Original Article

Xylaria nigripes protects mice against cerebral ischemic injury by activating Nrf2/HO-1 pathway

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Abstract: Xylaria nigripes (XN) is a fungus used in traditional Chinese medicine and its extracts have been shown anti-oxidant and anti-inflammatory activities. This study was to determine the protective effect of XN against ischemic stroke induced neurotoxicity. Ischemic stroke mouse model was established by middle cerebral artery occlusion (MCAO). XN and 40 mol/Kg SnPP were injected intraperitoneally once daily for 7 days. The infarct volume and neurobehavioral score were evaluated. The levels of radical oxygen species (ROS), malondialdehyde (MDA) and cleaved caspase 3, nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), GFAP, NGFR, BDNF, NTRK1, and NTRK2 expression were assessed in the ischemic mouse brains. XN reduced neuronal injury, neurobehavioral deficits, and ROS and MDA contents in MCAO mice, which was abrogated by HO-1 inhibitor SnPP. Moreover, XN treatment alleviated MCAO-induced reduction of the mRNA and protein levels of HO-1, Nrf2, and markers of neurons and astrocytes in ischemic brain. These results suggested that Nrf2/HO-1 pathway mediated the neuroprotective effect of XN following ischemic insult in the brain.

Keywords: Xylaria nigripes, MCAO, cerebral ischemia/reperfusion, Nrf2, HO-1

Introduction

Ischemic stroke is a primary cause of severe acquired disability worldwide [1]. Administration of the thrombolytic agent tissue plasminogen activator is still the only FDA-approved therapy for acute ischemic stroke [2, 3]. However, tissue plasminogen activator has a limited therapeutic window and efficacy [4] and carries the potential risk of hemorrhagic transformation [5], which reflects the incomplete understanding of the etiology of cerebral ischemia and incites the need for alternative treatment strategies. There is increasing evidence suggesting that post-ischemic oxidative stress and inflammation are significant contributing factors in the pathogenic process [6-9]. The levels of reactive oxygen species (ROS) are greatly elevated during reperfusion after ischemia and oxidative stress modulates cell survival signaling and cell death pathways to determine subsequent survival or death of neurons [10, 11]. The systemic administration of antioxidants or anti-inflammatory agents may alleviate ischemic damage in the brain [12-14]. Thus, agents with anti-oxidative or anti-inflammatory effects might be beneficial in the treatment of cerebral ischemia.

The nuclear factor-erythroid 2-related factor 2 (Nrf2) is expressed in most cell types of the brain and is a major regulator of several cytoprotective factors such as anti-oxidative enzymes and anti-inflammatory factors [15, 16]. Furthermore, the activation of Nrf2 can coordinately up-regulate the expression of several anti-oxidative enzymes including heme oxygenase-1 (HO-1). HO-1 is the rate-limiting enzyme converting heme to biliverdin, free iron, and carbon monoxide. HO-1 is rapidly upregulated under various stress conditions and exerts a protection against cellular stress and oxidative injury due to its anti-oxidative, anti-inflammatory, and anti-apoptotic properties [17]. Hence, the modulation of the Nrf2-dependent HO-1 pathway is considered an important target for the treatment of stroke.
Traditional Chinese medicine has been shown to be a complementary and alternative therapy for stroke due to its safety and effectiveness in alleviating symptoms [18, 19]. Several traditional Chinese medicines also produce a meaningful improvement in focal cerebral ischemia in animal studies [20, 21]. *Xylaria nigripes* (XN, also referred as *Wuling Shen*) has long been used for treating mental disorders like insomnia and morbid forgetfulness [22]. It has been shown that XN, especially its aqueous extract, could lower blood sugar levels in steroid-induced insulin resistant rats [23] and mitigate spatial memory impairment [24] and depression [25]. The XN aqueous extract also possesses a strong anti-inflammatory activity through inhibiting the expression of nitric oxide synthase and cyclooxygenase [26]. XN has also been shown to possess anti-oxidant activity in vitro [27]. This study aims to investigate whether and how XN protects against ischemic damages induced by middle cerebral artery occlusion (MCAO) in mouse brain.

**Materials and methods**

**Animals**

The C57BL/6J mice were obtained from the Experimental Animal Center at the Fourth Military Medical University, Xi’an, China. The animals were housed at 21 ± 2°C, 12 h light/dark cycle, and 60-70% humidity with free access to food and water for at least 1 week before surgery. Procedures for animals were reviewed and approved by the Ethics Committee for Animal Experimentation of the Fourth Military Medical University. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as stated in the European Community guidelines (EEC Directive of 1986; 86/609/EEC) and the US guidelines (NIH publication #85-23, revised in 1985).

