RESEARCH PAPER

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Pachymic acid prevents neuronal cell damage induced by hypoxia/reoxygenation *via* miR-155/NRF2/HO-1 axis

Yang Zhai[1,2,3], Bugu Liu[4], Lin Wu[1,2], Min Zou[5], Xiaoping Mei[6], Xueni Mo[1]*

[1] Guangxi University of Chinese Medicine, Nanning, China,
[2] Guangxi Key Laboratory of Chinese Medicine Foundation Research, Nanning, China,
[3] Department of International Medical, Guangxi International Zhuang Medicine Hospital, Nanning, China,
[4] Department of Neurology, The First Affiliated Hospital of Guangxi University of Chinese Medicine, Nanning, China,
[5] Department of Pediatrics, Guangxi International Zhuang Medicine Hospital, Nanning, China,
[6] Department of Endocrinology, Guangxi International Zhuang Medicine Hospital, Nanning, China,
** Email: moxunixhd422@163.com*

Pachymic acid (PA) plays a neuroprotective role during cerebral ischemia/reperfusion. However, the protective mechanisms of PA in cerebral ischemia/reperfusion have been not fully determined. This investigation aims to explore the neuroprotective role of PA in ischemia/reperfusion *via* miR-155/NRF2/HO-1 axis. The N2a cell line was induced by hypoxia/reoxygenation (H/R) to simulate the neuronal damage that occurs during cerebral ischemia/reperfusion. PA was used to treat H/R-induced N2a cells. An MTT assay was used to determine cell viability. The protein levels of Bcl-2, Bax, heme oxygenase-1 (HO-1) and nuclear factor E2-related factor 2 (NRF2) were measured *via* Western blot analysis. The level of apoptosis of N2a cells was determined by flow cytometry. The expression levels of miR-155 and NRF2 were quantified by real-time PCR. PA treatment inhibits the increase in apoptosis induced by H/R and also enhances the viability of cells exposed to H/R. PA reverses the increased expression of miR-155 caused by H/R. Furthermore, H/R does not change the expression of HO-1 and NRF2, but PA upregulates the expressions of HO-1 and NRF2. Additionally, NRF2 is the target of miR-155. Inhibiting miR-155 contributes to increased cell viability and decreased apoptosis *via* targeting the NRF2/HO-1 pathway. Overall, PA prevents neuronal cell damage induced by hypoxia/reoxygenation *via* miR-155/NRF2/HO-1 axis.

Key words: pachymic acid, miR-155, NRF2/HO-1 pathway, cerebral ischemia/reperfusion, neuroprotective effect

INTRODUCTION

Cerebral ischemia is a prevalent cause of disability and death (Stegner et al., 2019). Cerebral ischemia induced by disordered cerebral blood flow leads to complex metabolic disorders and cellular pathologies within the brain, which causes brain injury characterized by neuronal cell damage and cerebral infarction (Shin et al., 2020). It has been shown that rapid reperfusion to restore cerebral blood flow improves the symptoms induced by cerebral ischemia, whereas reperfusion might lead to more serious neuronal damage (Li et al., 2007; Jung et al., 2010; Bhaskar et al., 2018). Cerebral ischemia reperfusion results in massive neuronal death, such as apoptosis, which subsequently results in the destruction of nerve structure and the disruption of neuronal plasticity, both of which are associated with neurodegenerative events. Treatments based on neuroprotection strategies contribute to improved survival of and prognosis for patients with cerebral ischemia reperfusion injury in the clinic.

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Methods including hypoxic preconditioning, homocysteine or neuronal stem cells ameliorate cerebral ischemia reperfusion injury by preventing neuronal death (Gao et al., 2006; Zhao et al.; 2016, Yang et al., 2018), which suggests that inhibition of neuronal apoptosis is a potential method for the improvement of outcomes after cerebral ischemia reperfusion injury. Thus, it is necessary to investigate neuroprotective drugs for the treatment of neuronal damage in cerebral ischemia/reperfusion (Lv et al., 2020).

Pachymic acid (PA) is a potential neuroprotective drug that has attracted more attention in cerebral ischemia reperfusion injury. PA is a triterpenoid extracted from Poria cocos with low toxicity and anti-inflammatory effects (Lee et al., 2013; Arun et al., 2017; Kamalakannan-Preethi et al., 2020). Additionally, PA has emerged as a potential therapeutic target for ischemia. Jiang et al. (2021) suggested a mechanism in which PA prevents acute kidney injury caused by ischemia/reperfusion *via* NRF2-mediated ferroptosis. A previous study (Pang et al., 2020) showed that PA could promote blood flow and inhibit neuronal apoptosis during cerebral ischemia/reperfusion *via* the PI3K/AKT pathway, which indicated that PA plays a neuroprotective role during cerebral ischemia/reperfusion. The molecular mechanism of PA, although uncertain, in cerebral ischemia/reperfusion may provide the targets to selectively enhance its neuroprotective role.

miRNA, one kind of non-coding RNA, alters several cellular processes and histopathology. miRNA plays a significant role in cerebral ischemia/reperfusion by modifying the function of mitochondrion, including oxidative stress, metabolism and apoptosis (Hu et al., 2015). miR-155, located at chromosome 21, is involved in the molecular regulation of numerous diseases. As shown in previous studies (Jing et al., 2019; Zhang et al., 2020, Shi et al., 2020), miR-155 is a negative regulator of cerebral ischemia/reperfusion *via* targeting genes, including MafB, DUSP14 and GATA3 which mediate neuroinflammation, blood/brain barrier and apoptosis in brain injury induced by ischemia/reperfusion (Suofu et al., 2020). Obviously, miR-155 plays a significant role in the death and survival of neuronal cells from expression regulation to cellular function genes during cerebral ischemia reperfusion injury. Therefore, we hypothesize that miR-155 could be a neuroprotective target for drugs in cerebral ischemia reperfusion injury.

