NSA) [24] at 20 µM (supplemental Fig. 2), and a siRNA of GSDMD (siGSDMD fragment 829 (supplemental Fig. 3) was selected for their optimal inhibition effect. The peak time point of pyroptosis was subjected to the following inhibition experiments. AC-YVAD-CMK (Sigma-Aldrich, Darmstadt, Germany) or NSA (MedChemExpress, USA) dissolved in dimethylsulphoxide (DMSO) was added to the culture medium 4 h before OGD treatment [24, 25], siGSDMD-829 was transfected 48 h before OGD/R, and siGSDMD-NC was transfected as the control (siGSDMD-NC does not inhibit GSDMD). RNA oligonucleotides purchased from GenePharma (Shanghai, China) (supplemental Table) were transfected into cells using lipofectamine RNAiMax (Invitrogen, USA) according to the manufacturer instructions. Then, samples at the peak time point of cell pyroptosis were collected for subsequent experiments (Table 1).

**Transwell Co-Culture System for BV2 and HT22 Cells**

The transwell co-culture system was designed as previous reported [26]. To observe the effect of HT22 cells on BV2 cells after OGD/R, the upper layer was plated with HT22 cells and BV2 cells were cultured on the bottom well of the chamber. HT22 cells were treated with OGD/R, with or without pretreatment of AC-YVAD-CMK or NSA or siGSDMD-829 before OGD/R (as described previously). Similarly, in order to observe the effect of BV2 cells on HT22 cells after OGD/R, the BV2 cells were seeded on the upper layer, treated with OGD/R with or without pretreatment of AC-YVAD-CMK or NSA or siGSDMD-829 before OGD/R, and HT22 cells were plated on the bottom layer of the chamber. The semi-permeable membrane allows sharing of culture medium and its components between cells grown in the upper and lower chambers of the same transwell. The selection of time points for samples harvested from the lower chamber was in accordance with the peak time point of the corresponding cell pyroptosis observed in the foregoing experiment. The samples of the lower cells were collected for subsequent experiments.

**Table 1** The sequences of GSDMD siRNA and Negative control

| Gene              | Sequence (5’–3’)                        |
|-------------------|-----------------------------------------|
| Gsdmd-Mus-312     | F: CAUGUGUCAACCUUGCAAUUTT               |
|                   | R: AUGCAGGGUUACAAUUUGTT                 |
| Gsdmd-Mus-829     | F: GGAAUUCUCUCUGUCUCATT                 |
|                   | R: UAGGACAGGAAAGGAAUUCCATT              |
| Gsdmd-Mus-1477    | F: GCCAGAAGAAGUGGCGUATT                 |
|                   | R: UAGGCCCAUUUCUACCGT                   |
| Gsdmd-Mus-1582    | F: GUCAGUGUCAGCCAGAAATT                 |
|                   | R: UUCUCCGAGACUGACGATT                  |
| Negative control  | F: UUCUGUCACGUCUGACGATT                 |
|                   | R: ACGUGACAGGUGACGAGATT                 |
Statistical Analysis

Statistical analysis was performed using the GraphPad Prism Software. The ImageJ software was used to analyze the optical density of the western blot results and to calculate the number of pyroptotic cells. Values were presented as the means ± SEM with the homogeneity of variance. The Student t-test was used for the difference between the two groups of independent data. The comparison between multiple groups of samples was performed by one-way ANOVA. Sample sizes were chosen based on previous literature. Differences were considered statistically significant when \( P < 0.05 \) (*\( P < 0.05 \); **\( P < 0.01 \)).

Results

BV2 and HT22 Cells Underwent Cell Pyroptosis After OGD/R

The experimental results of the BV2 cell pyroptosis after OGD/R were as followed. The dye uptake method showed the highest proportion of pyroptotic cells appeared at 12 h after OGD/R (Fig. 1A, B). Western blot results demonstrated that GSDMD-N and cleaved-caspase-1 expression increased at 12 h after OGD/R, but the levels of GSDME-N and cleaved-caspase-11 were not upregulated at all time points after OGD/R (Fig. 1C–K). Immunofluorescence results showed that caspase-1 and GSDMD expression increased at 12 h after OGD/R (Fig. 1L–O). In addition, IL-1β, IL-18 and LDH in cell supernatant were significantly increased at 12 h after OGD/R. S The cell viability of BV2 cells was detected by MTT assay kit. The minimum cell proliferative ability appeared at 12 h after OGD/R. Data were represented as mean ± SEM. \( n = 3 \), *\( P < 0.05 \), **\( P < 0.01 \). N.S no significant difference.
A phenomenon that is 6 h earlier than BV2 cells (supplemental Fig. 5).