**Preparation of XN extract**

The XN materials were provided by Zhejiang Jolly Pharmaceutical Co., Ltd. Dried XN materials were subjected to water extraction according to the traditional uses of XN materials, as described previously [24, 28]. After drying by lyophilization, the XN extract powder was stored at 4°C until use. Test solutions were freshly prepared for each experiment with saline. The same volume of saline was used as a control.

**Induction of transient focal cerebral ischemia**

Focal cerebral ischemia was induced by MCAO with an intraluminal filament, as previously described [29, 30]. Briefly, mice were anesthetized with 2% isoflurane carried by 2 L/minute oxygen through a face mask. A six-0 nylon suture monofilament with a rounded tip (Shadong, Beijing, China) was inserted through a small incision on the right common carotid artery and moved forward into the internal carotid artery until a small resistance was felt. The filament was fixed with a silk suture knot on the common carotid artery. After 1 hour, the filament was slowly withdrawn to allow reperfusion. Regional cerebral blood flow (rCBF) was monitored using a PeriFlux System 5000 (Perimed AB, Stockholm, Sweden) in the ipsilateral cortex. MCAO was considered successful if rCBF sharply decreased below 30% of baseline; reperfusion was considered successful once rCBF recovered up to 80% of baseline. Otherwise, animals were excluded from analysis and sacrificed by anesthesia. The mice in the sham group underwent the same procedures of anesthesia and exposure of their carotid artery without MCAO. At onset of reperfusion, mice were received either saline, XN extracts at a low (L, 300 mg/kg) or high (H, 600 mg/kg) dose, or XN extracts (600 mg/kg) plus tin protoporphyrin-IX (SnPP, 15 mg/kg) intraperitoneally and once daily thereafter. Neurobehavioral score, the infarct volume ratio, and related molecular changes were assessed 7 days (Figure 1A) after reperfusion.

**Evaluation of neurological deficit**

Neurological deficits were assessed using a six-point scoring system [31]. Mice were scored as follows: zero, no apparent deficits; one, failure to fully extend left forepaw when pulled by the tail; two, left contralateral circling when pulled by the tail; three, circling or walking to the left; four, walking only if stimulated; and five, unresponsive to stimulation and with a depressed level of consciousness.

**Assessment of infarct size**

Immediately after the neurological deficit evaluation, mice were euthanized according to approved procedure, and their brains were dissected into six coronal slices (1 mm per slice) and immediately stained with 2% 2,3,5-triphenyltetrazolium chloride (T8877, Sigma-Aldrich, St
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Louis, MO) at 37°C for 10 minutes. The infarct volumes were evaluated with the following formula: (total contralateral hemispheric volume - total ipsilateral hemispheric stained volume)/total contralateral hemispheric volume × 100.

Measurement of ROS levels

Cerebral tissue ROS levels were assessed using OxiSelect™ In Vitro ROS/RNS Assay Kit (Cell Biolabs, San Diego, CA) according to manufacturer’s manual. 20-40 mg tissue was homogenized in 1 ml phosphate buffered saline on ice and centrifuged at 10000 g for 5 min at 4°C. The supernatant was subjected to ROS measurement immediately.

Figure 1. Xylaria nigripes improved neurological scores and reduced infarct volumes (A). A diagram of the design for the Xylaria nigripes treatment protocol and the assessment of the ischemic injury under Experiment I. Representative 2,3,5-triphenyltetrazolium chloride-stained thick brain sections (B) and quantitative evaluation of the infarction volume (C, n = 7/group). (D) Neurobehavioral scores for each group (n = 9/group). *P < 0.05.

Figure 2. Xylaria nigripes alleviated MCAO induced oxidative stress. Cerebral ROS (A) and malondialdehyde (B) levels were assessed by appropriate kits. N = 9/group. *, P < 0.05 compared to Sham; #, P < 0.05 compared to MCAO.
Determination of MDA levels

Cerebral content of MDA, the product of lipid peroxidation, was measured using a detection kit purchased from Beyotime (Shanghai, China) according to the manufacturer’s instructions. The absorbance of the supernatant was measured by spectrophotometry at 532 nm.

Quantitative real time (RT)-PCR

The ischemic hemispheres were homogenized in Trizol reagent (Invitrogen, Shanghai, China) and extracted with chloroform on ice. RNA pellets were precipitated with isopropanol, washed with precooled 75% ethanol, and dissolved in 10 μL DEPC-treated water. An aliquot was used for the determination of the amount of RNA. Reverse transcription was performed using the PrimeScript TM RT reagent kit with gDNA Eraser (TaKaRa, Dalian, China) according to the manufacturer’s protocol with 1 μg of total RNA as template. Quantitative RT-PCR was performed with SYBR Premix Ex TaqTM (TliRNaseH Plus, TaKaRa Corporation) and detected by the CFX96 Real-Time PCR Detection System (Bio-Rad Corporation, Hercules, CA). The following primers were used: Hmx1 (HO-1, NM_010442), forward, 5’-AAGCCGAGAATGCTGAGTTCA-3’, reverse, 5’-GCCGTGTAGATAGTGACAAAGGA-3’; Nfe-2l2 (Nrf2, NM_010902), forward, 5’-TTGGCAGAGATGCTGGTACTGCT-3’, reverse, 5’-AAACTTGCTCCATGTCCTGCTCTA-3’; Gfap (NM_001131020), forward, 5’-CGAAGAAAACCGCATCACCATTT-3’, reverse, 5’-CGAACGAAACCGGATAATCACAACCAC-3’;