Nuclear factor E2-related factor 2 (NRF2) emerges as a transcriptional regulator that manipulates the activation of cellular genes and proteins (Tonelli et al., 2018). Heme oxygenase-1 (HO-1), a rate limiting enzyme, reduces excessive free heme by combining with hemopexin to promote angiogenesis in cerebral ischemia reperfusion (Dong et al., 2018). Phosphorylated NRF2 translocates to the nucleus to activate HO-1, promoting cell survival under stress conditions (Kensler et al., 2007). The NRF2/HO-1 pathway is involved in self-protection of lung tissue in cerebral ischemia reperfusion *via* activation of VEGF-mediated angiogenesis (Fan et al., 2019). Moreover, Liu et al. (2019) determined that NRF2 shows neuroprotective effects in cerebral ischemia reperfusion *via* regulating the AQP4 protein in neuronal cells. These investigations indicate that NRF2 might improve neuronal cell survival in cerebral ischemia reperfusion *via* activating HO-1. Importantly, PA represses the inflammatory phenotype in sepsis *via* NRF2/HO-1 pathway (Cai et al., 2017), demonstrating a potential therapeutic role for PA in cerebral ischemia reperfusion injury by regulating the NRF2/HO-1 pathway.

We infer that NRF2 may be the target of miR-155 according to bioinformatics tools, which provide the NRF2-mediated possible mechanism between PA and miR-155: that PA can protect neuronal cells in cerebral ischemia reperfusion *via* miR-155-mediated NRF2/HO-1 pathway. In this study, neuronal cells induced by hypoxia/reoxygenation (H/R) were used to simulate neuronal damage that occurs in cerebral ischemia. These cells were treated with PA to investigate whether PA protects from neuronal damage in cerebral ischemia *via* miR-155/NRF2/HO-1 axis.

METHODS

Cell culture

Neuronal cells (N2a cells) were acquired from American Type Culture Collection (ATCC, USA). Cells were cultured with Dulbecco's modified eagle medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% streptomycin-penicillin (Sigma-Aldrich, USA). Control condition: N2a cells were incubated in the medium with 5% CO_2 at 37°C. H/R condition: N2a cells were incubated in 5% CO_2 and 95% N_2 with deoxygenated glucose-free Hanks' Balanced Salt Solution (Invitrogen, USA) for 8 h. Then the medium was replaced with DMEM containing 10% FBS and 1% streptomycin-penicillin, and cells were cultured at 5% CO_2 and 95% air for 24 h.

Cell transfection

Plasmids encoding miR-155 inhibitor, NC inhibitor, NRF2 siRNA and NC siRNA were purchased from Tiangen (Beijing, China). 125 µl Lipofectamine 2000 (Invitrogen, USA) was used to transfect cells. After

48 h of transfection, we determined the transfection efficiency.

Western blot analysis

Total protein was extracted from ground samples by RIPA lysis buffer (Solarbio, China). Subsequently, the concentration of protein in the extract was determined by the BCA protein kit (Abcam, Cambridge, UK). Total protein was separated using SDS-PAGE electrophoresis, and then separated protein was transferred to a PVDF membrane (Invitrogen, USA). The membrane was first incubated with the primary antibody overnight at 4°C and then with secondary antibody with HRP for 4 h at 4°C. Pierce™ ECL Western Blotting Substrate (Thermo Scientific, China) and Bio-Rad XR gel imaging analysis system (Bio-Rad, USA) were used to determine amount of protein present. All antibodies were purchased from Abcam, including Bcl-2 (ab182858, 1:2000), Bax (ab182733, 1:2000), HO-1 (ab52947, 1:2000), NRF2 (ab62352, 1:1000), GAPDH (ab8245, 1:10000) and goat anti-mouse IgG H&L (HRP, ab205719, 1:10000). Note: GAPDH was internal reference.

Real-time PCR

Total RNA was extracted with the Trizol reagent (Invitrogen, USA). Then, the OD value at 260 nm/280 nm was measured using a spectrophotometer to determine the purity of RNA. All-in-One™ miRNA qRT-PCR Detection System (GeneCopoeia, China) was used for PCR. The primers for miR-155 and NRF2 were designed and synthesized by Tiangen Co., LTD (Beijing, China). The reverse transcription and fluorescence quantification were studied by FastKing One Step RT-qPCR kit (SYBR) (Tiangen, China). Manufacturer instructions were followed for the reaction system and program. The internal reference genes were U6 and GAPDH. The relative expression of miR-155 and NRF2 was normalized by $2^{-\Delta\Delta Cq}$. Table 1 lists the primers of miR-155, NRF2, U6 and GAPDH.

Apoptosis assay

Apoptosis was studied by AnnexinV-FITC/PI fluorescence double-staining apoptosis kit (P-CA-201, Procell, China) and flow cytometry (BD Biosciences, USA). The extract from cells was supplemented with 500 µL 1×Annexin V Binding buffer, 5 µL Annexin V-FITC and 5 µL PI and incubated for 20 min in the dark. Then, the apoptosis rate was determined by flow cytometry and cellquest software (BD Biosciences, USA).

Table 1. Primer sequence.

