P2RY6 activation aggravates NLRP3-dependent microglial pyroptosis via down-regulation of the PI3K/AKT pathway in a mouse model of intracerebral hemorrhage

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Research Article

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Abstract

Background

Pro-inflammatory signals generated after intracerebral hemorrhage (ICH) trigger a form of regulated cell death known as pyroptosis in microglia. Although the p2Y purinoceptor 6 (P2RY6) plays a significant role in control of inflammatory reactions in central nervous system diseases, its roles in the development of neuroinflammation and microglial pyroptosis following ICH remain unclear.

Methods

Type IV collagenase was injected to induce ICH. Mice were then treated with MRS2578 and LY294002 to inhibit P2RY6 and phosphatidylinositol 3-kinase (PI3K), respectively. Bio-conductivity analysis was performed to examine PI3K/AKT pathway involvement. Immunostaining and quantitative polymerase chain reaction (qPCR) analyses were conducted to examine pyroptosis following P2RY6 inhibitor treatment. Western blot and immunostaining were performed to clarify the specific mechanisms underlying microglia pyroptosis and white matter injury (WMI). A modified Garcia test, corner turning test, and forelimb placement test were used to assess neurobehavioral function.

Results

Increases in the expression of P2RY6 and pyroptosis-related proteins were observed after ICH, peaking 24 h post ICH. P2RY6 was only expressed on microglia. Administration of MRS2578, a specific inhibitor of P2RY6, attenuated short-term neurobehavioral deficits and WMI while decreasing both microglia pyroptosis and cerebral edema. These changes were attended by decreases in pyroptosis-relatived proteins and pro-inflammatory cytokines. Bioinformatic analysis revealed an association between the PI3K/AKT pathway and P2RY6-mediated neuroinflammation. The effects of MRS2578 were partially reversed by treatment with LY294002, a specific PI3K inhibitor.

Conclusion

P2RY6 activation induces microglial pyroptosis, deficits in neurological function and WMI, partially via the P2RY6/PI3K/AKT/NLRP3 pathway. Consequently, targeting P2RY6 might be a promising approach to the therapy of ICH.

Background

Intracerebral hemorrhage (ICH) has been linked to significant morbidity and fatality rates, as well as companies with a poor prognosis after treatment. ICH contributes for approximately 15%-20% of all strokes and is more common in Asian populations compared with Western populations and there are no
Effective treatments for preventing further deterioration of neurological function [1]. Although neuroinflammation is widely believed to play a critical part in much pathophysiology of ICH, the specific molecular processes involved remain largely unclear [2].

Pyroptosis is an inflammation-inducing cell death process that has been closely associated with neuroinflammation after ICH [3]. Pyroptosis is characterized by cell enlargement, the formation of inflammatory and other toxic substances, and the perforation and rupture of the plasma membrane [4]. In addition, cellular pyroptosis serves a critical part in the progression of brain damage after ICH [5]. Suppression of pyroptosis might reduce secondary damage after ICH.

ICH-induced pyroptosis is caused by activated caspase-1 cleaving Gasdermin D (GSDMD) and producing a N-fragment that perforates the plasma membrane [3–6]. Immature inflammatory cytokines are cleaved simultaneously by functional caspase-1 to produce functional IL-1β and IL-18. During subsequent pyroptosis, cell membrane rupture leads to the spilling of cellular contents including inflammatory cytokines, producing an exaggerated inflammatory response [5]. Several investigations have shown that pyroptosis is an important factor in numerous acute diseases of the CNS, such as trauma-induced brain injury, cerebral ischemia, and spinal injury [7–9]. GSDMD is also significant in the neuroinflammation caused by pyroptosis; hence, regulation of GSDMD-mediated pyroptosis may attenuate neuroinflammation and secondary cerebral damage [10].

In recent years, white matter damage (WMI) has received increasing attention as poor prognosis in patients with ICH [11]. Hemorrhage frequently occurs in the nerve fiber-rich internal capsule and basal ganglia, where hemolysis products activate microglia and produce inflammatory factors that directly induce cell death and aggravate the severity of WMI [12]. Thus, reducing neuroinflammation may help to counter WMI and promote WM repair following ICH [13].

Microglia are considered a sentinel cell because of their ability to detect and respond to disturbances in homeostasis within the central nervous system [14]. Research has identified activation of microglia pyroptosis as a critical mechanism that can exacerbate neuroinflammation and WMI [7, 15]. A microglial pyroptosis-driven inflammatory response regulates the pathophysiology of secondary brain damage after ICH, suggesting that limiting the microglial pyroptosis can release inflammation and reduce brain injury after ICH [16].

