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ORIGINAL RESEARCH

Physalin A exerts neuroprotective effects: inhibition of OGD/R-induced cellular pyroptosis and inflammatory responses in nerve cells

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Abstract

A prevalent cerebrovascular disease like the acute cerebral infarction is caused by cerebral thrombosis or arteriosclerosis that can result in ischemic necrosis of brain tissues. Treatment drugs of higher efficiency are required to be developed for acute cerebral infarction. Physalin A (PA) is a major compound from *Physalis alkekengi L.* and has pharmacological properties. However, the neuroprotective effects of PA are less reported and the mechanism remains unclear. The acute cerebral infarction cell model was constructed by treating PC12 cells with oxygen-glucose deprivation/reoxygenation (OGD/R). The impacts of PA on cell viability were determined by performing 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) and lactate dehydrogenase (LDH) releasing assays. Immunoblot and Enzyme-linked Immunosorbent assay (ELISA) were conducted to examine PA effects on inflammation. PA effects on pyroptosis were detected by performing immunoblot and immunostaining. Moreover, immunoblot was further conducted to confirm the mechanism. In this study, the survival and inhibition of the pyroptosis of OGD/R-induced PC12 cells were regulated by PA. Furthermore, PA suppressed the inflammation. PA inhibited mitogen-activated protein kinase (MAPK) pathway activation, and the activation of MAPK pathway reversed the neuroprotective effect of PA. Therefore, PA exerts neuroprotective effects by suppressing OGD/R-induced cellular pyroptosis and inflammatory responses in nerve cells.

Keywords

Acute cerebral infarction; Physalin A (PA); OGD/R; Pyroptosis; MAPK pathway

1. Introduction

A prevalent cerebrovascular disease like the acute cerebral infarction is caused by cerebral thrombosis or arteriosclerosis that can result in ischemic necrosis of brain tissues [1, 2]. Although thrombectomy and drug therapy have been widely employed in treating cerebral infarction, therapeutic outcomes are however limited. Studies have shown that inflammation occurs throughout the course of thromboembolic stroke [3]. Cumulative inflammatory responses mediated by multiple pro-inflammatory cytokines, contribute to the onset and progression of ischemic injury and neurological diseases [4]. Inhibiting the inflammatory response is beneficial for inhibiting the progression of related diseases and is also the main strategy for drug development [5]. Treatment drugs of higher efficiency are required to be developed for acute cerebral infarction.

Physalin A (PA) is a major compound from *Physalis alkekengi L.* and has pharmacological properties such as anti-inflammatory, and antifungal *in vivo* and *in vitro* [6]. Previous experiments reveal that PA has a protective effect on cartilage [7]. In arthritis, PA alleviates chondrocyte inflammation by inhibiting MAPK and nuclear factor kappa-B (NF-κB) pathways, promotes anabolic metabolism, inhibits catabolism and reduces cartilage destruction, which make PA a promising drug for treatment of OA [8]. By inhibiting NF-κB pathway, PA inhibited carrageenan-induced foot edema in rats and aceto-induced capillary permeability in mice and inhibited the release of inflammatory factors [9]. PA also has anti-inflammatory effects. By inhibiting inhibitor of NF-κB (IκB)/NF-κB and c-Jun N-terminal kinase (JNK)/activator protein-1 (AP-1) signaling pathways, PA inhibits development of oxidative stress factors and inflammatory mediators in LPS-induced cells, and increases the levels of antioxidant factors such as superoxide dismutase (SOD) [10]. However, the neuroprotective effects of PA are less reported and the mechanism remains unclear.

This study aimed to clarify impact of PA on neuroprotection. The findings herein exhibited that PA inhibited activation of MAPK pathway, and blocked the pyroptosis of nerve cells and inflammatory factors release in OGD/R-induced nerve cells,

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thereby playing a neuroprotective role and supporting cells survival.

2. Materials and methods

2.1 Cell culture and treatment

Chinese Academy of Sciences provided the PC12 cells. Dulbecco's modified eagle medium (DMEM) was employed for cells culturing. PC12 cells were given treatment by PA (procured from sigma) for 24 h at the concentration of 0, 20, 40 and 80 μM. For OGD/R model construction, culturing of cells was done in glucose-deficient DMEM and serum. An anaerobic chamber was kept for 10 h at 37 °C without the oxygen supply (5% carbon dioxide (CO_2) and 95% nitrogen (N_2)), after being kept for 24 h in normal medium according to the previous study [5].