Primers	Sequences 5′ to 3′
miR-155 (forward)	ACACTCCAGCTGTAAACATCCTACACTCT
miR-155 (reverse)	CTCAACTGGTGTCGTGGA
NRF2 (forward)	GCTCAAACTTAGGGGCTCCG
NRF2 (reverse)	TCCGGGTGTGTTTGTTCCAA
U6 (forward)	ATTGGAACGATACAGAGAAGATT
U6 (reverse)	GGAACGCTTCACGAATTTG
GAPDH (forward)	AAAAGGGCCCTGACAACTCT
GAPDH (reverse)	TACATGACAAGGTGCGGCTC

Cell viability assay

MTT assay was used to determine the viability of neuronal cells. First, neuronal cells were seeded into 96-well plate and cultured with DMEM. Then, the MTT assay kit (Abcam, UK) was used according to manufacturer instructions to detect cell viability. Briefly, after 24 h, the cells were stained by MTT for 10 min, and the OD value at 570 nm was measured to quantify viability.

Dual luciferase reporter gene

The binding site between miR-155 and 3' UTR of NRF2 was analyzed *via* Targetscan7.2. pGLO reporter containing 3' UTR of NRF2 with the wild type or mutant predicted sit were designed and synthetized according to the prediction. NRF2 wild type contained the predicted site, but NRF2 mutant lacked the same site. The pGLO plasmid was the expression vector for both NRF2 wild type and NRF2 mutant. pGLO-NRF2 wild type plasmid or pGLO-NRF2 mutant plasmid were co-transfected with a miR-155 mimic into HEK293 cells *via* Lipofectamine 2000 (Invitrogen, USA). After 48 h, the luciferase activity in cells was measured *via* double luciferase reporter gene detection system (Promega). The relative luciferase activity was calculated by the following equation: relative luciferase activity = firefly-luciferase activity/renilla-luciferase activity.

Statistics and analysis

We used SPSS 22.0 (IBM, USA) and Graphpad 8.0 (USA) to calculate the statistics and visualize data. We used one-way ANOVA to analyze the differences among

groups and LSD-t test to determine pairwise comparison. Significance level was set to P<0.05. All comparisons were two-sided tests. 95% was considered as the confidence interval. The data came from three independent experiments for statistical analysis.

RESULTS

PA ameliorates the damage of H/R-induced neuronal cells

PA might play a considerable role in neuronal damage during cerebral ischemia/reperfusion. Neuronal cells induced by H/R were used to simulate the neuronal damage in cerebral ischemia/reperfusion. These cells were, respectively, treated with 5 μM PA, 10 μM PA and 20 μM PA to determine the neuroprotective mechanism of PA in cerebral ischemia/reperfusion. Fig. 1A shows that cell viability decreased under H/R condition (H/R group *vs.* control group, P<0.001) but that PA could reverse that trend (H/R+5 μM PA group *vs.* H/R group, P<0.05. H/R+10 μM PA group *vs.* H/R group, P<0.01. H/R+20 μM PA group *vs.* H/R group, P<0.001). As shown in Fig. 1B, H/R condition causes apoptosis (H/R group *vs.* control group, P<0.01), which is inhibited by PA (H/R+5 μM PA group *vs.* H/R group, P<0.05. H/R+10 μM PA group *vs.* H/R group, P<0.05. H/R+20 μM PA group *vs.* H/R group, P<0.01). Bcl-2 and Bax, two apoptosis regulators, were used to determine apoptosis at a molecular level. As illustrated in Fig. 1C, H/R condition decreases Bcl-2 and increases Bax (H/R group *vs.* control group, P<0.01.), but that trend is reversed by PA (H/R+5 μM PA group *vs.* H/R group, P<0.05. H/R+10 μM PA group *vs.* H/R group, P<0.05. H/R+20 μM PA group *vs.* H/R group, P<0.01).

