Experimental Neurology 272 (2015) 181–189

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Reduction of zinc accumulation in mitochondria contributes to decreased cerebral ischemic injury by normobaric hyperoxia treatment in an experimental stroke model

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ARTICLE INFO

Article history:
Received 26 January 2015
Revised 22 March 2015
Accepted 10 April 2015
Available online 17 April 2015

Keywords:
Zinc
Mitochondria
Oxygen
Ischemia
Brain
Penumbra

ABSTRACT

Cerebral ischemia interrupts oxygen supply to the affected tissues. Our previous studies have reported that normobaric hyperoxia (NBO) can maintain interstitial partial pressure of oxygen (pO_2) in the penumbra of ischemic stroke rats at the physiological level, thus affording significant neuroprotection. However, the mechanisms that are responsible for the penumbra rescue by NBO treatment are not fully understood. Recent studies have shown that zinc, an important mediator of intracellular and intercellular neuronal signaling, accumulates in neurons and leads to ischemic neuronal injury. In this study, we investigate whether NBO could regulate zinc accumulation in the penumbra and prevent mitochondrial damage in penumbral tissue using a transient cerebral ischemic rat model. Our results showed that NBO significantly reduced zinc-staining positive cells and zinc-staining intensity in penumbral tissues, but not in the ischemic core. Moreover, ischemia-induced zinc accumulation in mitochondria, isolated from penumbral tissues, was greatly attenuated by NBO or a zinc-specific chelator, *N,N,N′,N′*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN). NBO or TPEN administration stabilized the mitochondrial membrane potential in the penumbra after cerebral ischemia. Finally, ischemia-induced cytochrome *c* release from mitochondria in penumbral tissues was significantly reduced by NBO or TPEN treatment. These findings demonstrate a novel mechanism for NBO's neuroprotection, especially to penumbral tissues, providing further evidence for the potential clinical benefit of NBO for acute ischemic stroke.

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Introduction

Oxygen deficiency is one of the critical features of ischemic stroke due to insufficient cerebral blood supply to afflicted areas (An et al., 2012). Ischemic penumbra is an area compromised by partially reduced cerebral blood flow and disturbed energy metabolism surrounding the severely injury ischemic core (Astrup et al., 1981). It is defined as a tissue that could be "potentially reversible" with a timely intervention (Foley et al., 2010; Zhou and van Zijl, 2011; Song and Yu, 2014). We previously reported that normobaric hyperoxia (NBO) could maintain tissue oxygenation in the penumbra of cerebral ischemic rats (Liu et al., 2006). It has been demonstrated by us and others that NBO treatment exhibits neuroprotective effects in stroke animal models (Singhal et al., 2002; Henninger et al., 2007; Shin et al., 2007; Liu et al., 2009) and in patients in pilot studies (Singhal et al., 2005; Chiu et al., 2006), suggesting that NBO is a potential treatment for acute ischemic stroke. However, the mechanisms related to penumbral tissues rescue by NBO during cerebral ischemia are not fully understood.

Besides Ca^{2+}, zinc is another cation that is associated with neuronal injury (Shuttleworth and Weiss, 2011; Leng et al., 2014; Lai et al., 2014). Zinc is highly enriched in the central nervous system. Most zinc ions were reported to be stored in synaptic vesicles, which would be released from glutamatergic terminals of neurons during ischemia (Galasso and Dyck, 2007). Intracellular compartments and zinc binding proteins are involved in keeping zinc homeostasis (Sensi et al., 2009; Shuttleworth and Weiss, 2011). Among these, mitochondria are the key intracellular organelles for buffering zinc levels in neurons. In vitro studies demonstrated that zinc overload in mitochondria would induce multiple mitochondrial injuries (Sensi et al., 2000, 2003; Dineley et al., 2005; Gazaryan et al., 2007) and activate mitochondrial-mediated pro-apoptotic factors (Jiang et al., 2001). Zinc was also reported to activate

Abbreviations: NBO, normobaric hyperoxia; pO_2, pressure of oxygen; TPEN, *N,N,N′,N′*-tetrakis(2-pyridylmethyl)ethylenediamine; MCAO, middle cerebral artery occlusion; TTC, 2,3,5-triphenyltetrazolium chloride; DMSO, dimethyl sulfoxide; NG, Newport Green; MTG, Mito Tracker Green; BSA, bovine serum albumin; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; OGD, oxygen glucose deprivation.