The p2Y purinoceptor 6 (P2RY6), a member of the P2Y purinoceptor family, widespread distribution across the nervous system and plays key roles in numerous pathological processes, including immunological and inflammatory processes, as well as phagocytosis by microglia and macrophages [17–19]. When this receptor is activated, UDP, an endogenous ligand of P2RY6, stimulates the synthesis and secretion of several proinflammatory cytokines [20]. Leakage of endogenous UDP has been observed from injured cells in the areas of damage in patients with Parkinson's disease, resulting in aggravation of the microglia-mediated inflammatory response [21]. In an experimental mouse model of cerebral ischemia, inhibition of P2RY6 has been shown to worsen neurological impairments and brain damage by inhibiting microglial phagocytosis rather than inflammation [22]. Moreover, P2RY6 modulates the
JAK2/STAT3 signaling pathway, which promotes microglial activation and neuroinflammation in a model of chronic sciatic nerve crush injury [23].

Phosphatidylinositol 3-kinase, also known as PI3K, is a lipid kinase that mediates downstream activation of AKT, which has been described as a "second messenger" for cell growth and survival [24]. Previous research has demonstrated the anti-neuroinflammatory, antioxidative, and anti-apoptotic characteristics of neuronal PI3K/AKT signaling [25, 26]. Although current research indicates that PI3K/AKT signaling plays a role in inflamasome-mediated pyroptosis and WMI [27, 28], the potential therapeutic effects of inhibiting P2RY6 activity following ICH remain unclear. In our research, we uncovered that blocking P2RY6 has a neuroprotective benefit on microglial pyroptosis, as well as factors potentially mediating this relationship.

Materials and Methods

Animals

All mice were maintained at the Zhujiang Hospital Animal Centre, Southern Medical University, and consisted of 245 male C57BL/6 mice (weighing approximately 25 to 30 g) acquired from Sja Biotechnology (Guangdong, China). All mice are kept in a controlled setting that mimics their natural circadian rhythm, with a regular light cycle and a constant food and water. All procedures are in line with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. The Zhujiang Hospital, Southern Medical University, Institutional Ethics Committee approved all experimental operations (Approval No. LAEC-2022-036).

Experimental Design

To thoroughly evaluate whether P2RY6 inhibition exerts neuroprotective effects against microglial pyroptosis, we conducted a series of five experiments using an experimental ICH mouse model (Fig. 1).

Experiment 1

Eighteen mice were allocated at random into six groups of three each, including a sham-operated group and groups examined 3h, 6h, 12h, 24h, and 72h following ICH. Changes in P2RY6, NLRP3, caspase-1, ASC, and GSDMD expression were evaluated based on the results of western blotting. Six additional mice, average distribution in sham-operated and ICH 24h group, had their brains examined using a double immunofluorescence approach to determine the cellular expression of P2RY6.

Experiment 2

Eighty mice were allocated at random into five groups of sixteen each: sham-operated group, ICH + vehicle (DMSO), ICH + MRS2578 at 50, 150, and 450 micrograms per kilogram, respectively. Different drug concentrations of MRS2578 were intraperitoneally injected 30 min after ICH induction. Forty mice, 8 mice selected at random from each group, were assessed by neurological scores (forelimb placement test,
modified Garcia test and turning test) and brain edema, respectively. The most efficient dose of MRS2578 was picked for use in additional experiments based on these results. Nine additional mice were allocated at random into three groups of three each: sham-operated group, ICH + vehicle (DMSO) and ICH + MRS2578 (150 µg/kg). Hematoma volumes ought to be evaluated at 24 h following cerebral hemorrhage.

**Experiment 3**

In order to investigate the roles of P2Y6R post-ICH on microglial pyroptosis, neuroinflammatory response, and WMI. Twenty-seven mice were allocated at random into three groups of nine each: sham-operated group, ICH + Vehicle (DMSO), and ICH + MRS 2578 (150 µg/kg). After 24 h, immunofluorescence approach, WB and qPCR were applied on sections of the injury brain.

**Experiment 4**

In order to explore the influence that the PI3K/AKT signaling pathway has on microglial pyroptosis, the neuroinflammatory response, and WMI after ICH. Forty-eight mice were allocated at random into four groups of twelve each: sham-operated group, ICH + Vehicle (DMSO), ICH + MRS2578, and ICH + MRS2578 + LY294002 (2 µg/µl). Neurological function scores and brain edema ought to be evaluated respectively for the section of injury brain at 24 h after cerebral hemorrhage. Twelve mice, grouped as above, HE ought to be evaluated for peripheral tissue condition and cellular morphology of the injury brain at 24 h after cerebral hemorrhage.

**Experiment 5**

To further investigate the potential mechanisms by which P2RY6 activation is associated with microglial pyroptosis and WMI after ICH, mice were allocated at random into four groups of three each: sham-operated group, ICH + Vehicle (DMSO), ICH + MRS2578 (150 µg/kg), and ICH + MRS2578 + LY294002 (2 µg/µl). At 24 h after cerebral hemorrhage, we performed western blotting, immunofluorescence staining and ELISA on sections of the injured cerebral hemisphere.