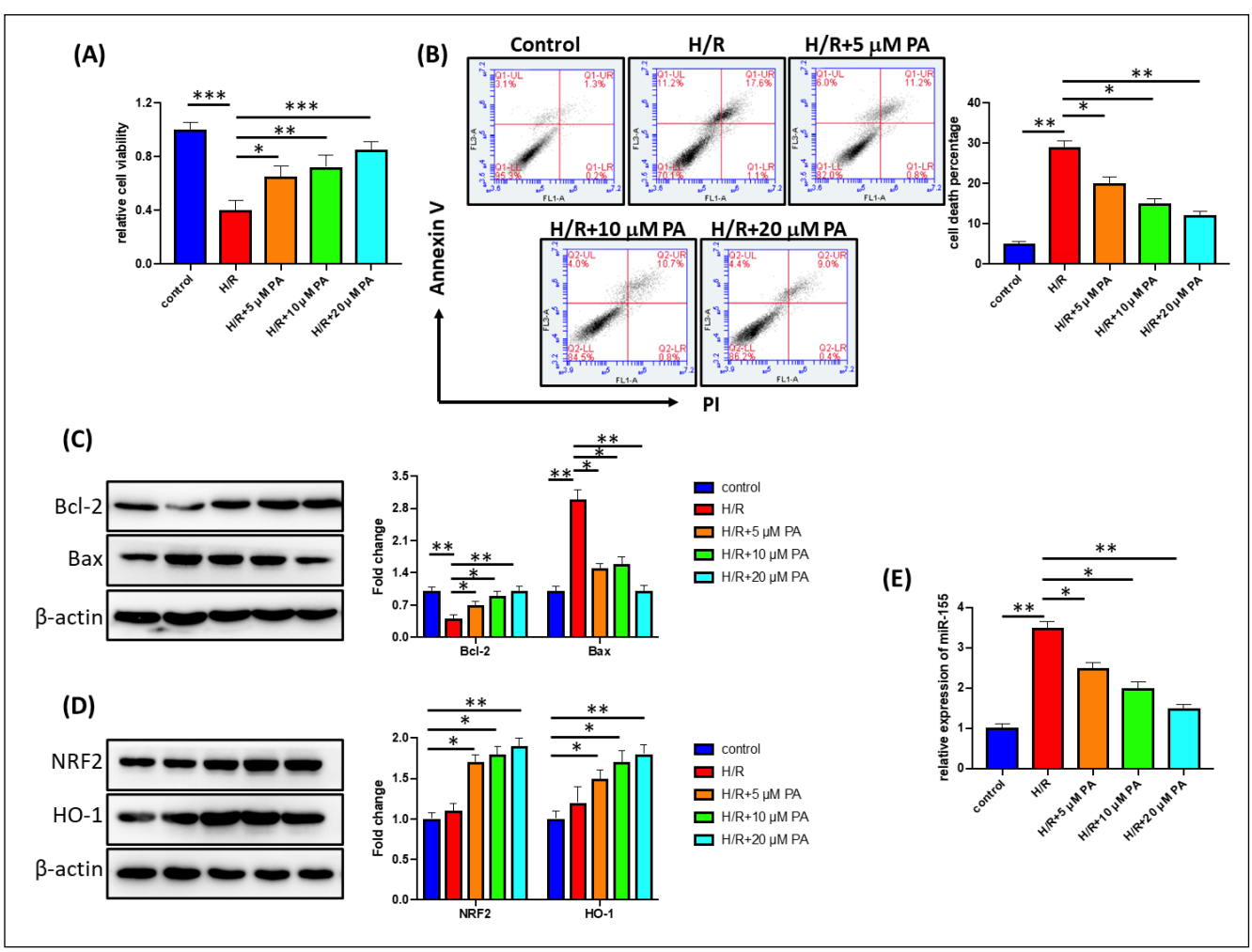

Fig. 1. PA ameliorated the damage of H/R-induced neuronal cells. (A) PA reversed the decrease of cell viability induced by H/R. (B) PA repressed apoptosis caused by H/R. (C) PA reversed the decreased Bcl-2 and increased Bax induced by H/R. (D) The expression of HO-1 and NRF2 was independent of H/R but raised by PA. (E) PA inhibited miR-155 upregulated by H/R.

NRF2/HO-1 pathway emerges as having a significant role in cerebral ischemia/reperfusion. In Fig. 1D, the H/R condition does not show different expression of HO-1 and NRF2 compared to the control condition (H/R group *vs.* control group, P>0.05), but PA upregulates the expression of HO-1 and NRF2 (H/R+5 μM PA group *vs.* H/R group, P<0.05. H/R+10 μM PA group *vs.* H/R group, P<0.05. H/R+20 μM PA group *vs.* H/R group, P<0.01). miR-155, one member of the microRNA family, exerts different effects on cerebral ischemia/reperfusion. Fig. 1E suggests that the expression of miR-155 is upregulated by the H/R condition (H/R group *vs.* control group, P<0.05), which is again inhibited by PA (H/R+5 μM PA group *vs.* H/R group, P<0.05. H/R+10 μM PA group *vs.* H/R group, P<0.05. H/R+20 μM PA group *vs.* H/R group, P<0.01). These results indicate that PA ameliorates the damage of H/R-induced neuronal cells.

Inhibited miR-155 improves the damage of H/R-induced neuronal cells

Fig. 1E reveals that miR-155 expression is induced by H/R condition but inhibited by PA. Therefore, we downregulated the expression of miR-155 *via* miR-155 inhibitor (Fig. 2A) to determine the role of inhibited miR-155 in neuronal damage (P<0.01). Fig. 2B, C show that inhibited miR-155 could reverse the decrease in cell viability and the increase in apoptosis that were caused by the H/R condition (P<0.05). Fig. 2D suggests that inhibited miR-155 reverses the decreased Bcl-2 (P<0.05) and increased Bax (P<0.01) caused by H/R condition. As illustrated in Fig. 2E, inhibited miR-155 upregulates the expressions of NRF2 (P<0.01) and HO-1 (P<0.05) in H/R-induced neuronal cells. These results implied that inhibited miR-155 improves the damage of H/R-induced neuronal cells.

