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Perillaldehyde attenuates cerebral ischemia–reperfusion injury-triggered overexpression of inflammatory cytokines via modulating Akt/JNK pathway in the rat brain cortex

Lixing Xu, Yuebi Li, Qiang Fu *, Shiping Ma *

Department of Pharmacology of Chinese Materia Medica, China Pharmaceutical University, Nanjing 210009, PR China

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ABSTRACT

Perillaldehyde (PAH), one of the major oil components in *Perilla frutescens*, has anti-inflammatory effects. Few studies have examined the neuroprotective effect of PAH on stroke. So the aim of our study is to investigate the effect of PAH on ischemia–reperfusion-induced injury in the rat brain cortex. Middle cerebral artery occlusion (MCAO) model was selected to make cerebral ischemia–reperfusion injury. Rats were assigned randomly to groups of sham, MCAO, and two treatment groups by PAH at 36.0, 72.0 mg/kg. Disease model was set up after intragastrically (i.g.) administering for 7 consecutive days. The neurological deficit, the cerebral infarct size, biochemical parameters and the relative mRNA and protein levels were examined. The results showed that the NO level, the iNOS activity, the neurological deficit scores, the cerebral infarct size and the expression of inflammatory cytokines including interleukin (IL)-1β, interleukin (IL)-6 and tumor necrosis factor (TNF)-α were significantly decreased by PAH treatment. PAH also increased the Phospho-Akt level and decrease the Phospho-JNK level by Western blot analysis. Meanwhile, the PAH groups exhibited a dramatically decrease of apoptosis-related mRNA expression such as Bax and caspase-3. Our findings shown that PAH attenuates cerebral ischemia/reperfusion injury in the rat brain cortex, and suggest its neuroprotective effect is relate to regulating the inflammatory response through Akt /JNK pathway. The activation of this signalling pathway eventually inhibits apoptotic cell death induced by cerebral ischemia–reperfusion.

1. Introduction

All therapies for cerebral ischemia–reperfusion injury have failed, leaving stroke as a leading cause of death, disability and waste of medical resources. Stroke is a terrifying ordeal that always despoils people of independent living by damaging language and movement without warning [1]. It caused by thrombotic or embolic occlusion and following recover of blood flow, which results in excitotoxicity, ionic imbalance, oxidative stress, inflammation and apoptosis. Inflammation is a key regulatory factor in pathological states and it usually contributes to blood brain barrier (BBB) disruption, mitochondria dysfunction and cytoskeleton damaged [2,3]. After cerebral ischemia–reperfusion, inflammatory response, including the rapid activation of resident microglial cells and infiltration of neutrophils and macrophages into the injured parenchyma, is involved in brain injury [4]. Microglia, as effector cells in brain damage and repair, are associated with central nervous system (CNS) pathology [5]. Microglial activation also occurs rapidly after targeted disruption of the BBB, followed by producing of cytokines containing IL-1β, IL-6 and TNF-α [6,7]. Therefore, we may put forward a hypothesis that controlling the expression of inflammatory factor might be a strategy for advancing stroke therapy.c-Jun N-terminal kinase (JNK), a serine/threonine protein kinase that phosphorylates c-Jun, is an available regulatory factor of inflammation [8]. Activation of PI3K-Akt signaling pathway can downregulate JNK activity through regulating the balance between upstream kinases and phosphatases, which plays a critical role in maintaining neurons structure integrity and advancing survival of neuronal cell [9,10]. The prevailing belief that cerebral ischemia triggers an intricate cascade of signaling pathway at molecular and cellular levels, but the regulation of inflammation by PI3K-Akt and JNK pathway is extremely important in the central nervous system (CNS) and in the periphery [11].

Perillaldehyde (PAH) is a major component in the essential oil extracted from perilla Herba and has been found exhibiting

* Corresponding authors at: Department of Pharmacology of Chinese Materia Medica, China Pharmaceutical University, Tongjiaxiang 24, Nanjing, Jiangsu 210009, PR China. Fax: +86 25 3271505 (S. Ma).

E-mail addresses: fuqiang@cpu.edu.cn (Q. Fu), spma@cpu.edu.cn (S. Ma).