Inhibition of Caspase-1 Alleviated BV2 and HT22 Cell Pyroptosis and GSDMD Activation After OGD/R

Based on the above experimental results, BV2 cells at 12 h and HT22 cells at 6 h after OGD/R were used to observe the inhibitory effects of caspase-1 and GSDMD.

After pretreatment of AC-YVAD-CMK, a caspase-1 inhibitor, the proportion of pyroptotic BV2 and HT22 cells was significantly decreased (Fig. 2A, B, I, J). AC-YVAD-CMK reduced caspase-1 cleavage and activation (supplemental Fig. 1), and down-regulated the processing of GSDMD-N in cells after OGD/R (Fig. 2C–E, K–M). Production of IL-1β, IL-18, and LDH in cell supernatant of BV2 cells was also significantly decreased in cells pre-treated with AC-YVAD-CMK (Fig. 2F–H) and production of IL-1β, IL-18, and LDH in cell supernatant of HT22 cells was also significantly decreased in cells pre-treated with AC-YVAD-CMK (Fig. 2N–P). Notably, the levels of IL-1β, IL-18 and LDH in HT22 cells were less than that in BV2 cells.

Inhibition of GSDMD Alleviated BV2 and HT22 Cell Pyroptosis After OGD/R

After pretreatment of NSA, an inhibitor of GSDMD, which knocked down cellular GSDMD expression (Fig. 3C–E, K–M), the proportion of pyroptotic BV2 and HT22 cells...
was significantly decreased (Fig. 3A, B, I–J). Production of IL-1β, IL-18, and LDH in cell supernatant was also significantly decreased in cells pre-treated with NSA (Fig. 3F–H, N–P).

In addition, cells were transfected with siGSDMD-829, which knocked down cellular GSDMD expression (Fig. 4C–E, K–M), the proportion of pyroptotic BV2 and HT22 cells was significantly decreased (Fig. 4A, B, I–J). Production of IL-1β, IL-18, and LDH in cell supernatant was also significantly decreased in cells pre-treated with NSA (Fig. 4F–H, N–P). Notably, the levels of IL-1β, IL-18 and LDH in HT22 cells were less than that in BV2 cells.

**HT22 and BV2 Cells Undergoing OGD/R Aggravated Pyroptosis of Adjacent Non-OGD/R-Treated Cells**

Using the transwell co-culture model, HT22 cells were seeded in the upper layer of the chamber and underwent OGD/R treatment for 6 h, then HT22 cells were co-cultured with BV2 cells for additional 12 h. Greater proportion of pyroptotic cells (Fig. 5A, B), increased GSDMD-N expression (Fig. 5C–E), higher level of IL-1β, IL-18 and LDH (Fig. 5F–H) and decreased cell viability (Fig. 5I) were observed in the co-cultured BV2 cells as compared with BV2 cells cultured alone and were treated by OGD/R for 12 h.

Similar results were found in the transwell co-culturing experiments, in which BV2 cells were seeded in the upper layer and underwent OGD/R for 12 h, then co-cultured with HT22 cells in the lower layer for additional 6 h. Compared
with HT22 cells treated by OGD/R for 6 h, the HT22 cells co-cultured with OGD/R-treated BV2 cells showed greater proportion of pyroptotic cells (Fig. 5J–K), increased GSDMD-N expression (Fig. 5L–N), higher level of IL-1β, IL-18 and LDH (Fig. 5O–Q) and decreased cell viability (Fig. 5R). However, levels of secreted IL-1β, IL-18 and LDH found in HT22 culture medium could be contributed from co-cultured BV2 cells and therefore may not necessarily reflect the changes of HT22.

**Inhibition of Caspase-1 in HT22 Cells Before OGD/R Alleviated Pyroptosis of Adjacent BV2 Cells, and Vice Versa**

Using the transwell model, HT22 cells were pretreated with AC-YVAD-CMK before OGD/R, followed by co-culturing with BV2 cells seeded in the lower layer of the chamber for additional 12 h. Reduction in pyroptotic cells (Fig. 6A, B), decreased GSDMD-N expression (Fig. 6C–E), lowered levels of IL-1β, IL-18 and LDH (Fig. 6F–H) were observed in the co-cultured BV2 cells as compared with BV2 cells that were co-cultured with OGD/R-treated HT22 without AC-YVAD-CMK pretreatment.

Similar phenomenon was found in the transwell co-culture model in which BV2 cells were pretreated with AC-YVAD-CMK before OGD/R, followed by co-culturing with HT22 cells in the same chamber for additional 6 h. Compared with HT22 cells in the transwell co-culture model, in which BV2 cells were not pretreated with AC-YVAD-CMK...
before OGD/R, the lower HT22 cells showed fewer proportion of pyroptotic cells (Fig. 6I, J), decreased GSDMD-N production (Fig. 6K–M) and lowered level of IL-1β, IL-18 and LDH in the culture medium (Fig. 6N–P). Similarly, levels of secreted IL-1β, IL-18 and LDH found in HT22 culture medium could be contributed from co-cultured BV2 cells and therefore may not necessarily reflect the changes of HT22.

