Original Article
Paeoniflorin down-regulates the expression of NLRP1 and NLRP3 inflammasomes in rat hippocampal slices after oxygen-glucose deprivation

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Abstract: The bioactive glucoside, paeoniflorin, has been shown to have an anti-inflammatory effect on ischemic brain injury. In this study, we aimed to investigate the ability of paeoniflorin to regulate the expression of NLRP1 and NLRP3 inflammasomes in rat hippocampal slices. Different concentrations of paeoniflorin were applied to oxygen-glucose-deprived organotypic hippocampal slice cultures. Neuronal cell death was measured by propidium iodide staining, and the expressions of the components of NLRP1 and NLRP3 inflammasomes and their downstream protein were detected using western blotting and the enzyme-linked immunosorbent assay. Paeoniflorin exhibited a neuroprotective effect against oxygen-glucose deprivation (OGD)-induced neuronal cell death and demonstrated the ability to downregulate the levels of the components of NLRP1 and NLRP3 inflammasomes (NLRP1, NLRP3, ASC, and caspase-1) as well as their downstream proteins (interleukin [IL]-18, IL-1β, and caspase-3) in hippocampal slices. Our findings suggest that paeoniflorin has the ability to attenuate neuronal injury in ischemic stroke by downregulating the expression of NLRP1 and NLRP3 inflammasomes.

Keywords: Lschemic stroke, paeoniflorin, NLRP inflammasome, organotypic hippocampal slice culture, OGD

Introduction

Presently, stroke is the third leading cause of death, globally affecting 33 million individuals and causing 6.2 million deaths each year. Different types of stroke also cause serious long-term disability [1, 2]. Ischemic stroke occurs when the blood flow to the brain is interrupted by a thromboembolism in the cerebral artery. Various processes are involved in the pathophysiology of ischemic stroke, including energy failure, loss of cell ion homeostasis, acidosis, intracellular calcium overload, excitotoxicity induced by excitatory amino acids, free radical-mediated toxicity, the generation of arachidonic acid products, disruption of the blood-brain barrier, infiltration of leukocytes, activation of glial cells, and cytokine-mediated cytotoxicity [3]. Inflammation, which plays a significant role in the ischemic cascade, is characterized by the production and release of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-18, and IL-6 during the activation of neurons, astrocytes,

microglia, and endothelial cells; this process eventually leads to neuronal and glial cell death during ischemic stroke [4]. Emerging evidence suggests that the nucleotide binding and oligomerization domain (NOD)-like receptor (NLR) pyrin domain-containing 1 (NLRP1) and NLRP3 inflammasomes in neurons and glial cells, which act as pattern recognition receptors (PRRs) in the innate immune system, play an important role in detecting cellular damage and mediating inflammatory responses to brain injury during cerebral ischemia [5, 6]. NLRs are composed of three domains: an N-terminal protein-protein interaction domain for signal transduction, a central NACHT domain for oligomerization, and a C-terminal leucine-rich repeat (LRR) that confers ligand recognition. NLRPs are classified according to their N-terminal domains, which contain a pyrin domain (PYD) [7, 8]. The NLRP inflammasomes are large multi-protein complexes composed of the NLRP receptor, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and precursor caspase-1 and/or X-linked
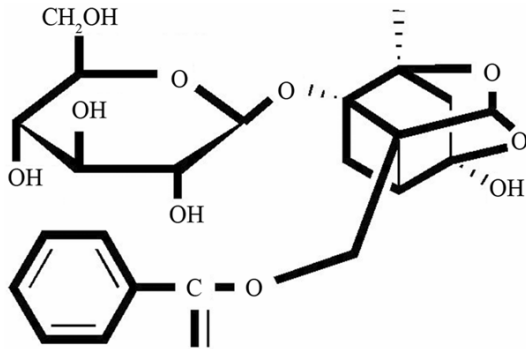
Figure 1. The chemical structure of Paeoniflorin.

inhibitor of apoptosis (XIAP) [9]. The activation and oligomerization of the NLRP1 and NLRP3 receptors result in the formation of the NLRP1 and NLRP3 inflammasomes. This process activates cleavage of precursor caspase-1 into cleaved caspase-1, which cleaves precursor IL-1β and precursor IL-18 into mature pro-inflammatory cytokines. Mature IL-1β and IL-18 can then bind to their respective receptors in an autocrine or paracrine manner. Additionally, cleaved caspase-1 can initiate cell death through apoptosis and pyroptosis [10].