**Induction of the intracerebral hemorrhage mouse model**

The experimental mouse model of ICH was induced using type IV collagenase (Sigma, USA), as previously described [29]. Healthy male C57BL/6 mice weighing about 25 to 35 g received an intraabdominal injection of 1.25% tribromoethanol aspirated with a 5 ml syringe, and anesthesia was gradually induced. Following attachment of the microinjector to the stereotactic apparatus, the fontanelle was positioned, the scale of each axis of the stereotactic apparatus was recorded, and the puncture point was made 1 mm anterior 3.0 mm to the right of the fontanelle (depending on the weight of the mouse). The skull drill was centered on the puncture point and carefully honed to create a 1.0 mm-diameter bone window. A microsyringe was used to withdraw 1 µl of collagenase IV (0.04 U). The tip of the injection needle was adjusted in relation to the dura mater, and penetration was initiated 3 mm vertically, although this varied according to mouse weight.

**Neurobehavioral function tests**
Neurobehavioral scores was assessed by two researchers who have no knowledge of the experimental settings. Twenty-four hours following ICH induction, neurobehavioral function was evaluated using a modified Garcia test and turning test [30], which consists of assessments of the following seven sections: autonomous movement, axial feeling, tactility, symmetry of the limbs, lateral rotation, forelimb moving and gripping capability. Scores for per test ranged from 0 to 3 or 1 to 3, with total possible scores ranging from 3 to 21. The higher the scores, the more it was thought to indicate a higher level of neurological function. During turning test, mouse subjects in the turn test are free to make a left or right turn as they try to escape as they approach a rotation angle of 30 degrees. Each mouse underwent 10 trials, with at least 30 s of rest between trials. In the forelimb placement test, the experimenter gripped each mouse by the trunk, permitting the forelimbs to dangle autonomously. Ten separate trials were performed with each front limb, as well as the proportion of tests in which the mice correctly put their limb on the surface in response to tactile stimulation was recorded.

Assessment of cerebral edema

Edema in the brain was assessed by weighing brain water content 24 hours following cerebral hemorrhage, as previously described [30]. The injury brain ought to be dissected into 4 millimetres coronal sections, then segmented into sound sides and affected sides of basal ganglia and cerebral cortex. Control group was provided by the cerebellum. WW was determined by instantly weighing samples, after that samples were dried for 48 hours at 100°C to get the DW. The ratio of water content in the brain is calculated by dividing the DW by the WW multiplied by 100%.

Western blot analysis

In accordance with a previously described method [7], deep sedation was administered to the mice and intracardially injected with 60 milliliters of frozen saline. Then, the injury tissue was gathered and frozen at -80 degrees for usage. RIPA lysis buffer (CW bio, China) was used to lysis prepared brain tissues, then Centrifuge, adding protein loading buffer and boiling 10min. The prepared protein were added to SDS-PAGE gels and electrophoresed. Following electrophoresis, the samples were transferred to PVDF membranes and complete immersion of the membrane in 5% bovine serum albumin solution for 2 h, and then hatched at 4°C for 15 to 18 hours with the corresponding antibody. The primary antibodies consist of anti P2RY6 (Affinity Biosciences, USA), ASC (Proteintech, USA), NLRP3 (Abcam, USA), GSDMD (Abmart, USA), caspase-1 (Abmart, USA), PI3K/p-PI3K (Abcam, USA), AKT/p-AKT (Abcam, USA), MBP (Novus, USA), NF200 (Sigma, USA), of which dilution ratio was 1:2000. Selecting the corresponding secondary antibody at the appropriate dilution ratio and incubation the sample for 1 to 2 hours. These bands were visualized using NineAlliance imaging system (UVitec, UK) and probed with an Immobilon Western HRP Substrate (Millipore, USA). Image J was applied to analyze immunoblots for differences in protein density.

Immunofluorescence staining

As previously described [29], while under general anesthesia, mice were given an intracardial injection of pre-cold PBS and then 60 milliliters of 4% paraformaldehyde. After removing the injury brain, it was
immersed twice in 30% sucrose at 4°C for 48 hours. Brain specimens were obtained, frozen at −80°C, and sectioned into 10 micrometres coronal slices with the cryostat (Leica, Germany). For immunofluorescence staining, cerebral slices were stained by the following primary antibodies: anti-Iba-1 (Servicebio, China), GFAP (Proteintech, China), NeuN (Abcam, USA), MBP (Novus, USA), and NF200 (Sigma, USA), of which dilution ratio was 1:300. Subsequently, the tissue samples were incubated for 15–18 hours at 4°C, following which two hours at 37 degrees Celsius with the corresponding secondary antibodies (1:500, Thermo Fisher Scientific, USA). The cells were stained with DAPI (Solarbio, China). Nikon fluorescence microscope (Nikon-Ti2E, Japan) was used to visualize and photograph images. The micrographs were analyzed with Image J.

**Quantitative polymerase chain reaction (qPCR) analyses**

Following decapitation under deep anesthesia, an area around the perihematomal tissue weighing approximately 20 mg was gathered, then isolating total RNA from the injury brain using the AG RNAex Pro RNA Reagent (Accurate Biotechnology, China). A reverse transcription kit, SYBR Green Pro Taq HS, was used to extract genomic DNA (gDNA) from the RNA samples and synthesize complementary DNA (cDNA). RT-PCR was performed in a Bio-Rad sequence detection system. GAPDH served as the internal control.