Figure 3. *Xylaria nigripes* up-regulated Nrf2/HO-1 expression and inhibited caspase 3 activation. The mRNA levels of Nrf2 (A) and HO-1 (B) were analyzed by quantitative real-time PCR. (C) Western blot showed the protein levels Nrf2, HO-1, and cleaved caspase 3. (D) Quantitative analysis of Nrf2, HO-1, and cleaved caspase 3 protein levels. *, P < 0.05 compared to Sham; #, P < 0.05 compared to MCAO.
Nfr (NM_033217), forward, 5'-CCCAACCC-TGTGCAGATGA-3', reverse, 5'-CAGGACATTAGCCCGACTCC-3'; Bdnf (NM_001316310), forward, 5'-GCCTTTGGATACCGGGACTT-3', reverse, 5'-CAGGACATTAGCCCGACTCC-3'; Ntrk1 (NM_001033124), forward, 5'-ATCAACCGCCCTGTATT-3', reverse, 5'-TGCCCTCAGTAGGGGA-AAGA-3'; Ntrk2 (NM_001025074), forward, 5'-CAGCCCTCACGTCACTTCG-3', reverse, 5'-GA-CTGGGCTCTAAACCGGGA-GAA-3'; Gapdh (NM_001289726), forward, 5'-CCACTACGCTCAGTGTT-3', reverse, 5'-GTTGAAATGCGAGAGACACC-3'. The relative gene expression level was calculated by the $2^{-\Delta\Delta C_{T}}$ method with Gapdh as the internal control.

**Western blot**

Cerebral tissue was homogenized in RIPA buffer supplemented with protease inhibitors cocktail (Roche, Shanghai, China). 40 µg total protein was resolved in an 8% SDS-PAGE gel and transferred onto a PVDF membrane, which was blocked in 5% nonfat milk in PBST, incubated with specified primary antibodies over night at 4°C, washed and incubated with proper HRP-conjugated secondary antibody at room temperature for 30 min before specific protein bands were visualized with Amersham ECL Western Blotting Detection Reagent (GE Life sciences, Shanghai, China). The images were scanned and analyzed with ImageJ software (NIH, Bethesda, MD). The primary antibodies against NRF2 (sc-722), HO-1 (sc-390991), GFAP (sc-33673), NGFR (sc-13577), BDNF (sc-65513), and β-actin (sc-69879) were purchased from Santa Cruz Biotech (Austin, TX), NTRK1 (ab76291) and NTRK2 (ab134155) were from Abcam (Cambridge, MA).

**Statistical analysis**

The software SPSS 16.0 for Windows (SPSS Inc., Chicago, IL) was used to conduct statistical analyses. All values, except for neurological scores, are presented as mean ± SD and analyzed by one-way analysis of variance. Between-group differences were detected with the post hoc Student-Newman-Keuls test. The neurological deficit scores were expressed as median (range) and analyzed using the Kruskal-Wallis test followed by the Mann-Whitney U-test with the Bonferroni correction. Differences were considered significant when $P < 0.05$.

**Results**

**XN reduced the effects of transient cerebral ischemia**

MCAO caused significant increase of infarct volume ($P < 0.01$) (Figure 1B, 1C) and neurologic deficiency ($P < 0.01$) (Figure 1D) 7 days after reperfusion compared to sham, which were alleviated by XN treatment ($P < 0.05$ compared to MCAO) (Figure 1B-D).
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**Figure 5.** Inhibition of HO-1 activity blocked the neuroprotective effects of *Xylaria nigripes* in MCAO mice. Representative 2,3,5-triphenyltetrazolium chloride-stained thick brain sections (A) and quantitative evaluation of the infarction volume (B) of mice undergone sham operation, MCAO, MCAO treated with 600 mg/kg XN, MCAO treated with 600 mg/kg XN and 15 mg/kg SnPP (n = 7/group). (C) Neurobehavioral scores for each group (n = 9/group). *, P < 0.05 compared to Sham; #, P < 0.05 compared to MCAO; ^, P < 0.05 compared to MCAO+XN.