Paeoniflorin (PF) (**Figure 1**), which is a bioactive monoterpene glucoside extracted from the root of *Paeonia lactiflora* Pall., has been reported to exhibit neuroprotective effects, such as anti-inflammatory properties against ischemia-induced brain damage. Previous studies indicate that PF can prevent apoptosis, scavenge free radicals, regulate cerebral energy metabolism, activate adenosine A1 receptor, and facilitate the translocation of protein kinase C, suggesting that these abilities may be related to the neuroprotective effects of PF [11-15]. Therefore, to investigate the mechanisms of the anti-inflammatory effect of PF and its relevance to NLRP1 and NLRP3 inflammasomes in ischemic brain tissue, we slightly modified the protocols of organotypic hippocampal slice culture (OHSC), which were successfully developed [16] to obtain hippocampal slices, and exposed the tissue slices to oxygen-glucose deprivation (OGD) conditions to mimic cerebral damage during ischemic stroke.

## Methods

Animals and materials

Neonatal male Sprague-Dawley (SD) rats were obtained from the Laboratory Animal Center of Fujian University of Traditional Chinese Medicine (Fuzhou, China). The principles of laboratory animal care were followed, and the study was approved by the Ethics Committee of Fujian University of Traditional Chinese Medicine.

Hank's Balanced Salt Solution (HBSS) and minimum essential medium (MEM) were obtained from HyClone. Horse serum was purchased from Gibco. Propidium iodide (PI) fluorescent dye was purchased from Sigma-Aldrich. Enzyme-linked immunosorbent assay (ELISA) kits were used to detect IL-18 and IL-1β levels (Shanghai Westang Bio-tech Co., LTD). The following primary antibodies were used: NLRP1, ASC, caspase-1, and caspase-3 (Abcam), and NLRP3 (Novus Biologicals). Horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from EMAR and Transgen Biotech. PF (HPLC ≥ 98.5%) was purchased from Shanghai Yuanye Co., LTD.