2.2 Western blotting

Radio immunoprecipitation assay (RIPA) lysate was used to fully lysate cells to extract protein, which was quantitated by Bovine serum albumin (BCA) reagent, fractionated *via* sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and further moved onto poly (vinylidene fluoride) membranes. 5% milk was used for 1 h to block the membranes. Membranes were then treated overnight at 4 °C by primary antibodies comprising of β-actin (1:3000; ab8226), p38 (1:1000, ab170099), p-p38 (1:1000, ab17886), JNK (1:500, ab110724), p-JNK (1:500, ab215208), extracellular regulated protein kinases (ERK) 1/2 (1:1000, ab184699), p-ERK1/2 (1:1000, ab201015), p65 (1:500, ab32536), p-p65 (S536, 1:500, ab76302), interleukin (IL)-18 (1:1000, ab243091), IL-1beta (1:500, ab216995), caspase 1 (1:1000, ab207802), Gasdermin D-N (GSDMD-N) (1:1000, ab215203), and NOD-like receptor thermal protein domain associated protein 3 (NLRP3) (1:1000, ab263899, abcam). Membranes were then treated for 1 h at room temperature with secondary antibodies and photographed after chemiluminescence.

2.3 Cell viability assay

1000 cells/well of PC12 cells were placed in 96 wells plate and kept for 2 days. Afterwards, the cells were treated for 4 h with MTT and dissolved in 200 μL dimethyl sulfoxide (DMSO). OD490 value was then recorded. For LDH releasing assays, the LDH releasing degree was measured by the kit from Abcam (ab102526, Abcam, Cambridge, UK). Absorbance measurement was made at 450 nm.

2.4 ELISA assay

IL-6 (ab178013), IL-1β (ab214025) and tumor necrosis factor-α (TNF-α) were monitored *via* ELISA (ab181421, Abcam, Cambridge, UK) following manufacturers' instructions. Standard reagents and samples were added and kept at 4 °C for 20 h. Microplate reader (168-1130, Bio-Rad, Hercules, CA, USA) was employed to record the absorbance at 450 nm.

2.5 Immunofluorescent staining

4% paraformaldehyde (PFA) in 5% bovine serum albumin (BSA) was used to fix and block the cells followed by incubation with PI (1:3000, HY-D0815, MCE) and Hoechst33342 (1:2000, ab145597, abcam) for 20 min. After rinsing three times, fluorescent microscope was utilized to capture the images.

2.6 Statistics

The data were analysed through GraphPad 8.0 (National Institutes of Health, Bethesda, MD, USA) software and presented as mean $\pm$ standard deviation (SD). Statistical significance for the two groups was determined by student's t test. Multiple comparisons were made through one-way analysis of variance (ANOVA) and Tukey's *post hoc* test. Statistically significant difference was considered for $p < 0.05$.

3. Results

3.1 PA contributing to OGD/R-induced PC12 cells survival

To determine PA effects on acute cerebral infarction, we first constructed a model of acute cerebral infarction using PC12 cells. PC12 cells were used to induce model *in vitro* by OGD/R. Through MTT assays, we revealed that PA had modest effects on PC12 cells viability at low concentration (10, 20 and 40 μM), and the high concentration (80 μM) of PA suppressed their viability (Fig. 1A). We next used the low concentration of PA to conduct the *in vitro* assays. Furthermore, we noticed that PC12 cells viability was suppressed by OGD/R treatment, while PA (10, 20 and 40 μM) reversed the suppressing of cell viability produced by OGD/R treatment (Fig. 1B). Consistently, PA treatment also reversed the promoted PC12 cells cytotoxicity caused by OGD/R treatment, confirmed *via* LDH releasing assays (Fig. 1C). Therefore, PA promoted the survival of OGD/R-treated PC12 cells.

3.2 PA suppresses the inflammation of OGD/R-induced PC12 cells

Afterward, PA effects on inflammatory response of OGD/R induced PC12 cells were detected. From ELISA assays, we detected secretions of inflammatory factors including IL-1β, IL-6 and TNF-α. We noticed that secretions of these factors in PC12 cells were induced by OGD/R (Fig. 2A). However, PA treatment attenuated the OGD/R induced secretions of these inflammatory factors in PC12 cells (Fig. 2A). Similarly, immunoblot assays also showed that PA treatment reversed the enhanced NF-κB p65 phosphorylation levels caused by OGD/R treatment (Fig. 2B). Collectively, PA inhibits OGD/R-induced PC12 cell inflammation.