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vasodilative effect and anti-inflammatory effects [12,13]. In consideration of previous studies, we thought that PAH might be a feasible or practicable attempt to treat stroke, which associates with inflammatory mechanisms more or less. So the aim of our study is to evaluate the neuroprotective effects of PAH in rat with acute ischaemic stroke and to explore the relationship between immunoinflammatory variables and acute neurological deficits.

2. Materials and methods

2.1. Preparation of drugs

Perillaldehyde, purchased from Changsha Kaimei Essence & Flavor Co., Ltd. (Hunan, PR China), was diluted in 0.18% and 0.36% with 0.03% sodium carboxymethyl cellulose (CMC-Na).

2.2. Experiment animals

Male Sprague–Dawley rats (weighing 230–270 g) were purchased from Comparative Medicine Centre of Yangzhou University (Yangzhou, China). Rats were kept in cages and maintained under standard housing conditions with a12-h light/12-h dark cycle, the room temperature was kept at 25 ± 1 °C and the humidity was maintained at 60–65%. All rats were provided with water and standard chow ad lib for the whole experiment procedure. All the experiments and animal care were performed strictly in accordance with the Provision and General Recommendation of Chinese Experimental Animals Administration Legislation and were approved by the Science and Technology Department of Jiangsu Province.

2.3. MCAO

After acclimatization to the environment for 7 days, sixty animals were randomly divided into 4 groups: sham- operated control (sham), MCAO and PAH groups at low (18 mg/kg) and high (36 mg/kg) dosages. The first two groups were intragastrically (i.g.) administered with CMC-Na for 7 days, while the other groups were administered with drugs.

After drug delivery, middle cerebral artery occlusion (MCAO) was made using the intraluminal suture technique with reference to origin of the right middle cerebral artery by Longa. Briefly, rats were anesthetized with intraperitoneal injection of chloral hydrate (350 mg/kg, i.p.). The right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were exposed through a short incision. CCA and ECA were ligated and ICA was temporarily clamped by using bulldog clamp. To occlude the middle cerebral artery, a 3–0 monofilament nylon suture with the tip rounded was inserted into the external carotid artery and advanced into the internal carotid artery approximately 18–20 mm beyond the carotid bifurcation until mild resistance was felt. Rectal temperature was maintained at 36.6–37.5 °C with a heating blanket throughout the procedure. Rats were subjected to 2 h of focal ischemia and the monofilament nylon suture was pulled out gently to accomplish 24 h reperfusion thereafter.

2.4. Neurobehavioral deficit evaluation

Eight rats in each group were quantified at 1 h and 24 h after reperfusion by using a five-point scale: 0, no (normal); 1, slight (fail to flex left forepaw fully); 2, moderate (counterclockwise circling); 3, severe (lean to the left); and 4, very serious neurobehavioral deficit (unconsciousness, fail to walk autonomously and response to noxious stimulus) [14].

2.5. Measurement of infarct volume and determination of infarction rate

Three rats in each group were decapitated for TTC staining. The fresh brains were stored immediately at 20 °C for 15 min after removal of cerebellum and then were cut into 2-mm thick coronal slices. Slices were placed into a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) and then warmed in a thermostatic Water Bath at 37 °C for 30 min. Slices treated with paraformaldehyde (4%) were refrigerated overnight at 4 °C. The measured slices were photographed, and the area of ischemic brain injury was calculated by a blinded observer with Image J software, expressed as infarct area percentage (%).

2.6. ELISA detection of IL-1β, TNF-α and IL-6 concentrations

Three rat serums in each group were taken immediately after neurobehavioral deficit evaluation. The concentration of cytokines was determined using commercially available ELISA assays, following the instructions supplied by the manufacturer (R&D, Minneapolis, MN, USA) and quantified by a microplate reader (450 nm). The results were shown as picograms per milliliter (pg/ml).