OHSC

The Stopinni method [16] was followed to obtain hippocampal tissue slices. Each animal was decapitated after being washed with 70% medicinal alcohol and the brain was immediately removed and immersed in ice-cold HBSS. The cerebral pia mater was then removed using ophthalmic forceps. Subsequently, the brain was transferred to a pre-cooled buffer tray for placement in a tissue vibratome (Campden Instruments, model 5000 mz), and the tray was filled with ice-cold HBSS. Three intact brain slices (350-μm thickness) were obtained using the vibratome with a frequency and amplitude of blade motion of 50 Hz and 1.0 mm, respectively. The hippocampus in each brain slice was carefully separated using ophthalmic forceps. All slicing and separating procedures were carried out at a temperature of no more than 4°C. After separation, the hippocampal slices were collected and were placed in semi-porous cellulose membrane inserts (Millicell-CM, 0.4 μm, Millipore). The inserts were then placed in a 6-well plate with each well containing 1 ml of pre-warmed culture medium (50% MEM, 25% HBSS, 25% heat-inactivated horse serum supplemented with 25 mM HEPES, 6.5 g/L glucose, 2 mM L-glutamine, and 1% v/v penicillin/streptomycin). The pH of the culture medium was adjusted to 7.2~7.4. The 6-well plates were placed in an incubator at 37°C with a 5% CO_2-enriched atmosphere for 14 days; the culture medium was changed three times a week.
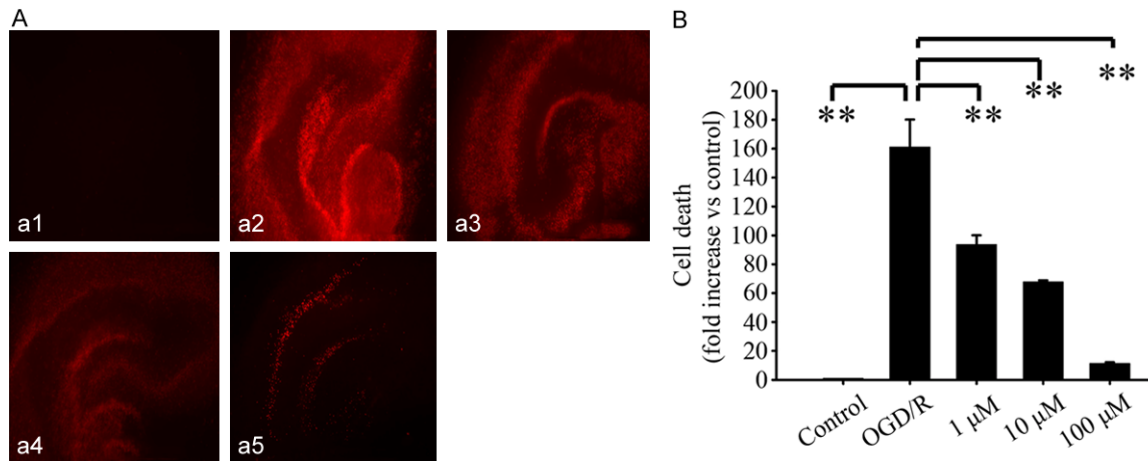
Figure 2. Paeoniflorin relieves cell death in rat hippocampal slices subjected to OGD after PI staining. Organotypic hippocampal slice cultures in the drug treatment groups were treated with or without (OGD group) Paeoniflorin at the concentrations of 1, 10 and 100 μM after being subjected to 45 minutes of OGD; Control group was kept during the same period of time with sufficient supply of normal culture medium containing oxygen and glucose at the absence of Paeoniflorin. After the final incubation, the hippocampal slices were stained by PI and briefly incubated for 20 minutes, images were then taken at magnification of 40× and exposure time of 30 ms and analyzed by using the software ImageJ. A. Illustrates images of PI uptake. B. Represents the semiquantitation of PI images. Data were represented as mean ± SE (n = 6 slices in each group) and normalized with respect to the values of Control group that were considered as 1. **$P < 0.05$, compared with the OGD group.

OGD and drug treatment

After 14 days of culture, the hippocampal slices were randomly divided into control, OGD, and PF-treatment groups. Firstly, OGD assays were performed in the OGD and PF-treatment groups. All slices were carefully washed with PBS at least three times and the culture medium was replaced with PBS. Subsequently, the slices in the OGD and PF-treatment groups were incubated under 95% N_2/5% CO_2 by using a tri-gas incubator for 45 min. After the OGD phase, the PBS in the 6-well plates was replaced with fresh culture medium. PF was then added to the culture medium of the PF-treatment groups at a concentration of 1 μM, 10 μM, or 100 μM; PF was not added to the control and OGD groups. All slices were then incubated at 37°C in 5% CO_2 for 24 h.

PI staining

The measurement of PI uptake has been used for detecting OGD-induced cell death in hippocampal slices [17]. After the final incubation, the hippocampal slices were loaded with 10 μg/ml PI and were incubated for 20 minutes. Images were obtained using a Leica DMI4000B inverted fluorescence microscope at magnification of 40× and exposure time of 30 ms. The

semiquantitative analysis of PI uptake was performed using Image J software. The data were normalized to the values of the control group, which were considered as 1.