3.3 PA inhibited the pyroptosis of OGD/R-induced PC12 cells

The detections were made whether PA affected pyroptosis of OGD/R treated PC12 cells. High expression of pyroptosis markers including caspase-1/p20, GSDMD-N and NLRP3 in

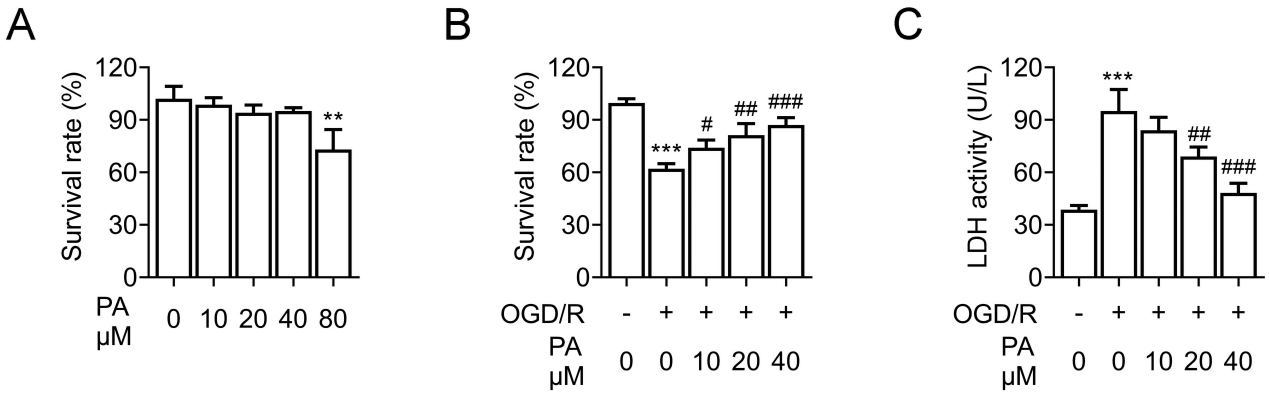

FIGURE 1. PA contributing to survival of PC12 cells induced by OGD/R. (A) MTT assays exhibited viability of PC12 cells upon PA treatment for 24 h at 10, 20, 40 and 80 μM concentrations. (B) MTT assays showed PC12 cells viability with or without OGD/R upon PA treatment for 24 h at 10, 20 and 40 μM concentrations. (C) LDH releasing assays showed PC12 cells LDH levels upon PA treatment at concentration of 10, 20 and 40 μM for 24 h with or without OGD/R. Error bar indicates SD. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, OGD/R + PA *vs.* OGD/R. **$p < 0.01$, ***$p < 0.001$, OGD/R *vs.* control. OGD/R: oxygen-glucose deprivation/reoxygenation; PA: Physalin A; LDH: lactate dehydrogenase.