**ELISA**

Tissue protein content was measured using Mouse DuoSet ELISA kits for IL-1β and IL-18 (Cusabio, China). Mice were under anesthetized, and the left ventricle was perfused with pre-cooled normal saline at 24h following ICH. Homogenized tissue proteins from the animals used in the experiment were treated with a protein phosphatase inhibitor-containing lysis solution (100 ml of lysis buffer per 10 mg of tissue homogenate), and concentrations of interleukin-1β and interleukin-18 were tested without diluting the supernatant.

**Statistical analysis**

The mean and standard deviation (SD) are shown for all the data. SPSS was used for all statistical analyses. All statistics were first examined the normality with Shapiro–Wilk test. One-way analyses of variance (ANOVA) and least significance difference (LSD) were used to compare data that was normally distributed over a number of different groups. Kruskal-Wallis one-way ANOVA was used to examine non-normally distributed data, and LSD post hoc tests were applied for necessary further comparisons. P values less than 0.05 was regarded as statistically significant.

**Results**

**Mortality in experimental animal**

The mortality rate for all experimental mouse was 3.67% (9/245). In this investigation, no mice that underwent sham operations died. The death rates between each groups in the trial were not significantly
different. Exhibiting ICH models could see obvious haematoma. No statistically significant variations in the absence of side effects were seen across the therapy groups.

The increased trend of P2RY6 expression in brain after ICH

To explore P2RY6 alterations in brain tissue, we examined changes in the expression levels of P2RY6 protein in damage tissue at different time points following cerebral hemorrhage. Our analysis indicated that compared to the sham group P2RY6 expression obviously increased in the ICH group. This trend was most noticeable 24 h after ICH and diminished gradually thereafter (Fig. 2A). Immunofluorescence staining uncovered that P2RY6 was only localization in microglia but not in astrocytes or neurons (Fig. 2B).

Effect of P2RY6 inhibition on neurobehavioral deficits, hematoma size and cerebral edema after ICH

In order to establish an optimal dose required to inhibit P2RY6, MRS2578 was administered at doses of 50, 150, and 450 µg/kg. ICH + MRS2578 was compared to ICH + vehicle in terms of neurofunction. In corner turning test, the modified Garcia test and forelimb placement test, the scores of ICH + MRS2578 group were higher compared to ICH with vehicle group(Fig. 3A). While significant improvements in neurological function and brain water content were observed relative to sham-operated group at all doses, the most notable reductions in ICH-induced neurological damage, cerebral edema were MRS2578 (Fig. 3A, B). In ICH + MRS2578 group, hematoma size was smaller than cerebral hemorrhage group (Fig. 3C). In summary, the best dose of MRS2578 was 150µg/kg and apply this dose for subsequent experiments.

Effect of P2RY6 Inhibition on Microglia Activation and neuroinflammation following ICH

To explore the effects of P2RY6 inhibition, activated microglia was marked with Iba-1 staining, while neutrophil expression was tracked by MPO labeling. we observed Iba-1-positive cells obviously growing around perihematomal tissue, although this change was attenuated following MRS2578 administration (Fig. 4A). Meanwhile, the MPO-positive cells around the damage cerebral hemisphere also increased considerably after ICH, while P2RY6 inhibition via administration of MRS2578 significantly reversed this tendency (Fig. 4B).

Effect of P2RY6 Inhibition on microglial pyroptosis following ICH

The results of qPCR analysis demonstrated the quantites of P2RY6 mRNA expression rose significantly at 24 hours post-ICH, concurrent with the upregulation of pyroptosis-related protein and pro-inflammatory factors mRNA quantites of ASC, NLRP3, caspase-1,IL-1β and IL-18(Fig. 5A). Western blot analyses show
that changes in P2RY6, pyroptosis-related proteins and pro-inflammatory factors expression consistent with mRNA trend. However, P2RY6 inhibition downregulated the protein expression and mRNA of pyroptosis-associated and pro-inflammatory factors (Fig. 5B, C). Since GSDMD is the executor of pyroptosis[10], we should focus on its changing trends. Surprisingly, trends of bands and relative densities between the groups were consistent with P2RY6, pyroptosis-related proteins and inflammatory cytokines (Fig. 5D, E).

**Effect of P2RY6 inhibition on WMI after ICH**

The western blot bands and related density results show that MBP and NF200 protein expression both decreased around the hematoma area compared with sham group 24h after ICH, while following MRS2578 treatment, bands and relative density of MBP and NF200 increased around the haematoma (Fig. 6A, B). The corresponding results for images and mean fluorescence intensity suggested that MRS2578 treatment attenuated in MBP and NF200 mean fluorescence intensity while reducing the extent of WMI (Fig. 6C, D).