Western blotting

Protein samples obtained from the hippocampal slices were subjected to sodium dodecyl sulfate-polyacrylamide (8~12%) gel electrophoresis (SDS-PAGE) in SDS-PAGE electrophoresis buffer (Beyotime). The proteins were then transferred to nitrocellulose membranes by using a Bio-Rad transfer apparatus with Western Transfer Buffer (Beyotime) containing 10% (v/v) ethanol for 2 h at 80~100 V. The membranes were blocked with 5% non-fat milk for 2 h, washed briefly, and incubated with the respective primary antibodies (ASC, NLRP1, NLRP3, caspase-1, and caspase-3) at 4°C overnight. Subsequently, the membranes were incubated with the appropriate HRP secondary antibody for 1 h at room temperature. After washing the membranes, protein immunoreactive bands were developed using the enhanced chemiluminescence (ECL)-Plus kit (Beyotime) and were detected using a camera with the ChemiDoc Imaging System (Bio-Rad). The intensities of the bands were quantified using Image J software. The data were normal-

ized to the respective β-actin bands and to the values of control group, which were considered as 1.

ELISA

Following collection and centrifugation of the culture medium, the supernatant was retained for measuring IL-18 and IL-1β by using commercially available ELISA kits. The concentrations of IL-18 and IL-1β proteins were normalized to the values of the control group, which were considered as 1.

Statistical analysis

All data are expressed as the means ± SE. Statistical analysis was performed using SPSS software. The analysis was conducted by performing the one-way ANOVA test followed by the LSD or Games-Howell post hoc test. *P* values < 0.05 were considered statistically significant. The figures were generated with SigmaPlot software.

Results

PF inhibits neuronal cell death in hippocampal slices exposed to OGD

Firstly, we utilized the PI staining method to measure OGD-induced cell death in hippocampal slices in the different groups. After the OGD phase, the hippocampi in the OGD group displayed strong PI uptake in comparison to the untreated hippocampi in the control group (**Figure 2A**, **2B**). Treatment with the three different concentrations of PF inhibited the increase in PI uptake in a significant and dose-dependent manner. Moreover, 100 μM of PF demonstrated the strongest ability to block PI uptake compared with treatment with 1 μM and 10 μM of PF.

PF downregulates the expression of the NLRP1 and NLRP3 inflammasome components

Next, we analyzed the expression of the components of NLRP1 and NLRP3 inflammasomes (NLRP1, NLRP3, ASC, and caspase-1) in the hippocampal slices from the different groups. The expression of these components was significantly increased after the OGD process and drug treatment, whereas PF treatment decreased the expression of the various components to different extents (**Figure 3**). The levels

of precursor/cleaved caspase-1 and NLRP3 were markedly decreased in the three PF treatment groups (**Figure 3A**, **3B**). Notably, the expression levels of NLRP3 and precursor caspase-1 were lower in the PF-treatment groups than in the control group. The levels of NLRP1 and ASC expression decreased in the PF-treatment groups at concentrations of 10 μM and 100 μM, whereas 1 μM of PF minimally decreased the expression of these proteins (**Figure 3C**, **3D**). Similarly, the levels of NLRP1 and ASC were lower in the group that was treated with 100 μM of PF compared with the untreated control group.

PF reduces the levels of proteins downstream of NLRP1 and NLRP3 inflammasomes

We further investigated the effect of PF on downstream proteins that are regulated by NLRP1 and NLRP3 inflammasomes, such as IL-18, IL-1β, and caspase-3. Similar to the results with the inflammasome components, the levels of IL-18 and IL-1β were significantly increased in the OGD group, whereas they were decreased in the PF-treatment groups (**Figure 4A**, **4B**). Notably, IL-18 was significantly reduced in the 10-μM and 100-μM PF-treatment groups and IL-1β was significantly reduced in the 100-μM PF group, whereas treatment with 1 μM PF only moderately decreased these proteins. Low expression of cleaved caspase-3 was detected in the 10-μM and 100-μM PF treatment groups in comparison to the control group; however, no significant difference was detected in the 1-μM PF treatment group compared with the control group (**Figure 4C**).