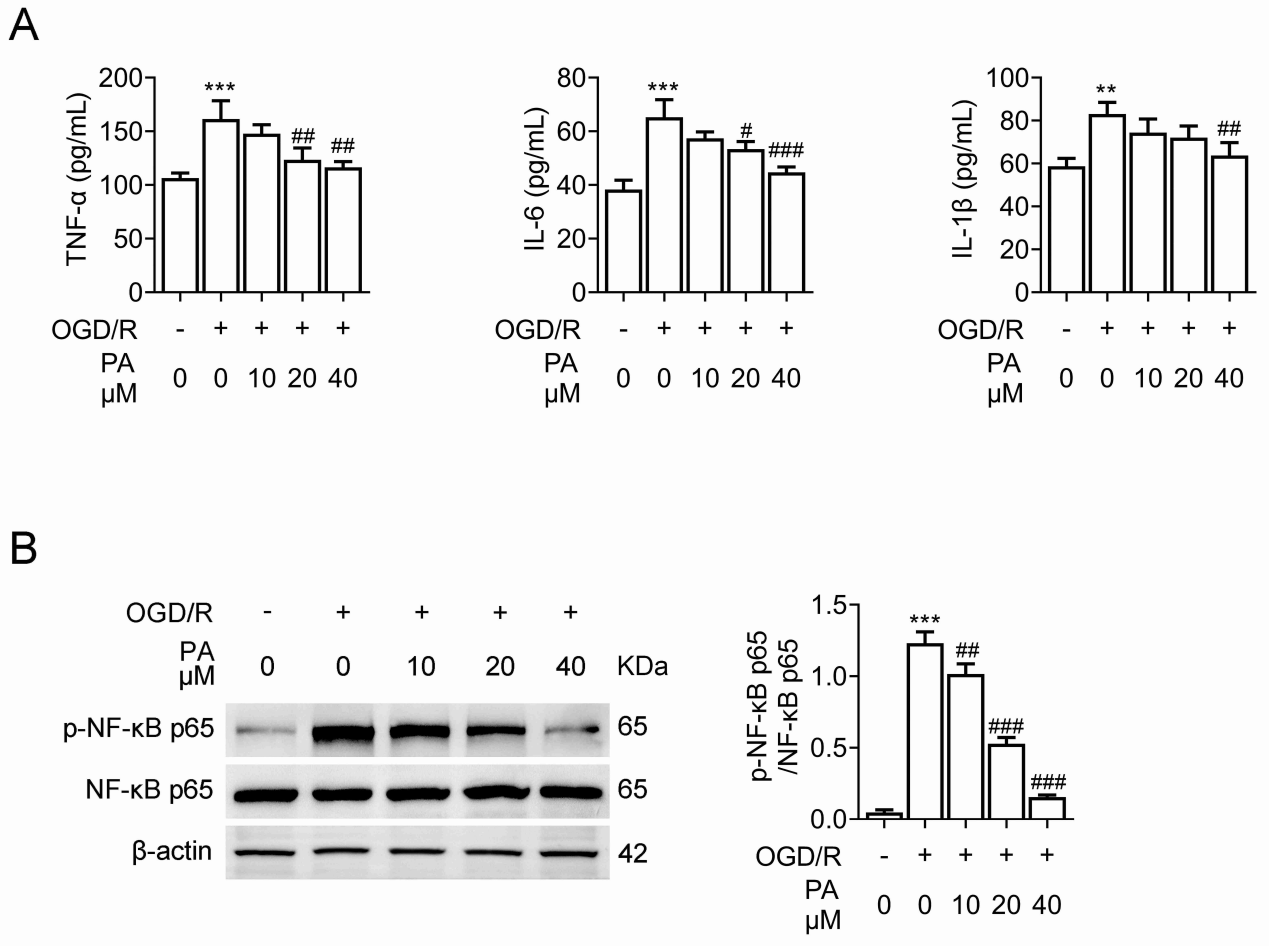

FIGURE 2. PA suppresses the inflammation of PC12 cells induced by OGD/R. (A) ELISA assays showed the secretion of IL-1β, IL-6 and TNF-α in PC12 cells with or without OGD/R upon the treatment of PA for 24 h at 10, 20 and 40 μM concentrations. (B) The phosphorylation levels and expression of NF-κB p65 in PC12 cells were depicted with or without OGD/R through immunoblot assays upon the PA treatment at concentrations of 10, 20 and 40 μM for 24 h. Error bar indicates SD. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, OGD/R + PA *vs.* OGD/R. **$p < 0.01$, ***$p < 0.001$, OGD/R *vs.* control. OGD/R: oxygen-glucose deprivation/reoxygenation; PA: Physalin A; TNF-α: tumor necrosis factor-α; IL: interleukin; NF-κB: nuclear factor kappa-B.

PC12 cells were found through immunoblot assays. Furthermore, the high expressions of inflammatory factors including IL-1β and IL-18 were found in PC12 cells induced by OGD/R (Fig. 3A). However, PA treatment suppressed these proteins expressions in OGD/R treated PC12 cells (Fig. 3A). We further performed the immunostaining assays, and OGD/R treatment induced PC12 cells pyroptosis (PI-positive cells), whereas PA treatment reversed the induction of OGD/R triggered PC12 cells pyroptosis (Fig. 3B). Therefore, OGD/R-induced PC12 cells pyroptosis was inhibited by PA.

3.4 PA inhibited MAPK pathway activation in OGD/R-induced PC12 cells

We subsequently detected mechanism underlying PA relieving PC12 cells OGD/R injury. It was noticed that OGD/R treatment promoted the phosphorylation of ERK1/2, p38, as well as JNK in PC12 cells (Fig. 4). However, PA treatment reversed the enhanced expression of ERK1/2, p38 and JNK phosphorylation in OGD/R treated PC12 cells (Fig. 4). These data suggested that PA inhibited MAPK pathway activation in PC12 cells treated by OGD/R.