**Analyses of differentially expressed genes and enrichment of biological pathways based on preliminary screening**

Primary microglia were cultured in a medium containing serum, and microglia were divided into two groups: uLPS (10 µg/mL) vs. uLPS (10 µg/mL) + MRS2578 (5 µM). The miRNA and expression profile dataset (GSE195866) was downloaded from the GEO database, and the downloaded data were analyzed using R 4.0.4 software to filter out differentially expressed genes (Fig. 7A). Differential mRNA expression and pathway enrichment were determined based on analyses of pathway enrichment was performed using data from KEGG, and the P-value set to < 0.05. This analysis revealed significant enrichment of the PI3K/AKT pathway, JAK/STAT3 pathway, chemokine receptor pathway, and inflammatory bowel disease pathway. Notably, PI3K/AKT signalling pathway had the greatest number of genes enrichment with varying expression levels (Fig. 7B).

**Effects of inhibition PI3K on neurological function and brain damage following MRS2578 treatment**

To explore the inhibition of P2RY6 has a preventive impact against microglial pyroptosis, which is linked with PI3K/AKT signalling pathway, we blocked PI3K via LY294002 before MRS2578 treatment in our mouse model of ICH. Improvements in neurological function and brain water content induced by administration of MRS2578 were partly reversed by the administration of LY294002 (Fig. 8A, B). Hematoxylin and eosin (HE) staining suggested that MRS2578 improved the morphological integrity of perihematomal cells, while LY294002 reversed this phenomenon (Fig. 8C).
Potential mechanisms underlying the protective effect of P2RY6 inhibition

To further explore the molecular mechanisms of the benefits of P2RY6 inhibition, western blot results show that expressions of PI3K and AKT increased after MRS2578 treatment compared to ICH + Vehicle. In addition, bands and relative densities of caspase-1, ASC, GSDMD, and NLRP3 were largely grew up in ICH + MRS2578 + LY294002 group (Fig. 9A, B). ELISA analyses suggested MRS2578 treatment reduced levels of inflammatory indicators IL-1β and IL-18; however, treatment with LY294002 reversed this effect (Fig. 9C, D). Quantitative immunofluorescence experiments indicated that levels of MPO and GSDMD expression were lower in cerebral hemorrhage group than in ICH with MRS2578 treatment group, although compared to ICH with MRS2578 treatment group, the ICH + MRS2578 + LY294002 group showed significantly increased MPO expression. These results suggests that administration of LY294002 counteracted the protective effect of MRS2578 (Fig. 9E, F).

PI3K/AKT signalling pathway in P2RY6 induced WMI following ICH

To investigate whether the effects of P2RY6 inhibition on WMI are dependent on the PI3K/AKT pathway, we inhibited PI3K activation after MRS2578 treatment with LY294002. The western blot bands and related density results show that while decreases in MBP and NF200 protein expression were observed following ICH, levels of both proteins increased following MRS2578 treatment; however, the levels of MBP and MF200 protein both reduced in e cerebral hemorrhage with MRS2578 and LY294002 treatment group (Fig. 10A, B). Results for immunofluorescence images and mean fluorescence intensity suggested that MBP and NF200 were in line with trends in the above-mentioned expression of proteins (Fig. 10C, D).

Discussion

In the current research, we clarified the role of P2RY6 in microglial pyroptosis with an experimental mouse model of ICH. We also explored a crucial role for P2RY6 in reducing neuroinflammation and WMI and demonstrated that PI3K/AKT signalling pathway is concerned with this procedure. First, our results illustrated that P2RY6 expression were gradually growing following cerebral hemorrhage, peaking at 24h, and was only located in microglia. Notably, treatment with the P2RY6 specific inhibitor MRS2578 improved neurological deficits and cerebral edema after ICH. Second, inhibition P2RY6 reduced pyroptosis-related protein expression, inflammatory factor release, and neutrophil infiltration. Third, treatment with P2RY6 antagonists attenuated ICH-induced WMI. In addition, blocking PI3K abolished the protective roles of MRS2578 on neurological function and WM integrity while exacerbating microglial pyroptosis. These results suggest that P2RY6 is essential for controlling WMI and neuroinflammation and that P2RY6 may be an effective target for enhancing neurological function after ICH (Fig. 11).
According to a growing number of research, in the pathophysiology of brain damage that results from cerebral hemorrhage, neuroinflammation is an essential factor [2, 31, 32]. The neuroinflammatory response in the CNS is distinguished by a complicated immune–inflammatory cascade involving several cell types and cellular connections [33]. Microglial activation is the first step in the inflammatory response, which is then amplified by neutrophil infiltration and subsequent disruption of the integrity of normal cellular forms and functions [8, 34]. Many researches have shown that suppressing neuroinflammatory responses after ICH can prevent brain damage and improve neurological function [31, 35, 36].