Discussion

In this study, OHSC, which is a classic method that had been frequently used in neuroscience studies, was slightly modified to obtain individual and intact hippocampal slices. In comparison to in vitro neuronal cultures, OHSC provides a better approximation of vivo conditions. Brain slices are an ideal platform for the investigation of molecular pathways underlying neuronal dysfunction because they maintain many aspects of in vivo biology, including functional synaptic circuitry with intact brain architecture, which allows precise and multiple experimental interventions of the extracellular conditions. Moreover, this technique is convenient for successful explantation of nervous tissue, and more
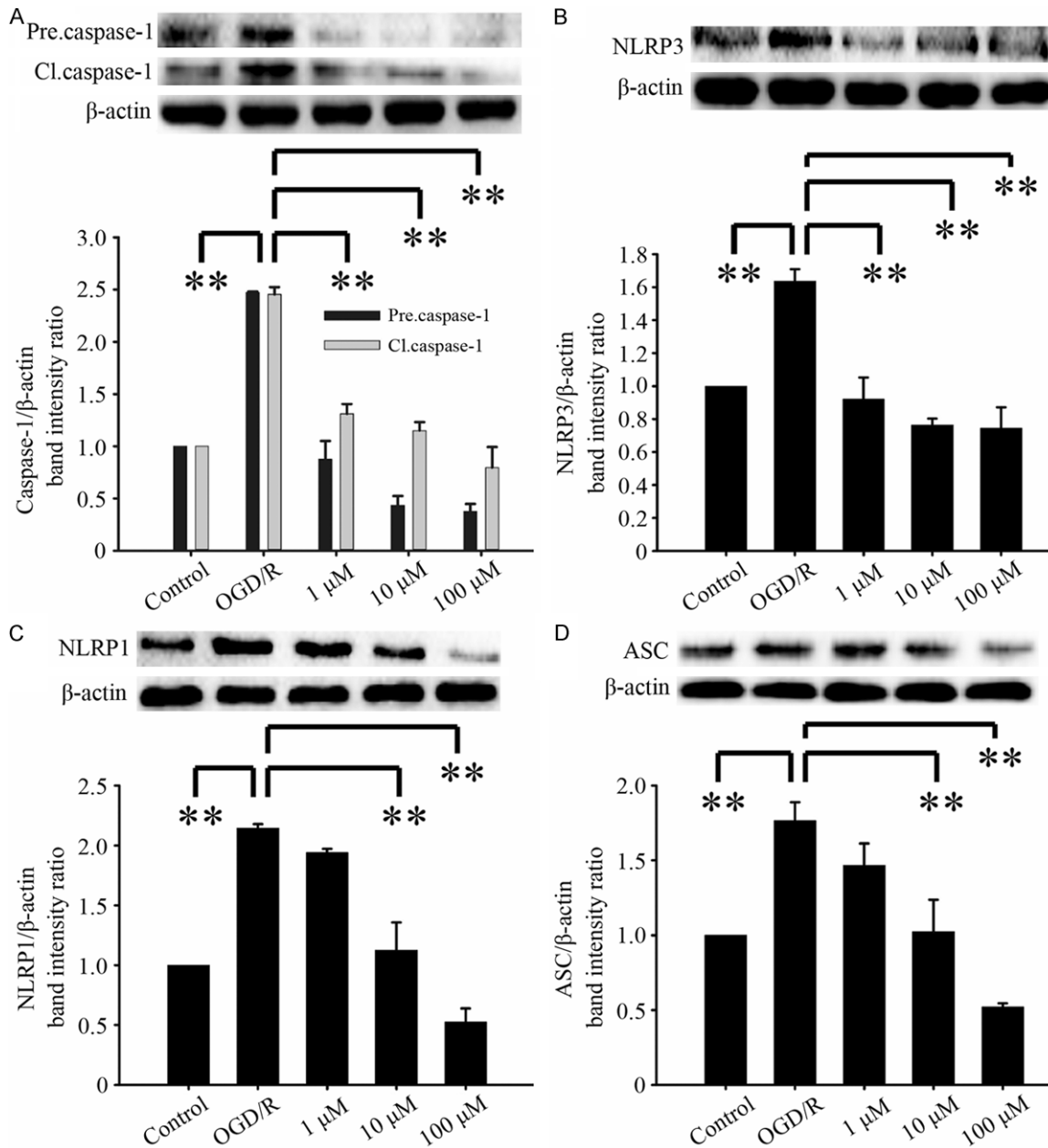
Figure 3. Paeoniflorin downregulates the expressions of NLRP1 and NLRP3 inflammasome in an OGD model of ischemic stroke based on rat organotypic hippocampal slices cultures. A-D. Respectively represent immunoblots and quantification and illustrate increases in the levels of precursor capsase-1 (pre.caspase-1) and cleaved caspase-1 (cl.caspase-1), NLRP3, NLRP1 and ASC in hippocampal slices following 45 minutes of OGD. Paeoniflorin reduced the activation levels of NLRP3, NLRP1, ASC, pre.caspase-1 and cl.caspase-1, in different extent compared with OGD group. Data were represented as mean ± SE (n = 6 slices in each group) and normalized with respect to the values of Control group that were considered as 1. $^{**}P < 0.05$, compared with the OGD group.