2.7. Oxidative stress-related biochemical index measurement

Eight rat brain cortexes in each group were homogenized in 10% physiological saline and then centrifuged, and then the supernatants were examined as soon as possible to determine the levels of Nitric oxide (NO) and the activities of inducible nitric oxide synthase (iNOS) using corresponding test kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.8. Western blot analysis

Three rat brain cortexes in each group were thawed on ice, weighed rapidly and homogenized in ice-cold tissue lysis buffer (w:v 1:5). The lysed tissue was centrifuged after 30 min pyrolysis at 12,000 r for 20 min at 4 °C. The total protein concentration of the supernatant was determined using bicinchoninic acid (BCA) protein assay kit (Beyotime, Haimen, China) according to the manufacturer's instructions. After heating at 100 °C for 5 min, the proteins were separated on SDS–PAGE gels and transferred to the polyvinylidenedifluoride (PVDF) membranes (Bio-Rad, Laboratories, Inc., California) for immune blotting. The membrane was then blocked with blocking solution (5% skimmed milk) at room temperature for 2 h and followed by an overnight incubation at 4 °C with anti-Akt, Phospho-Akt, Phospho-JNK monoclonal antibodies (1:1000 dilution, Cell Signaling Technology, MA, USA). After wash in phosphate-buffered saline (PBS)-Tween, the membranes were incubated with secondary antibodies (1:5000 dilution, Cell Signaling Technology, MA, USA), followed by three washing in TBS-T. Immunoreactivity was detected with enhanced chemiluminescence detection system (Beyotime, Haimen, China) and visualized on X-ray film (Kodak, Shanghai, China). β-actin (1: 1000; Cell Signaling Technology, MA, USA) was used as control.

2.9. Real-time quantitative reverse transcription polymerase chain reaction (Q-PCR)

Eight rat brain cortexes in each group were used for Q-PCR analysis. Total RNA was extracted from the thawed brain cortex using the RNAiso Reagent kit (Vazyme Biotech, Nanjing, China). cDNA was generated by reverse transcription of total RNA using reverse transcription kit (TransGen Biotech, Beijing, China). The sequences of forward and reverse oligo nucleotide primers were designed using Primer 5 software. The primers used were the following:

Bax primer, sense: 5′-CGA TGA ACT GGA CAA CAA CAT GGA G-3′, antisense: 5′-GCA AAG TAG AAA AGG GCA ACC-3′; caspase-3 primer, sense: 5′- ACA TCC TCA CTC GTG TTA ACC GGA A-3′, antisense: 5′-CAC GGG ATC TGT TTC TTT GC-3′; β-actin primer, sense: 5′-ACC ACC ACA GCT GAG AGG GAA ATC G-3′, antisense: 5′-TTC TCC AGG GAG GAA GAG G-3′. Quantitative real-time PCR was performed on CFX96 System (Bio-Rad, USA) by using AceQTMqPCR SYBR Green Master Mix (Vazyme Biotech, Nanjing, China). The PCR cycle conditions were 95 °C for 5 min, followed by 40 cycles of 10 s at 95 °C and 30 s at 55 °C and the amplification specificity was evaluated with a melting curve analysis.

2.10. Statistical analysis

All results presented in the paper were shown as the mean ± S.D. and subject to a one-way analysis of variance (ANOVA), followed by Tukey's test for inter-group comparisons. For all the analyses used by the GraphPad Prism 5.0 software statistical package (GraphPad Software, San Diego, CA, USA), a probability value less than 0.05 was considered statistically significant.

3. Results

3.1. Effect of PAH on neurological scores

The neurologic deficit scores 1 h and 24 h after reperfusion were presented in Fig. 1A. As results shown, no deficits were observed in the sham. Compared to the sham, the scores of MCAO group were significantly increased. While the scores in groups treated with PAH were all significantly decreased compared to MCAO.

3.2. Effect of PAH on the rate of cerebral infarction

According to the results of TTC staining, and PAH significantly decreased the infarct volume. It indicated that PAH could relief the cerebral ischemia–reperfusion injury (Fig. 1B and C).

3.3. Effects of PAH on serum pro-inflammatory cytokine levels

As shown in Fig. 2A–C, the serum levels of IL-β, IL-6 and TNF-αin MCAO group significantly increased compared to sham. However, pretreatment with PAH (18, 36 mg/kg) significantly attenuated ischemia/reperfusion injury-induced upregulation of pro-inflammatory cytokine levels.