However, the current study results show that ICH activates P2RY6 in brain tissue, which is abundantly expressed on microglia [17, 19]. Collagenase triggers cell death 3–6 hours after ICH, and any form of cell injury is followed by a substantial release of UDP-rich energy material [37]. Purinergic signaling is pivotal to many progressions in wide of pathological conditions, including immune cell recruitment, inflammation, and neurotransmission [20, 37, 38]. As mentioned previously, P2RY6 is involved in the pathophysiology of many neurological diseases and has a variety of different consequences. In the context of TMCAO, P2RY6 inhibition worsens neurological function by inhibiting microglial phagocytosis, rather than by promoting neuroinflammation. However, neuroinflammation aggravates neurological damage following ischemic or hemorrhagic stroke [34, 39–41], despite reports of some differences according to stroke type. Ischemic stroke is primarily characterized by neuronal apoptosis, whereas hemorrhagic stroke results in demyelination of nerve fibers [26, 42–44]. In Parkinson disease and neuropathic pain, inhibition of P2RY6 has been shown to attenuate microglia-induced neuroinflammation [20–22]. However, little is known regarding the relationships among P2RY6, microglial pyroptosis-mediated neuroinflammation, and WMI in the context of ICH; thus, we investigated these functions in an experimental brain hemorrhage model.

In our research, an upward trend in P2RY6 protein expression was obviously detected around haematoma as early as 6 hours following cerebral hemorrhage, with expression peaking at 24 h. These results shown that P2RY6 performs a dominant function within the early phases of brain damage. P2RY6 expression in the CNS is restricted to microglia, suggesting its involvement in inflammatory processes [45]. Consistent with prior research, our immunofluorescence experiments identified strong expression of P2RY6 in the microglia of the perihematomal tissue. Hence, we came up with the hypothesis that P2RY6 is an essential component in the overall neuroinflammatory response after ICH. Pyroptosis, which is defined as Gasdermin-mediated cell death, is frequently followed by the rupture of the cytoplasmic membrane into pores and generates inflammatory chemicals, which results in a cascade response that exacerbates inflammation [3, 46]. The inflammasome NLRP3 is responsible for the typical pyroptosis that boosts cell necrosis by activation NLRP3-inflammasome and pro-inflammatory cytokine [41, 47, 48]. The cleaved GSDMD generates a gap in the cytoplasmic membrane, allowing intracellular inflammatory substances to drain, thereby aggravating ICH-induced neuroinflammation [49]. Pyroptosis could induce cells to produce large amounts of inflammatory factors, which trigger an inflammatory response [50, 51]. The findings of our investigation provide the first proof that administering P2RY6 inhibitors after ICH decreases amount of microglia and macrophages, as well as the expression of proteins that are
increased during pyroptosis. According to these findings, P2RY6 inhibition attenuates the pyroptotic effects of microglia after ICH.

Hemorrhagic ICH is common in brain white matter, such as the basal ganglia, which are densely packed with white matter fibers [13, 43, 44]. Consequently, motor dysfunction caused by WMI is among the most significant consequences of ICH [52]. Therefore, we investigated the effects of P2RY6 inhibition on WMI. Initially, we discovered that cerebral hemorrhage results in aggravation WMI. In addition, we uncovered that inhibiting P2RY6 with MRS2578 dramatically reduced WM damage after ICH. By assessing cerebral hemorrhage prognosis in patients, WMI is a pivotal element [43, 53]. According to previous research, neuroinflammation is another major contributor to the aggravation of WMI after ICH [11, 54], indicating that targeting microglia may represent an effective treatment for both neuroinflammation and WMI resulting from ICH.

Subsequently, we investigated the potential mechanisms and signaling pathways associated with P2RY6-mediated microglial pyroptosis and WMI. In the GEO database, we identified two sets of in vitro samples of BV2 microglia in a model of LPS-induced inflammation, one of which was sequenced after adding 5 µM MRS2578. After screening for differentially expressed genes, these genes were enriched using pathways from the KEGG database to search for differential pathways. The PI3K/AKT pathway was highly enriched among the differentially expressed genes.

In the current study, endogenous PI3K/AKT signaling pathway expression was downregulated in our experimental mouse model of ICH, suggesting that such process is closely related to the progression of cerebral damage following ICH [55, 56]. To further understand the connection between the neuroprotective effects of P2RY6 and PI3K/AKT signaling, we suppress the activity upon the endogenous PI3K/AKT signaling pathway using the PI3K inhibitor LY294002. According to our findings, MRS2578 significantly suppressed the effects of P2RY6 on microglial pyroptosis and WMI while decreasing the expression of MBP and NF200. Additionally, LY294002 partially reversed the effect of MRS2578 on neurological prognosis. These results further suggest that the involvement of P2RY6 in charge of microglial activation and WMI were via the PI3K/AKT pathway after ICH. Ultimately, our results verify that P2RY6 enhances post-ICH neuroinflammation and WMI.