Inhibition of GSDMD in HT22 Cells Before OGD/R Alleviated Pyroptosis of Adjacent BV2 Cells, and Vice Versa

Using the transwell model, HT22 cells were pretreated with NSA before OGD/R, followed by co-culturing with BV2 cells seeded in the lower layer of the chamber for additional 12 h. Reduction in pyroptotic cells (Fig. 7A, B), decreased GSDMD-N expression (Fig. 7C–E), lowered levels of IL-1β, IL-18 and LDH (Fig. 7F–H) were observed in the co-cultured BV2 cells as compared with BV2 cells that were co-cultured with OGD/R-treated HT22 without NSA pretreatment. Similar phenomenon was found in the transwell
co-culture model in which BV2 cells were pretreated with NSA before OGD/R, followed by co-culturing with HT22 cells in the same chamber for additional 6 h. Compared with HT22 cells in the transwell-co-culture model, in which BV2 cells were not pretreated with NSA before OGD/R, the lower HT22 cells showed fewer proportion of pyroptotic cells (Fig. 7I, J), decreased GSDMD-N production (Fig. 7K–M) and lowered level of IL-1β, IL-18 and LDH in cell supernatant of YVAD+DMSO+Transwell HT22 cells was decreased compared with that in Transwell HT22 cells. β-actin was used as a loading control. F–H ELISA results showed that the level of IL-1β, IL-18 and LDH in cell supernatant of YVAD+DMSO+Transwell BV2 cells was decreased compared with Transwell BV2 cells. I, J Representative immunofluorescence staining of HT22 cells by dye uptake method (I) and histogram of the percentage of pyroptotic HT22 cells (J). Compared with Transwell HT22 cells (as described in Fig. 7), there was less pyroptotic proportion in HT22 cells which were layed in the lower layer and cultured for 6 h with the solution of upper BV2 cells pretreated with YVAD before OGD/R (indicated as YVAD+DMSO+Transwell in Figure). 0.1% Triton X-100 was used as a positive control. Magnification, ×100. Scale bar, 100 µM. K–M Representative immunoblot and its quantification of GSDMD protein in HT22 cells. The GSDMD-N expression in YVAD+DMSO+Transwell HT22 cells was decreased compared with that in Transwell HT22 cells. β-actin was used as a loading control. N–P ELISA results showed that the level of IL-1β, IL-18 and LDH in cell supernatant of YVAD+DMSO+Transwell HT22 cells was decreased compared with that in Transwell HT22 cells. Data were represented as mean ± SEM. n = 3, *P < 0.05, **P < 0.01. N.S no significant difference.

Using the transwell model, HT22 cells were pretreated with siGSDMD-829 before OGD/R, followed by co-culturing with BV2 cells seeded in the lower layer of the chamber for additional 12 h. Reduction in pyroptotic cells (Fig. 8A, B), decreased GSDMD-N expression (Fig. 8C–E), lowered levels of IL-1β, IL-18 and LDH (Fig. 8F–H) were observed in the co-cultured BV2 cells as compared with BV2 cells that were co-cultured with OGD/R-treated HT22 without siGSDMD-829 pretreatment. Similar phenomenon was found in the transwell co-culture model in which BV2 cells were pretreated with siGSDMD-829 before OGD/R, followed by
co-culturing with HT22 cells in the same chamber for additional 6 h. Compared with HT22 cells in the transwell co-culture model, in which BV2 cells were not pretreated with siGSDMD-829 before OGD/R, the lower HT22 cells showed fewer proportion of pyrototic BV2 cells (Fig. 8I, J), decreased GSDMD-N production (Fig. 8K–M) and lowered level of IL-1β, IL-18 and LDH in the culture medium (Fig. 8N–P). Similarly, levels of secreted IL-1β, IL-18 and LDH found in HT22 culture medium could be contributed from co-cultured BV2 cells and therefore may not necessarily reflect the changes of HT22.

**Discussion**

To our knowledge, it is first study to comprehensively explore the mechanisms and interactions of microglial and neuronal cell pyroptosis after I/R. The results here showed that in the simulated I/R environment in vitro (the OGD/R model), both BV2 and HT22 cells underwent pyroptosis, and the onset of pyroptosis of HT22 cells was 6 h earlier than that of BV2 cells. Our data also showed no significant changes in caspase-11 and GSDME expression in BV2 and HT22 cells after OGD/R. Inhibition of caspase-1 or GSDMD...
alleviated BV2 and HT22 cell pyroptosis after OGD/R, and GSDMD activation was suppressed by caspase-1 inhibitor. HT22 or BV2 cells undergoing OGD/R aggravated pyroptosis of adjacent BV2 or HT22 cells, respectively, which was relieved by inhibition of caspase-1 or GSDMD.