3.4. Regulation effect of PAH on oxidative stress

The content of NO was significantly increased in MCAO group compared to the sham, while significantly decreased in the PAH treatment groups compared to MCAO group (Fig. 2D). PAH also reverse the abnormal increase of iNOS activity by ischemia–reperfusion (Fig. 2E).

3.5. Effect of PAH on Phospho-Akt level

The effects of PAH on activating of Akt were determined by Western blot analysis (Fig. 3A). Cerebral ischemia/reperfusion significantly decreased Akt phosphorylation in ischemic brain. Rats

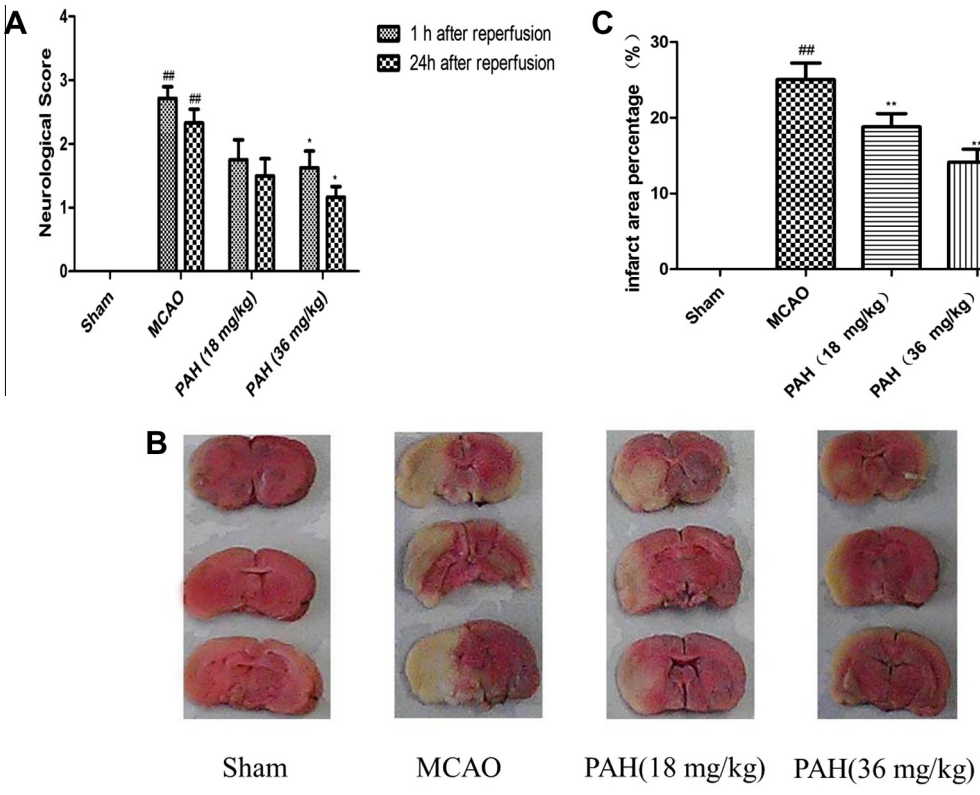

Fig. 1. (A) PAH obviously decreased the neurologic deficit scores 1 h and 24 h after reperfusion. (B) The infarct area were shown as white color, and groups treated PAH significantly reduced the infarct volume. (C) The area percentage of cerebral infarction in the groups treated with PAH decreased significantly by Image J software. Data were expressed as mean ± standard deviation (S.D.), $n \geqslant 3$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. sham group, $^{*}p < 0.05$, $^{**}p < 0.01$ vs. I/R group.