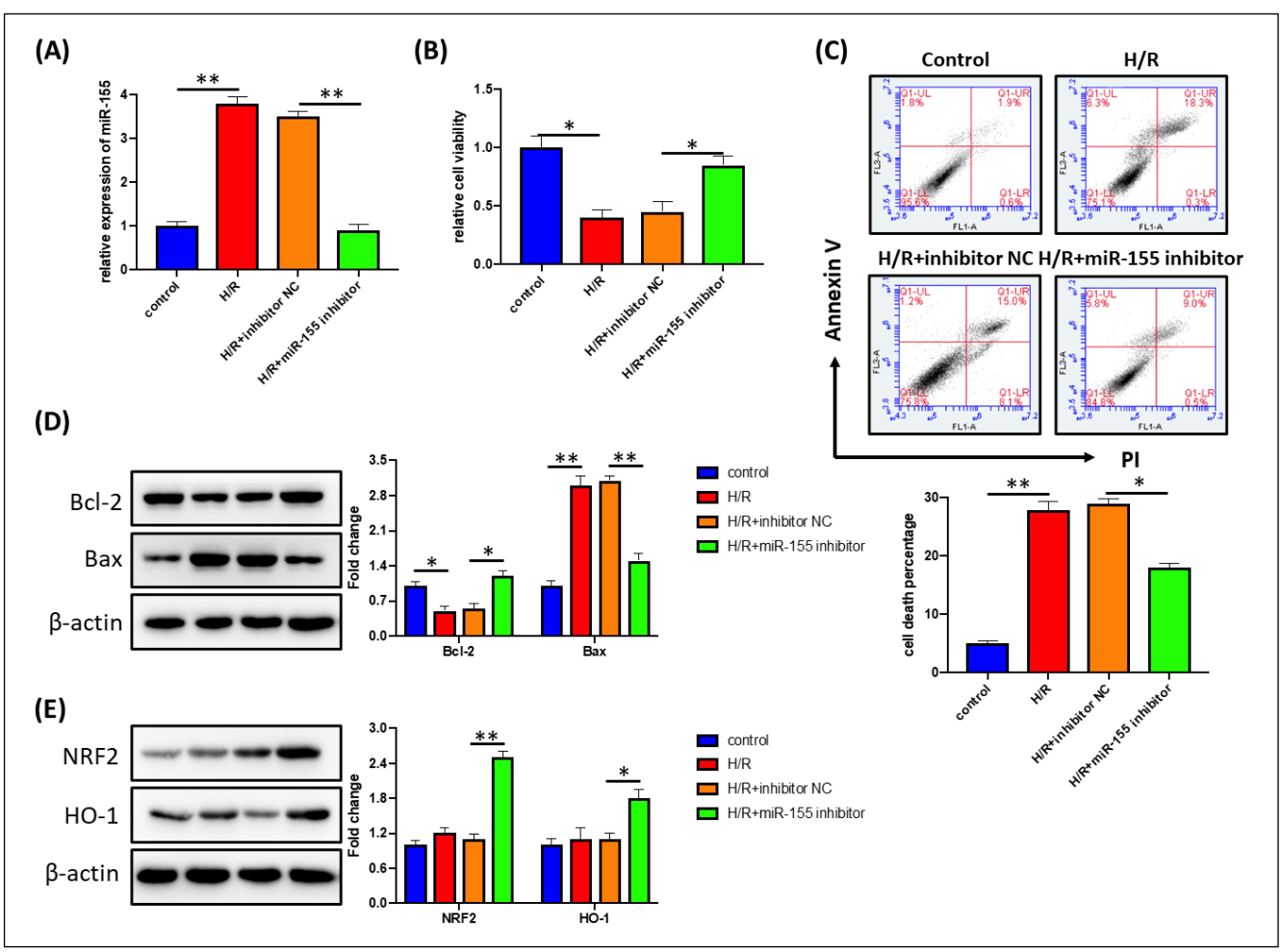

Fig. 2. Inhibited miR-155 improved the damage of hypoxia/reoxygenation-induced neuronal cells. (A) miR-155 inhibitor decreased the expression of miR-155 increased by H/R. (B) Inhibited miR-155 raised cell viability repressed by H/R. (C) Inhibited miR-155 reduced apoptosis induced by H/R. (D) Inhibited miR-155 reversed the decreased Bcl-2 and increased Bax induced by H/R. (E) The expression of HO-1 and NRF2 was independent of H/R but raised by inhibited miR-155.

NRF2 is the target of miR-155

As shown in Fig. 2E, the level of NRF2 protein increases with the decrease of miR-155, which indicates that miR-155 might be involved in the expression of NRF2. Targetscan7.2, a bioinformatic tool, predicts that there is a binding relationship between miR-155 and the 3' untranslated region (UTR) of NRF2 (Fig. 3A). Double luciferase reporter assay is used to determine the binding site between miR-155 and 3'UTR of NRF2. As illustrated in Fig. 3B, when miR-155 mimic and NRF2 wild type are present (including binding site at 3'UTR), the relative luciferase activity is increased compared with negative control (P<0.05). This finding suggests that miR-155 could bind to the 3' UTR of NRF2 *via* the predicted site. Fig. 4C, D show that the miR-155 mimic inhibits the expression of NRF2 (P<0.05) but that miR-155 inhibitor promotes NRF2 expression (P<0.05), which means that NRF2 is negatively regulated by miR-155. To summarize, NRF2 is the target of miR-155, and its expression is negatively regulated by miR-155.

PA protects neuronal cells via downregulating miR-155

Treatment with 10 μM PA was used to determine the relationship between miR-155 and PA in neuronal damage of cerebral ischemia/reperfusion. While the expression of miR-155 is inhibited by PA in neuronal cells (P<0.01), miR-155 mimic upregulates the expression of miR-155 in cells (P<0.001) (Fig. 4A). Fig. 4B, C show that the miR-155 mimic reverses the increase in cell viability (P<0.05) and the decrease in apoptosis (P<0.05) that were caused by PA. Moreover, the finding that PA inhibits Bax (P<0.01) and promotes Bcl-2 (P<0.05) is offset by the miR-155 mimic (P<0.05), as shown in Fig. 4D. As illustrated in Fig. 4E, the miR-155 mimic represses the activation of the NRF2-HO-1 pathway caused by PA (both P<0.05). These results mean that PA protects neuronal cells *via* downregulating miR-155.