This study had several limitations. First, although P2RY6 exerts various biological functions [57], we focused solely on its anti-pyroptotic effects and its role in WMI following ICH. However, microglial P2Y6R may interact with other potent receptors implicated in the immunological and inflammatory response after ICH, and more study is necessary to uncover other neuroprotective roles of P2RY6 in the context of post-ICH brain injury, as well as potentially relevant signaling mechanisms. Second, our experiments were conducted in male mice alone, necessitating further studies of P2RY6 involvement in female mice [58, 59]. Third, age plays a significant role in predicting functional outcomes for patients with numerous diseases [60, 61]. Unfortunately, in this research, we just only investigated the function of P2RY6 in young mice models of ICH. Hence, additional experiment is required to investigate the probable pathways by which P2RY6 exerts effects in older mouse models of ICH. Future studies should also aim to examine the
contribution of microglial polarization after ICH, as well as the long-term efficacy and probable molecular mechanisms by which treatment with the P2RY6 inhibitor MRS2578 may attenuate brain damage following ICH.

**Conclusion**

The present results demonstrate that P2RY6 worsens neuroinflammation and WMI by downregulating the expression of the PI3K/AKT signalling pathway in experimental intracerebral hemorrhage in mice, partly by increasing microglial pyroptosis. Our results also suggest that inhibition of P2RY6 improves neurological function, meaning that this may be a promising strategy for minimizing neuroinflammatory reactions and WMI after ICH.

**Declarations**

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**Authors’ contributions**

This work was conducted with the cooperation of the authors. QHW conceived and jointly designed the study with FL, LYL, and HRT. LYL, HRT, and SFZ conducted this study and wrote the manuscript; SFZ, ZQD, GWW, JFP, DYL and LBH conducted data acquisition and data analysis; LYL, HRT, SFZ, ZQD, GWW, JFP, DYL, LBH, FL and QHW revised the manuscript. All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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**Availability of data and materials**

All of the data used in the study are available from the corresponding author upon reasonable request.

**Ethics approval and consent to participate**

All animal studies were approved by the Ethics Committee of Zhujiang Hospital of Southern Medical University.

**Consent for publication**

Not applicable
Competing interests

The authors declare no conflicts of interest.

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References


14. Fatoba O, Ito Kazu T, Yamashita T. **Microglia as therapeutic target in central nervous system disorders.** *Journal of Pharmacological Sciences* 2020, **144**:102-118.


17. Anwar S, Pons V, Rivest S. **Microglia Purinoceptor P2Y6: An Emerging Therapeutic Target in CNS Diseases.** *Cells* 2020, **9**.


19. Liu GD, Ding JQ, Xiao Q, Chen SD. **P2Y6 receptor and immunoinflammation.** *Neurosci Bull* 2009, **25**:161-164.


24. Hawkins PT, Stephens LR. **PI3K signalling in inflammation.** *Biochim Biophys Acta* 2015, **1851**:882-897.


34. Ma F, Sun P, Zhang X, Hamblin MH, Yin KJ. Endothelium-targeted deletion of the miR-15a/16-1 cluster ameliorates blood-brain barrier dysfunction in ischemic stroke. *Sci Signal* 2020, **13**.


42. Tuo QZ, Zhang ST, Lei P. **Mechanisms of neuronal cell death in ischemic stroke and their therapeutic implications.** *Med Res Rev* 2022, **42**:259-305.


44. Li J, Xiao L, He D, Luo Y, Sun H. **Mechanism of White Matter Injury and Promising Therapeutic Strategies of MSCs After Intracerebral Hemorrhage.** *Front Aging Neurosci* 2021, **13**:632054.


51. Frank D, Vince JE. **Pyroptosis versus necroptosis: similarities, differences, and crosstalk.** *Cell Death Differ* 2019, **26**:99-114.


57. Illes P, Rubini P, Ulrich H, Zhao Y, Tang Y. **Regulation of Microglial Functions by Purinergic Mechanisms in the Healthy and Diseased CNS.** *Cells* 2020, **9**.


60. Gong Y, Hua Y, Keep RF, Hoff JT, Xi G. **Intracerebral hemorrhage: effects of aging on brain edema and neurological deficits.** *Stroke* 2004, 35:2571-2575.


**Abbreviations**

P2RY6: p2Y purinoreceptor 6; WMI: white matter injury; GSDMD: Gasdermin D; IL: interleukin; ICH: intracerebral hemorrhage; CNS: central nervous system; ELISA: enzyme-linked immunosorbent assay; WW: wet weight; DW: dry weight; KEGG: Kyoto Encyclopedia of Genes and Genomes; DMSO: dimethyl sulfoxide; WB: western blot; HE: hematoxylin and eosin; MBP: myelin basic protein; NF-200: neurofilament-200; Iba-1: ionized calcium binding adaptor molecule; GFAP: glial fibrillary acidic protein; NeuN: neuronal specific nuclear protein; qPCR: quantitative polymerase chain reaction; PI3K: phosphatidylinositol 3-kinase; UDP: uridine diphosphate; GAPDH: glycer-aldehyde-3-phosphate dehydrogenase.

**Figures**
Figure 1

Methodology and animal groupings for experiments.