3.5 MAPK pathway activation reversing the PA neuroprotective impact in PC12 cells treated by OGD/R

We then used activator of MAPK pathway, Virodhamin, to activate this pathway in OGD/R-treated PC12 cells. It was depicted *via* MTT assays that Virodhamin treatment reversed viability of OGD/R-treated PC12 cells caused *via* PA treatment (Fig. 5A). Consistently, Virodhamin treatment also revealed the suppression of cytotoxicity of these cells caused from PA treatment (Fig. 5B). Further through ELISA assays, we found

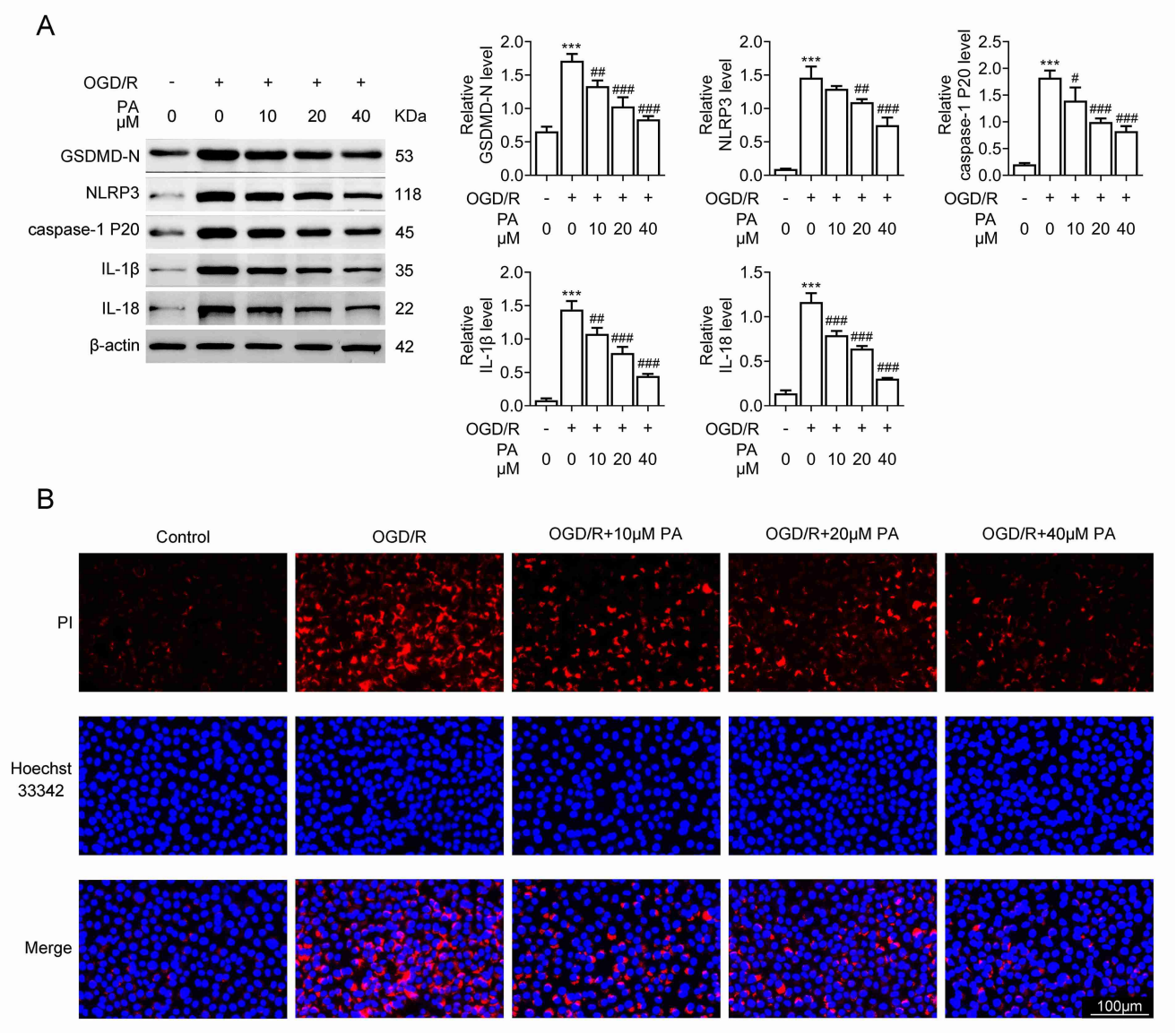

FIGURE 3. PA inhibited the pyroptosis of OGD/R-induced PC12 cells. (A) Immunoblot assays showed proteins expression in PC12 cells with or without OGD/R upon PA treatment for 24 h at 10, 20 and 40 μM concentrations. (B) Immunostaining assays showed the expression of Propyl iodide (PI) and hoechst in PC12 cells with or without OGD/R upon PA treatment at concentrations of 10, 20 and 40 μM for 24 h. Error bar indicates SD. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, OGD/R + PA *vs.* OGD/R. ***$p <$ 0.001, OGD/R *vs.* control.