Inhibited miR-155 prevents neuronal cells from hypoxia/reoxygenation via upregulating NRF2

How did miR-155 regulate neuronal cells induced by H/R? It has been confirmed that NRF2 is the target of miR-155, which underscores that miR-155 has a regulatory role *via* NRF2 in H/R-induced neuronal cells. As shown in Fig. 5A, B, inhibited miR-155 ameliorates neuronal injury by increasing cell viability (P<0.05 *vs.* inhibitor NC group) and decreasing apoptosis (P<0.05 *vs.* inhibitor NC group), whereas the trend is reversed by NRF2 silence (P<0.05 *vs.* siNC group). Fig. 5C suggests that NRF2 silence offsets the influence (P<0.05 *vs.* siNC group) of the miR-155 inhibitor, which causes Bcl-2 to be upregulated and Bax to be downregulated. Fig. 5D shows that NRF2 silence (P<0.01 *vs.* inhibitor NC group) prevents the miR-155 inhibitor from upregulating the

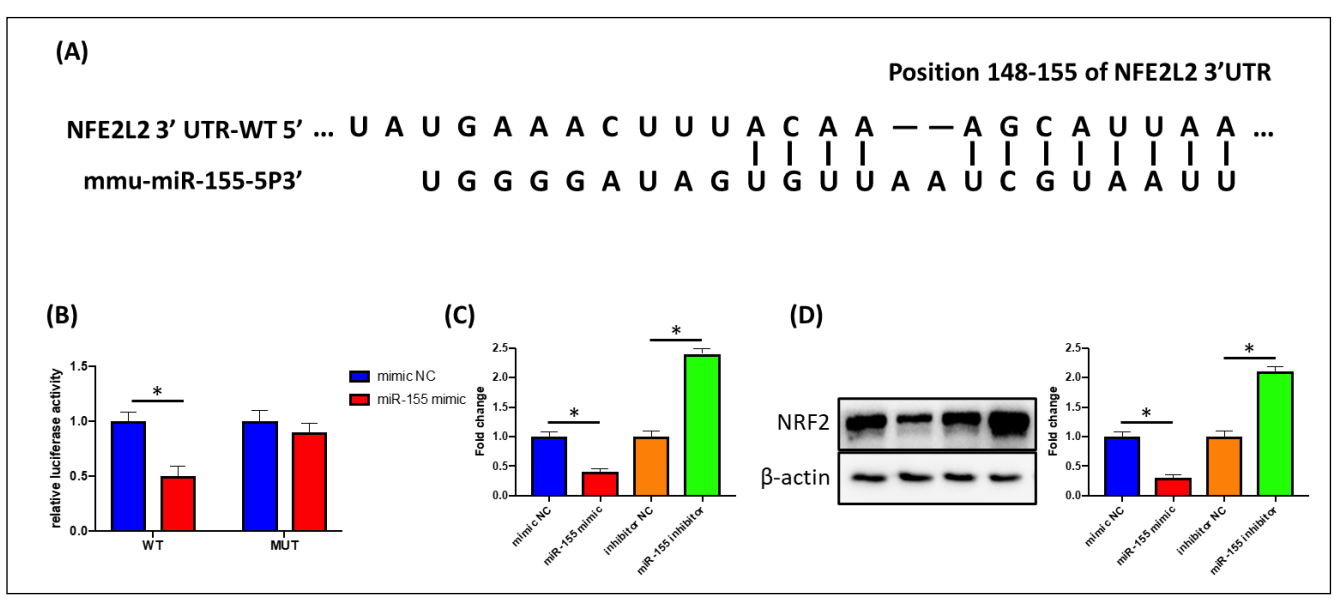

Fig. 3. NRF2 was the target of miR-155. (A) The predicted site between miR-155 and NRF2 *via* Targetscan7.2. (B) Double luciferase reporter assay determined miR-155 bind to NRF2. (C) miR-155 mimic downregulated NRF2 mRNA but miR-155 inhibitor upregulated that. (D) miR-155 mimic downregulated NRF2 protein but miR-155 inhibitor upregulated that.