specifically, hippocampal slices [16, 18-21]. Nevertheless, although ischemic stroke is most likely an 'age-dependent' disease, long-term (over two weeks) culture of adult hippocampal slice is impossible [22, 23], and it is accepted that slices from 8- to 15-day-old rats

are able to obtain the best organotypic organization [16]. In addition, OGD-induced neuronal injury based on OHSC in neonatal rats has been used to study neurological diseases, including ischemic stroke [24]. Under normal physiological conditions, cerebral tissue requi-
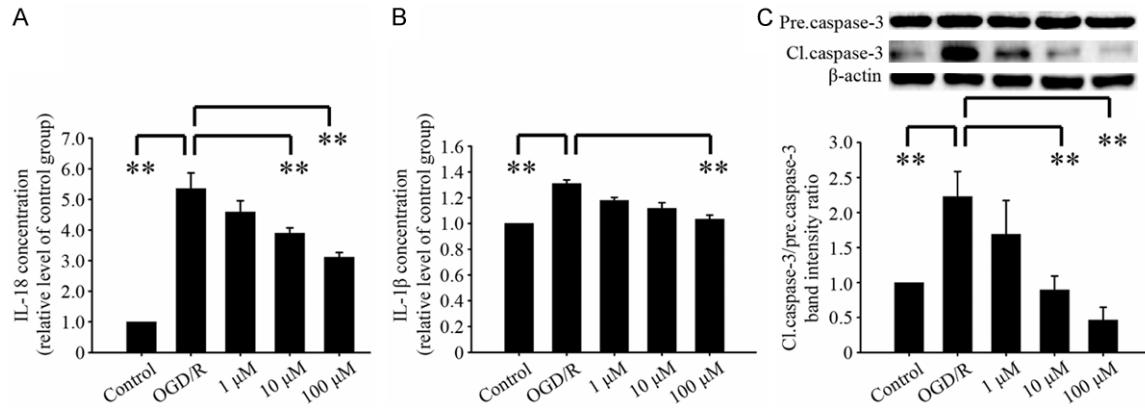
Figure 4. Paeoniflorin reduces the levels of the downstream proteins of NLRP1 and NLRP3 inflammasome in rat organotypic hippocampal slice cultures following 45 minutes of OGD. A, B. Represent the detected concentrations of pro-inflammatory cytokines IL-18 and IL-1β. C. Represents immunoblots and quantification of precursor caspase-3 (pre.caspase-3) and cleaved caspase-3 (cl.caspase-3). Paeoniflorin reduced the levels of IL-18, IL-1β and cl.capsase-3 in different extent compared with OGD group. Data were represented as mean ± SE (n = 6 slices in each group) and normalized with respect to the values of Control group that were considered as 1. **$P < 0.05$, compared with the OGD group.