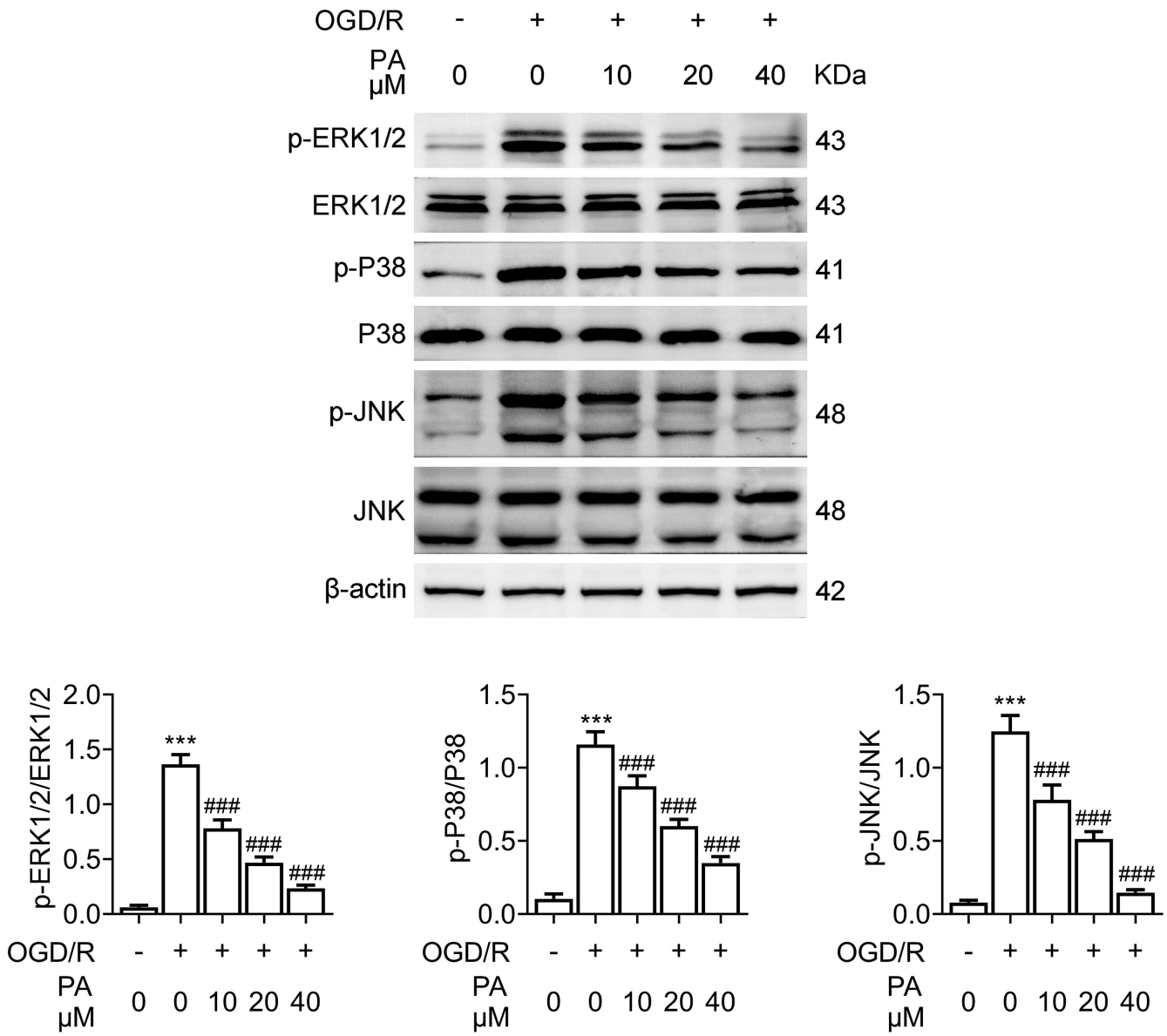

FIGURE 4. PA inhibited MAPK pathway activation in OGD/R-induced PC12 cells. Immunoblot assays depicted the phosphorylation levels and expression of ERK1/2, p38 and JNK in PC12 cells upon PA treatment with or without OGD/R at concentrations of 10, 20 and 40 μM for 24 h. Error bar indicates SD. ###$p < 0.001$, OGD/R + PA *vs.* OGD/R. ***$p < 0.001$, OGD/R *vs.* control. ERK: extracellular regulated protein kinases.