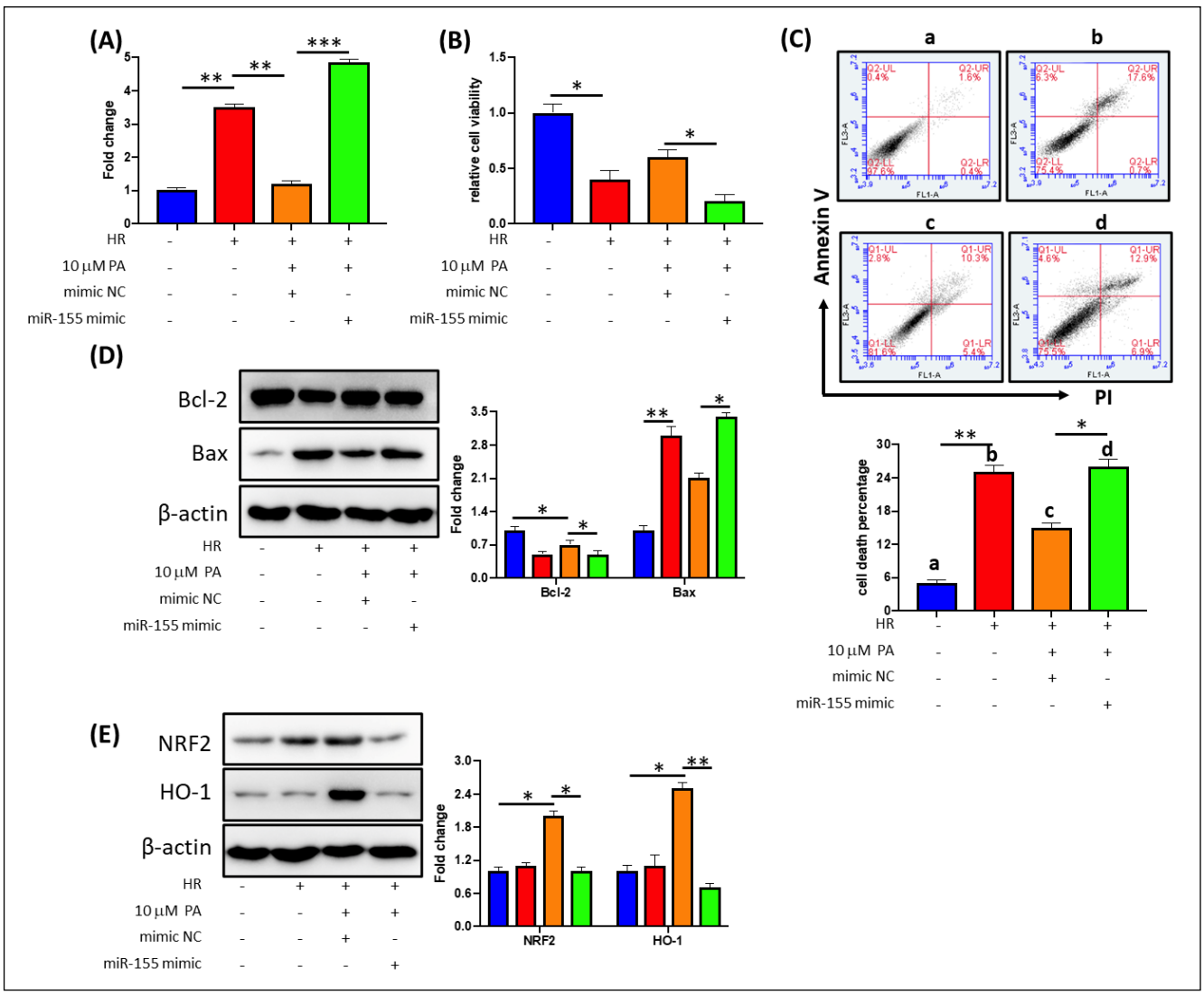

Fig. 4. PA protected neuronal cells *via* downregulating miR-155. (A) PA decreased miR-155 but miR-155 mimic increased that. (B) miR-155 mimic inhibited cell viability promoted by PA. (C) miR-155 mimic reversed the reduction of apoptosis caused by PA. (D) miR-155 mimic reversed the PA-decreased Bcl-2 and PA-increased Bax. (E) The PA-induced expression of HO-1 and NRF2 was reduced by miR-155 mimic.

expressions of NRF2 ($P<0.05$ *vs.* siNC group) and HO-1 ($P<0.01$ *vs.* siNC group). To conclude, inhibiting miR-155 prevents neuronal cells in the H/R condition from up-regulating NRF2.

DISCUSSION

Cerebral ischemia/reperfusion commonly aggravates neuronal damage. Mahura (2003) concluded that reactive oxygen species (ROS), an oxygen free radical induced by ischemia/reperfusion, triggers the inflammation and apoptosis of neuronal cells. Dornbos and Ding (2012) suggested that exercise pre-conditioning could alleviate the injury caused by ischemia/reperfusion by promoting neurovascular formation and inhibiting the neuroinflammation and neuronal apoptosis. A previous investigation (Sun et al., 2014) found that gallic acid regulates mitochondrial dysfunction-mediated neuronal damage to improve the damage caused by cerebral ischemia/reperfusion. Pickell et al. (2020) summarized that histone deacetylase inhibitors serve as potential therapies for cerebral ischemia/reperfusion due to their neuroprotective effects. These previous findings indicate that neuroprotection is as an important mechanism for the management of cerebral ischemia/reperfusion. Neuroprotection prevents brain function from