I.p., intraperitoneal. I.c.v., intracerebral ventricular.
Figure 2

The expression of the P2RY6 protein at various time points and cellular location following ICH. (A) Typical bands of western blot and related density analyses of P2RY6 protein levels around damage brain tissue following ICH. n = 3 for each group. \*P < 0.05 vs. sham. (B) Typical double immunofluorescence experiments revealed that P2RY6 co-react on the microglial marker Iba-1 but not with the astrocytic marker GFAP and the neuronal marker NeuN, n = 3 for each group. Scale bar = 50 μm.
Figure 3

The P2RY6 inhibitor MRS2578 exerts neuroprotective effects following ICH. (A) The modified Garcia test, corner turning test, forelimb placement test were accessed to evaluate neurological function under different MRS2578 dose. (B) Brain edema at 24 hours post-ICH was evaluated by measuring the content of water in the brain. (C) Values are shown for the ipsilateral basal ganglia (IPSI-BG), contralateral basal ganglia (contra-BG), ipsilateral cortex (IPSI-CX), contralateral cortex (contra-CX), and cerebellum. n = 8 for each group. *P < 0.05 vs. sham; #P < 0.05 vs ICH + Vehicle.
Inhibition P2RY6 releases the activation of microglia and peripheral inflammatory factors. (A and B) With the MRS2578 treatment following ICH, the immunofluorescence pictures and analysis results show that MPO and Iba-1 with the quantification positive cells of P2RY6 Iba-1 and MPO. n = 3 for each group. Scale bar = 50 μm.*P < 0.05 vs. sham; #P < 0.05 vs ICH + Vehicle.
Figure 5

Inhibition P2RY6 attenuates microglial pyroptosis and its inflammatory proteins expression. (A) Quantification of P2RY6, ASC, NLRP3, caspase-1, IL-18, and IL-1β mRNA relative levels. (B and C) P2RY6, ASC, caspase-1, NLRP3, MPO, IL-18 and IL-1β with western blot bands and relative densities. (D and E) The western blot bands and relative density of GSDMD. n = 3 for each group. *P < 0.05 vs. sham; #P < 0.05 vs. ICH + Vehicle.
Figure 6

Inhibiting P2RY6 attenuates white matter injury following ICH. (A) Bands of western blot and relative density show the protein level of MBP and NF200 in the hematoma region. (B) Representative immunofluorescence images and mean fluorescence density in double immunofluorescence staining experiments for NF200 and MBP in the perihematomal region at 24 h after ICH. Scale bar = 50 μm. All groups above n = 3 for each group and *P<0.05 vs. sham, #P < 0.05 vs. ICH + Vehicle.
Volcano mapping and pathway analysis of differentially expressed miRNAs. (A) volcano map of miRNAs with varied levels of expression. Significantly upregulated and downregulated genes are represented by red and blue dots, respectively. Grey dots indicate non-differentially expressed genes. (B) Map of KEGG pathway enrichment analysis.

Figure 7
Figure 8

Effects of inhibition PI3K on neurological function and brain damage following MRS2578 treatment. (A) Scoring results in the modified Garcia test, left forelimb placement test and left turning test. (B) Between the different groups of brain water content. All groups above $n = 6$ for each group and *$P < 0.05$ vs. sham, # $P < 0.05$ vs. ICH + Vehicle, @ $P < 0.05$ vs. ICH + MRS2578. (C) HE were applied to assess different groups of histocyte morphology around the haematoma. $n = 3$ for each group.
Figure 9

Effects of inhibition PI3K on microglial pyroptosis and pro-inflammatory factors after MRS2578 treatment. (A) Representative bands of western blotting. (B) Relative density results of PI3K/p-PI3K, AKT/p-AKT, NLRP3, ASC, N-GSDMD, C-caspase-1, MPO and TNF-α. (C and D) Levels of IL-18 and IL-1β expression around cerebral haematomas measured by ELISA. (E and F) Immunofluorescence images for MPO and GSDMD, as well as quantitative assessments of MPO-positive and GSDMD-positive cells. Scale bar = 50 μm. All groups above n = 3 for each group and *P<0.05 vs. sham, #P < 0.05 vs. ICH + Vehicle, @P < 0.05 vs. ICH + MRS2578. For proteins with the same molecular weights such as PI3K/p-PI3K and AKT/p-AKT, following western blot membrane stripping, we reused the identical blots for the restaining process.
**Figure 10**

Inhibition of P2RY6 exerted protective effects against WMI via PI3K/AKT pathway. (A and B) Representative bands of western blotting results show the protein expression bands and relative density of MBP and NF200. (C and D) Immunofluorescence images and mean fluorescence intensity of four groups. Scale bar = 50 μm. All groups above n = 6 for each group and *P<0.05 vs. sham, #P < 0.05 vs. ICH + Vehicle, @P < 0.05 vs. ICH + MRS2578.
Figure 11

Schematic of potential molecular mechanism. PI3K/AKT signalling pathway, which is responsible for the anti-inflammatory and anti-WMI actions of P2RY6 suppression following ICH.