that Virodhamin treatment reversed the suppressed secretion of the indicated factors in these cells caused through PA treatment (Fig. 5C). Subsequently, we performed Immunoblot assays, and the data indicated that Virodhamin treatment reversed the suppression of pyroptosis markers expression in these cells caused by PA treatment (Fig. 5D). Finally, we noticed that Virodhamin reversed the suppression of PI expression in these cells as caused by PA treatment, suggesting promotion of pyroptosis (Fig. 5E). Therefore, the activation of MAPK pathway reversed the neuroprotective effect of PA in PC12 cells induced by OGD/R.

4. Discussion

Acute cerebral infarction is triggered through the acute blood flow interruption in the arterial blood tube of the brain, and the degeneration and necrosis of nerve cells in the corresponding brain blood supply area, resulting in neurological dysfunction in the corresponding brain area [11]. If patients with acute cerebral infarction are suitable for thrombolysis, they should receive timely thrombolysis treatment. Those who are not suitable for thrombolysis should be given aspirin as soon as possible for anti-platelet aggregation and neuroprotective therapy [12]. The drugs used in the acute stage of cerebral infarction mainly include anti-platelet aggregation drugs, anti-coagulation, defibrination, neuroprotective drugs and drugs that protect mitochondria, expand and improve blood circulation, and promote blood circulation and remove blood stasis. Here, OGD/R was employed to treat PC12 cells. It was revealed that PA plays a neuroprotective role by inhibiting the activation of MAPK pathway and inhibiting neuronal pyrop-