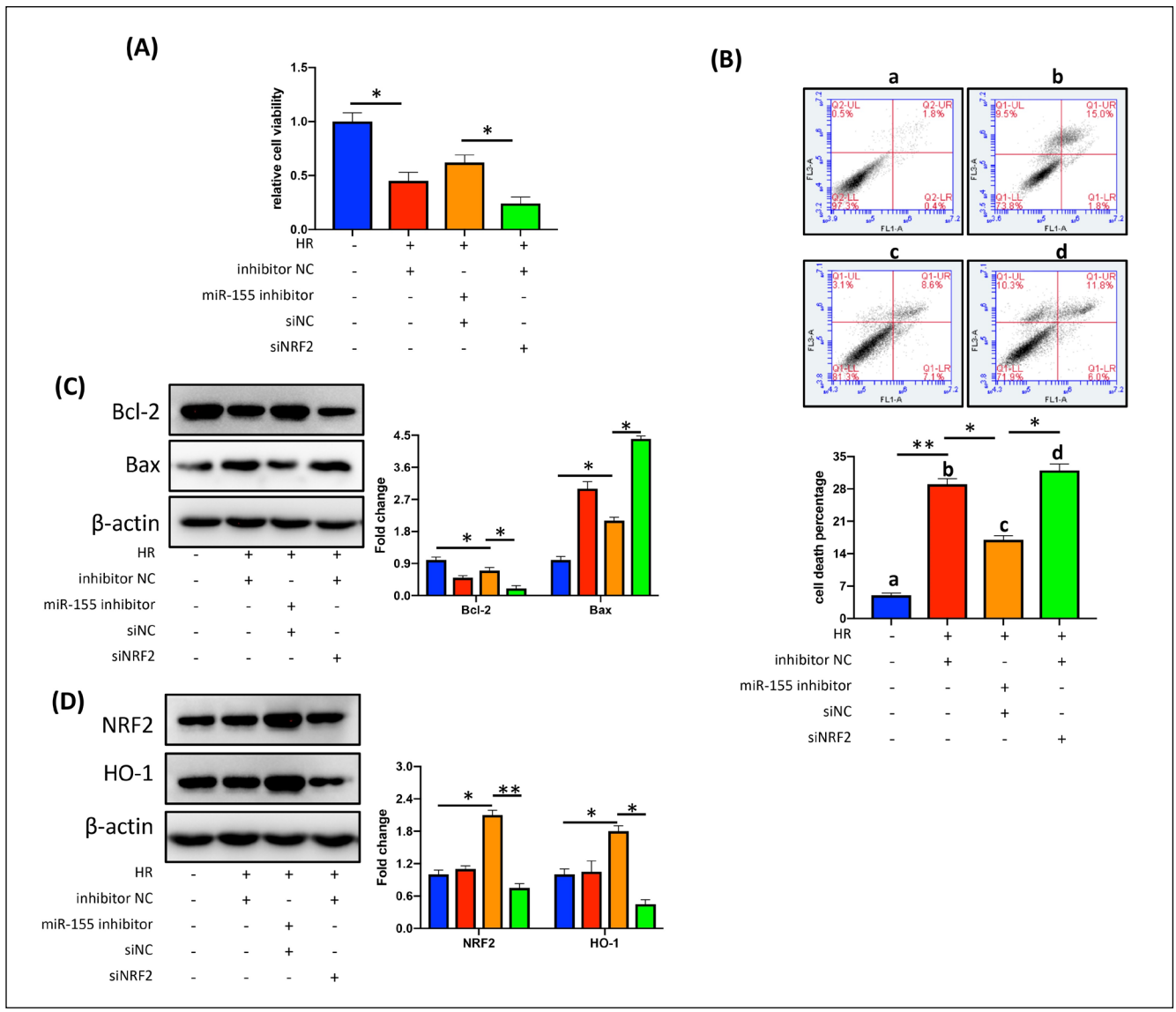

Fig. 5. Inhibited miR-155 prevented neuronal cells from hypoxia/reoxygenation *via* upregulating NRF2. (A) Silenced NRF2 repressed the increase of cell viability induced by miR-155 inhibitor. (B) Silenced NRF2 reversed apoptosis decreased by miR-155 inhibitor. (C) Silenced NRF2 reversed the miR-155 inhibitor-decreased Bcl-2 and miR-155 inhibitor-increased Bax. (D) The miR-155 inhibitor-increased expression of HO-1 and NRF2 was reduced by silenced NRF2.

diminishing during cerebral ischemia/reperfusion by reducing the risk of disability for patients with cerebral ischemia (Patel and McMullen, 2017).

We found that PA could enhance the survival of neuron cells induced by H/R *via* inhibiting apoptosis, which suggests that PA shows a potential neuroprotective effect in cerebral ischemia/reperfusion. PA possesses anti-ferroptosis and anti-neuronal apoptosis in ischemia injury (Pang et al., 2020; Jiang et al., 2021). Nonetheless, the concrete neuroprotective mechanism of PA remains unclear. We found that PA could suppress the increase of miR-155 caused by H/R. miRNA is a potential biomarker of ischemia, which contributes to the process of ischemia at molecular level *via* regulation mRNA during post-transcriptional processes (Forouzanfar et al., 2019). The phenomenon suggests that PA might manipulate the expression of miR-155 to regulate cellular process, including apoptosis and viability. Lopez-Ramirez et al. (2014) reported that miR-155 is the key negative regulator of the blood/brain barrier *via* promoting neuroinflammation, which means it was deeply involved in ischemia process. In cerebral ischemia/reperfusion, the inhibition of miR-155 could ameliorate inflammation and neuronal function (Zhang

et al., 2020; Shi et al., 2020; Suofu et al., 2020). Thereby, we assumed that miR-155 is the therapeutic target by which PA suppresses neuronal damage in cerebral ischemia/reperfusion.

How did miR-155 emerge as a regulator of cerebral ischemia/reperfusion? We downregulated the expression of miR-155 in neuronal cells induced by H/R to investigate the role of inhibited miR-155 in cerebral ischemia/reperfusion. We found that inhibited miR-155 reduces Bcl-2/Bax-involved apoptosis in H/R-induced neuronal cells. Moreover, inhibited miR-155 stimulates the expression of NRF2, which is the target of miR-155. A previous investigation (Ya et al., 2018) suggested that the activation of NRF2 possesses anti-oxidant and neuroprotective properties for cerebral ischemia/reperfusion. Dai et al. (2018) published the finding that NRF2, induced by isoquercetin, translocates from the cytoplasm to the nucleus to activate the NOX4 pathway, which mediates neuroprotection and oxidative stress in cerebral ischemia/reperfusion. NRF2/HO-1 pathway is involved in the process of cerebral ischemia/reperfusion. Phosphorylated NRF2 translocates to the nucleus and activates HO-1 to promote cell survival under stress conditions (Kensler et al., 2007). In this investigation, inhibited miR-155 prevents neuronal cells from H/R condition *via* NRF2/HO-1 pathway and Bcl-2/Bax-mediated apoptosis. Thus, we found that pachymic acid emerges as a protective agent for neuronal cells induced by H/R *via* targeting miR-155/NRF2/HO-1 axis.

We found that the protective effect of 10 μM PA is more obvious than that of 5 μM PA, but there is no difference between 10 μM PA and 20 μM PA in terms of neuroprotective effects. Is 10 μM the optimal dose of PA for cerebral ischemia/reperfusion? This question is worth studying in the future investigations. Moreover, NRF2/HO-1-mediated oxidative stress manipulates the inflammation in cerebral ischemia/reperfusion, which is not reflected in this investigation. Thus, we plan to pursue the anti-inflammation properties of pachymic acid in cerebral ischemic/reperfusion *via* NRF2/HO-1-mediated oxidative stress in the future.

Pachymic acid has emerged as neuroprotective role in cerebral ischemia/reperfusion *via* targeting miR-155/NRF2/HO-1 axis, which indicates that pachymic acid might be a reliable and rational drug for the treatment of cerebral ischemia.

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