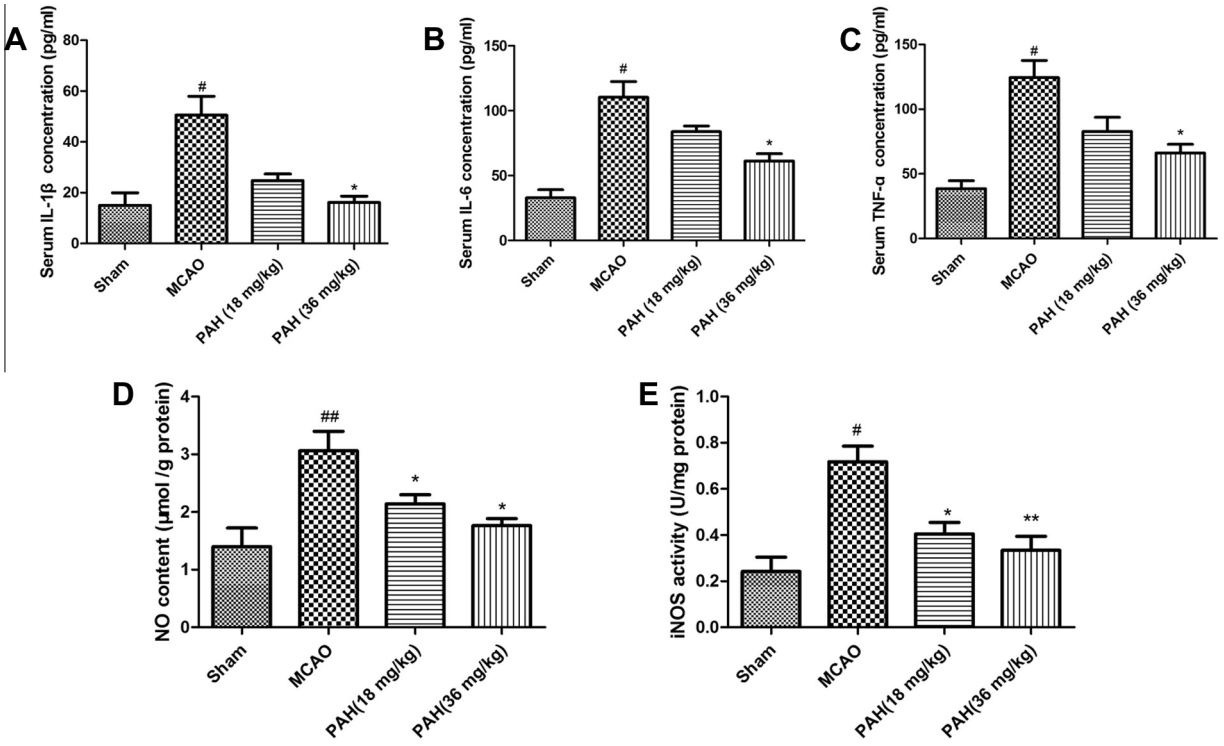

Fig. 2. Effects of PAH treatment on serum IL-1β (A), IL-6 (B) and TNF-α (C) levels in LPS-induced mice. According to the bar graph, PAH could inhibit abnormal expression of inflammatory factor induced by ischemia–reperfusion. (D) NO level in PAH groups dramatically decreased by NO assay kit detecting. (E) PAH could significantly reverse iNOS activity in the cortex by iNOS assay kit detecting. Data were expressed as mean ± standard deviation (S.D.), $n \geqslant 3$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. sham group, $^{*}p < 0.05$, $^{**}p < 0.01$ vs. I/R group.