**Figure 6.** The alleviation of MCAO induced oxidative stress by *Xylaria nigripes* was abrogated by HO-1 inhibition. Cerebral ROS (A) and malondialdehyde (B) levels were assessed by appropriate kits. N = 9/group. *, P < 0.05 compared to Sham; #, P < 0.05 compared to MCAO; ^, P < 0.05 compared to MCAO+XN.

**XN alleviated MCAO-induced oxidative stress**

MCAO strongly elevated ROS (**Figure 2A**) and MDA (**Figure 2B**) levels in cerebral tissue after ischemia/reperfusion compared to sham (P < 0.01). Low dose XN (300 mg/kg) reduced about 15% ROS and 30% MDA while high dose XN (600 mg/kg) about 30% ROS and 45% MDA in MCAO mice (**Figure 2**).

**XN elevated Nrf2/HO-1 expression and reduced cleaved caspase 3 levels in the ischemic brain**

Nrf2 (**Figure 3A**) and HO-1 (**Figure 3B**) mRNA levels were drastically reduced in MCAO mouse brains. Accordingly, Nrf2 and HO-1 proteins were substantially decreased while cleaved caspase 3 level markedly increased in ischemic mouse brains (**Figure 3C, 3D**). XN reversed the changes of Nrf2, HO-1, and processed caspase 3 levels in a dose-dependent manner (**Figure 3**).

**HO-1 mediated the neuroprotective effects of XN**

The reduction of infarct size in MCAO mouse brains by XN was significantly overridden by specific HO-1 inhibitor SnPP (**Figure 5A, 5B**). SnPP also abrogated the recovery of neurological function by XN in MCAO mice (**Figure 5C**).
The antioxidant activity of XN in ischemic brain was mediated by HO-1

In MCAO mouse brain, SnPP antagonized the antioxidant effects of XN, increasing ROS level by about 30% (Figure 6A) and MDA level by about 25% (Figure 6B).

Discussion

In the present study, we showed that XN (300 mg/kg or 600 mg/kg) IP injection at the onset of reperfusion after focal cerebral ischemia and thereafter daily once for 7 days significantly reduced infarct volume and relieved neurologic deficits, increased the expression of Nrf2/ HO-1 and markers for neurons and astrocytes, and inhibited ROS, MDA, and cleaved caspase 3 levels after MCAO, which was abrogated by specific HO-1 inhibitor SnPP.

Ischemic pathogenesis involves a series of biochemical events including oxidative stress, inflammatory responses, and programmed cell death, which is rapidly initiated within minutes after the onset of brain ischemia [32, 33]. Studies have revealed a therapeutic window of approximately 6 hours between the onset of ischemia and irreversible neuronal death [34, 35]. The long lasting neuroprotective effect of some reagents could be observed 7 days after reperfusion [36, 37]. The current results will serve as a basis for future studies into the application of XN as a potential therapy for cerebral ischemia.

It has been shown that the Nrf2 is a promising target for stroke treatments [38]. The Nrf2 antioxidant pathway is the primary cellular defense against the cytotoxic effects of oxidative stress. Aberrant or insufficient Nrf2 activity has been identified in neurodegenerative conditions [39]. Activation of Nrf2 plays a pivotal role in protecting against progressive ischemic damage and promoting recovery from stroke [40-43]. Knockout Nrf2 results in more significant loss of neuronal function and increase of infarct size and inflammatory damage after MCAO [40]. The neuroprotective effects of ursolic acid and S-allyl cysteine seen in wild type mice are lost in Nrf2-/- mice after MCAO [40, 41].

Nrf2 driven HO-1 transcription in astrocytes is critical for attenuating the oxidative stress and inflammation caused damages to neuronal functions [44, 45]. HO-1 gene knockout mice display much stronger inflammatory responses and are highly vulnerable to sepsis compared to wild-type mice [46]. Ginkgo biloba extract, omega-3 fatty acids, and 11-Keto-β-boswellic acid have been shown to protect against ischemic stroke through HO-1 mediated anti-oxidative response [47, 48]. Consistent with this, HO-1 gene knockout aggravates cerebral ischemic injury in mice [49].

Accumulating evidences support the notion that Nrf2/HO-1 axis is critical for the treatment of stroke [50-52]. The current data demonstrate that MCAO induces ROS and MDA production and the cleavage of Caspase 3 protein while inhibits Nrf2 and HO-1 expression [53, 54], which was substantially inhibited by XN treatment. Specific HO-1 inhibitor SnPP abrogated the neuroprotective effects of XN in MCAO mice, indicating that XN might exert neuroprotective effects against ischemic injuries by regulating the Nrf2/HO-1 axis.

Our findings suggest that XN treatment provided neuroprotective effects against cerebral ischemic injury in a mouse MCAO model through reducing oxidative stress and apoptotic activity. The neuroprotective effects of XN are mediated by the Nrf2/HO-1 anti-oxidative pathway.

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Disclosure of conflict of interest

None.

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