A number of studies have identified that the activation of the Gasdermin family proteins is the biological marker for pyroptosis [27–30] and the role of pyroptosis is important during ischemic injury of the brain [21–23]. Few studies have observed that GSDMD was activated in primary cultured cortical neurons or microglia or BV2 cells undergoing OGD or OGD/R [21–23], but the time point of GSDMD activation in these studies is different from that in our study, which may be due to the difference in cell type or culture condition. Our study observed the activation of GSDMD in BV2 and HT22 cells after OGD/R. The mechanisms of BV2 and HT22 cell pyroptosis after OGD/R were further explored in our study. The results showed that...
demonstrated that inhibition of caspase-1 could alleviate GSDMD activation, and inhibition of caspase-1 or GSDMD could reduce the level of IL-1β, IL-18 and LDH in both cell types after OGD/R, which is similar to the results reported by Poh et al. [21] and Tang et al. [23]. However, caspase-11 and GSDME activation in BV2 and HT22 cells following OGD/R were not found in our study. Fann et al. [34] reported that caspase-11 expression was increased in primary cultured cortical neurons after OGD/R, which is different from our results. As described above, the difference may be due to cell type or culture condition. Based on our findings, we speculate that activation of the canonical inflammasome pathway (caspase-1/GSDMD) is crucial for cell pyroptosis but caspase-3/GSDME pathway may not be involved in pyroptotic cell death following brain I/R. Whether the non-canonical inflammasome pathway (caspase-11/GSDMD) play a role in cell pyroptosis following brain I/R remains to be determined. In addition, in our study, for the first time we showed that necrosulfonamide (NSA) was able to inhibit GSDMD-induced pyroptosis following I/R. The results showed here strongly suggest that NSA could be an effective suppressor for the activation of GSDMD induced by OGD/R. Further investigation on the protective effect of NSA in I/R animal model is needed.

After cell pyroptosis, the release of cellular contents, including damage associated molecular patterns (DAMPs), and their binding to pattern recognition receptors (PRRs) on the adjacent cells result in inflammatory propagation [35, 36]. In addition, studies of inflammatory diseases have demonstrated that inflammasome components released from the pyroptotic cells could re-assemble into functional complexes or could be engulfed by neighboring phagocytic cells to further activate caspase-1, which helps amplify and prolong the inflammatory response [37, 38]. However, it is not clear yet whether the cell pyroptosis induced by I/R propagate inflammation. In this study, by using the transwell co-culture system, we observed that HT22 or BV2 cells undergoing OGD/R can not only produce pyroptosis phenotype, but also induce pyroptotic death of their neighboring BV2 or HT22 cells with more severe cell death phenotype than that of those cells treated with OGD/R alone. After inhibition of caspase-1 or GSDMD of HT22 or BV2 cells undergoing OGD/R, pyroptotic death of the neighboring BV2 or HT22 cells was alleviated. These results imply that the sequential induction of microglial and neuronal pyroptosis after cerebral I/R may result in an amplifying cascade of inflammatory response and prolonged tissue damage. In this study the transwell experiments were chosen to verify the mechanism of cell injury caused by the inflammation propagation after cell pyroptosis. The purpose could be achieved by the current experimental methods, so we did not observe the phenomena that the adjacent cells were the same as inhibition cells under the condition of OGD/R.

This article has some limitations. First, we only arrived at our conclusion by knockout method, we should add gain-of-function study to help confirm the mechanistic link between caspase-1 and GSDMD in OGD/R-induced pyroptosis in both neurons and microglia. Second, cell apoptosis or necroptosis induced by OGD/R has also been reported [39]. In our cell model, we only focused on the occurrence of pyroptosis and did not investigate whether or not apoptosis or necroptosis was also involved, which is warranted for further investigation in the future.

In summary, our study demonstrated that microglia and neurons underwent pyroptosis during mimic I/R injury in vitro, which was attributed to the activation of caspase-1/ GSDMD pathway without the involvement of other known pyroptosis pathways. Moreover, the cell pyroptosis occurred in neurons and microglia after I/R could trigger pyroptosis in other adjacent but non-OGD/R cells. Our findings suggest that caspase-1 and GSDMD are important therapeutic targets for cerebral I/R-induced tissue damage and inflammation (supplemental Fig. 6).

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Author Contributions ZFD, KP, YDW and WJL, conceived and designed the experiments. ZFD and QXP performed the experiments and analyzed the data. YDW and WJL contributed reagents/materials/analysis tools. ZFD wrote the first draft of the paper. WJL and YDW contributed to the writing of the paper. ZFD and QXP contributed equally to this work. All authors read and approved the final manuscript.

Data Availability Datasets analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

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