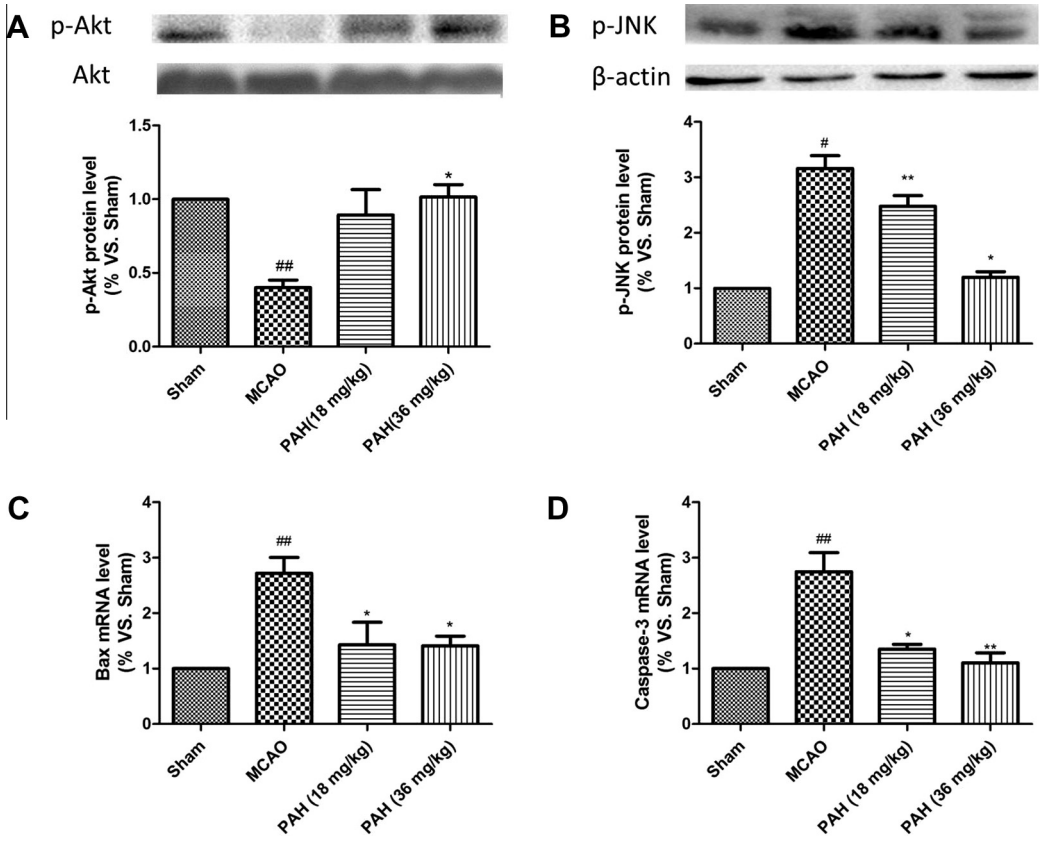

Fig. 3. The Western blot analysis shown that p-Akt (A) and p-JNK (B) protein levels significantly decreased in PAH groups. The Q-PCR results exhibited a meaningful decrease of Bax (C) and capase-3 (D) mRNA levels in PAH groups. Data were expressed as mean ± standard deviation (S.D.), $n \geqslant 3$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. sham group, $^{*}p < 0.05$, $^{**}p < 0.01$ vs. I/R group.

treated with PAH (18, 36 mg/kg) exhibited a significant increase in the phosphorylation of Akt.

3.6. Effect of PAH on Phospho-JNK level

The phosphorylated JNK level was determined by Western blot analysis (Fig. 3B). Cerebral ischemia/reperfusion might activate JNK signal pathway, which increased JNK phosphorylation in ischemic brain. Rats treated with PAH (18, 36 mg/kg) inhibited significantly the expression of Phospho-JNK.

3.7. Effect of PAH on levels of Bax and caspase-3

The levels of Bax and caspase-3 were determined by Q-PCR (Fig. 3C and D). Compare to sham, MCAO group presented a significant increase of mRNA levels of Bax and caspase-3. Pretreatment with PAH (18, 36 mg/kg) significantly inhibited this increase.

4. Discussion

In our findings, PAH alleviates cerebral ischemia–reperfusion injury by observing the decrease of neurological severity score and infraction rate. Additionally, PAH decreased the overexpression of IL-1β, IL-6 and THF-α by activating PI3K/Akt and inhibiting JNK pathway, which is supported by the upregulation of Phospho-Akt level and (the) downregulation of Phospho-JNK level in PAH groups. Furthermore, we evaluated the effects of PAH on levels of Bax and caspase-3, which shown that PAH might have anti-apoptosis effect. These results indicate that PAH attenuates cerebral ischemia–reperfusion injury-triggered overexpression of inflammatory factor through activating PI3K/Akt and inhibiting the JNK signal pathway.