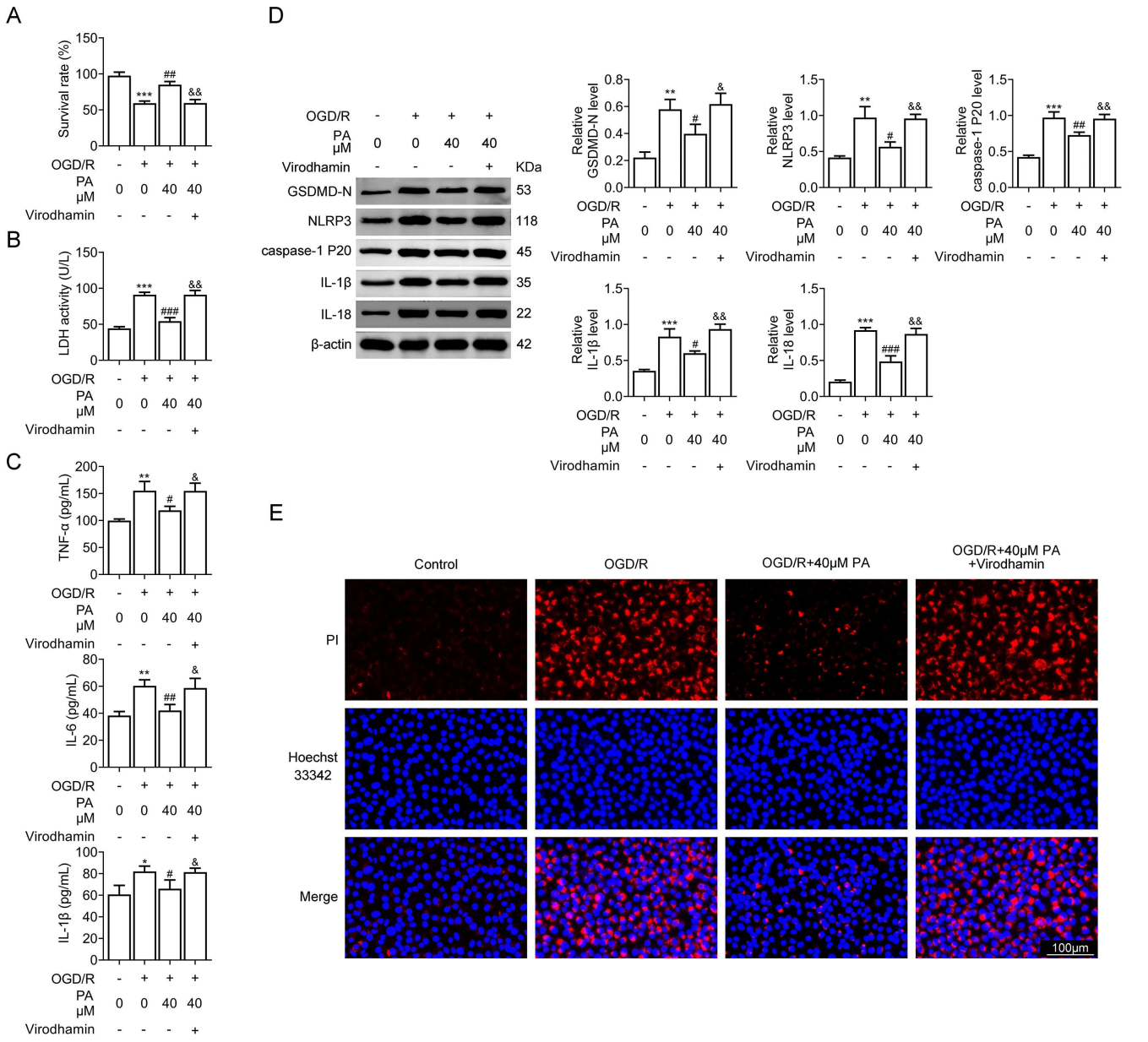

FIGURE 5. MAPK pathway activation reversing PA neuroprotective impact in PC12 cells induced by OGD/R. (A) MTT assays showed the PC12 cells viability on indicated treatment. (B) LDH releasing assays showed LDH levels and cell damage degree of PC12 cells. (C) ELISA assays revealed secretions of IL-1β, IL-6 and TNF-α in PC12 cells. (D) Immunoblot assays showed the expression of indicated proteins in PC12 cells. (E) Immunostaining assays depicted the expression of Propyl iodide (PI) and hoechst in PC12 cells. &$p < 0.05$, &&$p < 0.01$, OGD/R + PA + Virodhamin *vs.* OGD/R + PA. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, OGD/R + PA *vs.* OGD/R. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, OGD/R *vs.* control. LDH: lactate dehydrogenase.

tosis and inflammatory factors release in the OGD/R-treated nerve cells. Therefore, these findings demonstrate that PA is the potential antithrombotic drug.

Through a series of *in vitro* assays, we revealed that PA affected the viability, inflammation, pyroptosis of PC12 cells treated with OGD/R. PA is a main component of plant acid pulp, which has anti-tumor and anti-inflammatory effects. Pharmacological studies indicated PA can significantly inhibit the production of nitric oxide in macrophages induced by lipopolysaccharide, thus exerting anti-inflammatory effect [9, 13]. The mechanism may be linked to modification of IκB kinase-β and inhibition of NF-κB activity [13]. In arthritis, PA alleviates chondrocyte inflammation, promotes anabolic metabolism and reduces cartilage destruction by inhibiting MAPK and NF-κB pathways [7, 14]. Similarly, we here also revealed that PA suppressed inflammation of PC12 cells treated by OGD/R *via* targeting MAPK pathway.

Our results revealed that PA inhibited pyroptosis of OGD/R induced PC12 cells. Pyroptosis is programmed cell death (PCD) regulated by the inflammatory caspase, such as Caspase-1 [15]. It is accompanied by cell swelling, osmolysis, activation of the NLRP3 inflammasome, and pro-inflammatory cytokines release [16]. Previous works have depicted that ischemia induced pyroptosis can aggravate nerve

damage [17]. Therefore, inhibition of neuronal inflammation and scorch death can reduce the volume of brain obstruction.

In this OGD/R-induced cell model, PA inhibited the activation of MAPK pathway. MAPK pathway is an important signaling pathway *in vivo*, that has main role in regulating cell inflammation, apoptosis, differentiation, growth, proliferation and other physiological and pathological processes [18]. MAPK pathway is a crucial pathway, regulated by multiple factors and expressed in the central nervous system (CNS) [19]. ERK1/2, JNk, p38 are in MAPK family and their interaction with NF-κB, Nrf2, Akt, Caspase-3 and other pathways are tangled in signal transduction of the early pathologic mechanism of cerebral ischemia [19]. We confirmed that this pathway could act as a target for acute cerebral infarction.

5. Conclusions

In summary, we revealed that PA exerts neuroprotective effects by inhibiting OGD/R-induced cellular pyroptosis and inflammatory responses in nerve cells.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this published article.

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

AUTHOR CONTRIBUTIONS

HYY—performed the research. DL and YPW—analyzed the data. HYY and DL—wrote the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

ACKNOWLEDGMENT

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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