res persistent blood flow to obtain a sufficient supply of both glucose and oxygen for the maintenance of basic neurological functions. During ischemic stroke, the necessary blood flow is reduced to a very low level. Consequently, the formation and development of an ischemic core directly leads to irreversible cell death [25]. On the other hand, increasing evidence shows that a severe and detrimental inflammatory response occurs in the ischemic brain tissue within hours after ischemia [26]. We used the OGD assay, which is a reliable approach for investigating the mechanisms of neuroprotection after the process of ischemia [27], in the separated hippocampal slices in order to mimic ischemia in OHSCs.

A previous study showed that PF is able to quickly penetrates the blood-brain barrier (BBB) to reach the hippocampus [28]. Moreover, PF was shown to have neuroprotective effects against inflammation-induced neurological damage in the PI uptake assay, which can be used to determine necrotic cell death in hippocampal slices [29]. Herein, we applied PF concentrations of 1 μM, 10 μM, and 100 μM to investigate the basic neuroprotective ability of PF after ischemic damage. The PI uptake results indicate that PF has a significant protective effect, i.e., relieves OGD-induced neuron death in the hippocampus, in a concentration-dependent manner.

PF was previously shown to have an anti-inflammatory and protective effect against ischemia-induced brain damage in rats via inhibition of the mitogen-activated protein kinase (MAPK)- and nuclear factor kappa-B (NF-κB)-mediated cerebral inflammatory response [11]. In addition, NLRP1 and NLRP3 inflammasomes appear to play a notable role in the ischemic cascade involving the inflammatory response [30]. Therefore, we investigated the potential ability of PF to downregulate the expression of NLRP1 and NLRP3 inflammasomes, thereby protecting against the OGD-induced brain damage, in hippocampal slices. Our results showed that treatment with different concentrations of PF downregulated the components of NLRP1 and NLRP3 inflammasomes, such as NLRP1, NLRP3, ASC, and precursor/cleaved caspase-1, to different degrees. This suggests that the formation of NLRP1 and NLRP3 inflammasomes were significantly inhibited that PF may be capable of reducing the level of NLRP1 and NLRP3 inflammasomes after OGD.

Furthermore, it has been shown that the formation of NLRP1 and NLRP3 inflammasomes in ischemic stroke will eventually activate precursor caspase-1 to cleaved caspase-1, which is able to produce both mature IL-18 and IL-1β to mediate neuronal cell death [5, 9, 10]. Additionally, cleaved caspase-1 was shown to cleave and activate precursor caspase-3, which can induce apoptosis [31]. As expected, our results show that the downregulation of cleaved caspase-1 decreased the downstream pro-

teins. The levels of IL-18, IL-1β, and cleaved capsase-3 were reduced in the groups treated with PF. However, 1 μM of PF did not notably reduce the levels of these proteins; although we believe that this was partly due to the low concentration of PF, this also suggests that these downstream proteins are mostly regulated by other mechanisms and that cleaved caspase-1 has only a partial contribution. In general, our findings suggest that PF can reduce the expression of pro-inflammatory cytokines (IL-18 and IL-1β) and an apoptosis protein (cleaved caspase-3) by inhibiting the formation and downregulating the expression of NLRP1 and NLRP3 inflammasomes, which can cleave and activate precursor caspase-1 in ischemic hippocampal slices.

In conclusion, taken together, our findings suggest that the neuroprotective effect of PF in OGD-induced hippocampus injury is linked to its ability to downregulate the expression of NLRP1 and NLRP3 inflammasomes, thereby indirectly affecting the levels of downstream proteins that have not yet been clarified [29]. Therefore, our results provide a new clue for elucidating the anti-inflammatory mechanisms of PF after ischemic stroke.

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Disclosure of conflict of interest

None.

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