Free radical generation and consequent activation of oxidative stress in stroke contributes to brain damage, which is accompanied by an activation of inflammatory responses [15]. It is widely accepted that oxidative stress is one of the causes of various kinds of diseases, including cancer, diabetes, atherosclerosis, neurodegenerative disorder, cardiovascular and cerebrovascular diseases. Previous research had demonstrated that oxidative stress is one of the mechanisms taking part in the process of cerebral ischemic injury [16]. When tissues are exposed to ischemia followed by reperfusion (I/R), free radicals are drastically generated in the early stage and react rapidly with excessive endogenous NO to generate neurotoxic peroxynitrite which ultimately leading to programed cell death. In models of inflammation, the generation of cytotoxic effect is a result of the production of large amounts of NO and NO-dependent reactions are important in modulating the inflammatory response [17,18].

In consideration of the pathogenesis of diseases caused by altered homeostasis, inflammation plays an important role in the process of essential immune response, which comes at the cost of a transient decline in tissue function. Inflammation also enables survival during infection or injury and maintains tissue homeostasis under a variety of noxious conditions [19]. Previous study has put forward the proposal that inflammation participated in each subtype of acute ischaemic stroke as a peculiar immuno-inflammatory pattern [20]. The first cells responding to brain ischaemia are glial cells, particularly microglia, which could develop into highly motile macrophages that infiltrate and take up residence in the developing brain and perform immunological functions [21]. With transcription of early pro-inflammatory cytokines such as IL1-β and TNF-α, microglia are able to activate additional inflammatory pathways leading to induction of nitric oxide, adhesion molecules and IL-6 [20].

Akt and JNK are two of the most important signaling pathway frequently dysregulated expression in stroke. JNK was originally identified as two protein kinases,p46 JNK1 and p54 JNK2, which specifically phosphorylate the transcription factor, c-Jun, at the Ser 63 and Ser 73 of its N-terminal transactivation domain [21]. JNK is activated by a host of NO production and this activation generally contributes to inflammation and the mediation of cell death [22,23]. Inactivation of JNK blocks phosphorylation of a wide range of transcription factors and keeps the expression of harmful proteins from attacking both the CNS and the periphery. Current studies have shown that the inhibitor of JNK, SP600125, performs neuroprotective action on transient brain ischemia/reperfusion-induced neuronal death in rat partly by suppressing the activation of nuclear substrate (c-Jun) and inactivating non-nuclear substrate (Bcl-2) induced by ischemic insult [24]. Akt is an important cellular survival protein due to its inhibition of JNK activity. According to our result, during cerebral ischemia–reperfusion, the JNK signaling pathway is highly activated, inducing the lesion and dysfunction of neurons; after treatment with PAH, the activation of Akt could be recovered, subsequently inhibits the abnormal phosphorylation level of JNK, and eventually exhibits anti- apoptosis effect.

Members of the Bcl-2 family play crucial roles in regulating apoptosis, which contain pro-survival family members such as Bcl-2, pro-apoptotic BAX/BAK family members such as Bax, and pro-apoptotic BH3-only proteins. Bax either directly or indirectly induce the release of proteins from the space between the inner and outer mitochondrial membranes, and then promotes the activation of caspase-3 and -9 [25]. After stroke, dephosphorylates Akt, phosphorylates JNK, the protein levels of the Bcl-2 family are changed, the expression level of Bax lose control and dramatically increase, followed by translocation from the cytosol to the mitochondrial membrane [26,27]. The inhibition of apoptosis signaling pathways at least might be an intelligent strategy for moderating cerebral ischemia–reperfusion injury and this view is supported by our data analysis which presented the decrease of the mRNA level of Bax and caspase-3 after PAH treatment.

In summary, PAH exhibits neuroprotective effects on cerebral ischemia–reperfusion injury in the rat brain cortex. In this present study, PAH could reduce microglial activation, oxidative stress injury and its anti-inflammatory effect decreases the overexpression of inflammatory factor through activating PI3K/Akt and inhibiting the JNK pathway in the rat brain cortex. Additionally, PAH could inhibit neuronal death via controlling the level of Bcl-2 family and caspase-dependent pathway. These laboratory findings may represent only the tip of the iceberg, a complete picture of inflammation regulation is not available currently and need to be further investigated.

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