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http://dx.doi.org/10.1016/j.expneurol.2015.04.005
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mitochondrial outer membrane channels (Bonanni et al., 2006) and cytochrome *c* discharged from mitochondria (Calderone et al., 2004) in transient global ischemic animals. Our previous study has reported that a combination of zinc and hypoxia synergistically leads to high astrocytic cell death in vitro, suggesting the augmentation of zinc cytotoxicity under ischemic conditions (Pan et al., 2013). Therefore, we hypothesize that NBO may prevent zinc accumulation in the mitochondria of penumbral tissues and reduce zinc toxicity to mitochondrial functions under ischemic conditions, affording neuroprotection during recovery from acute ischemic brain injury.

In the current study, we investigated the role of NBO in reducing ischemia-induced mitochondrial injury in the penumbra via preventing zinc accumulation in mitochondria in a transient ischemic rat model.

Materials and methods

Animal model and experimental group

Animal procedures for this study were approved by the Institutional Animal Care and Use Committee of Xuanwu Hospital of Capital Medical University (Beijing, China). Male Sprague–Dawley rats (280–300 g) were subjected to middle cerebral artery occlusion (MCAO) surgery as descried previously (Zhao et al., 2014). Right MCAO was induced using the modified intraluminal filament method (Tajiri et al., 2013; Qi et al., 2014). The animals underwent 90 min of MCAO and then were reperfused for 22.5 h by careful withdrawal of the filament. Successful MCAO was assessed by circling to the non-ischemic side (left) at the end of ischemia then further confirmed by 2,3,5-triphenyltetrazolium chloride (TTC) staining at the end of reperfusion (see *Infarct measurement* below).

Twenty-eight rats with successful MCAO were divided at random into four groups: normoxia, NBO, DMSO, and TPEN ($n = 7$ in each group). Three rats with unsuccessful MCAO were excluded from this study. No rats died due to surgical or stroke complications.

NBO and TPEN treatment

Ten minutes after the onset of MCAO, the animals were put into an anesthesia box, which delivered 100% oxygen (NBO, 5 L/min) and lasted until the start of reperfusion (Liu et al., 2006). The normoxic rats underwent similar treatment except for ventilation with air (21% O_2, 5 L/min).

A specific zinc chelator, *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN, Sigma-Aldrich), was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) and then further diluted into physiological saline with 20% DMSO. TPEN (15 mg/kg) was administrated by intraperitoneal injection at 30 min prior to MCAO onset. In our previous study, we found that 15 mg/kg of TPEN by intraperitoneal injection 30 min before MCAO were able to reduce infarct volume and block zinc activities following ischemia (Zhao et al., 2014). Therefore, intraperitoneal injection TPEN (15 mg/kg) 30 min before the onset of MCAO was chosen in this study. The vehicle DMSO (20% in saline) was given to rats as the control group.

Tissue collection, infarct measurement, and penumbra identification

Rats were decapitated after 22.5 h of reperfusion. Brains were quickly removed and sectioned into four 2-mm thick slices from an 8-mm thick region 5 mm away from the tip of the frontal lobe. The first and third slices were prepared for isolating cerebral mitochondria. The fourth slice was snap-frozen in liquid nitrogen and stored at −80 °C for zinc staining.

The second slice was stained with 1% TTC solution at 37 °C for 20 min. The infarct volume was measured using the TTC-stained slice as described previously (Swanson et al., 1990). The non-infarcted area in the ipsilateral brain was subtracted from that in the contralateral brain. The infarct volume was calculated as a percentage of the volume of the contralateral brain. In TTC assay,7 rats were used in each group to confirm the success of MCAO model and to calculate infarct volume. Then 4 rats were randomly chosen out of 7 rats for other assays in each group.

Penumbral tissue is operationally defined as the ischemic area that will undergo apoptosis without treatment but is rescued with treatment (Ford et al., 2012; Zhu et al., 2012). The differences in infarct area between NBO (TPEN) groups and normoxic (DMSO) groups were considered as the penumbra (Fig. 1A) as our previous studies described (Liu et al., 2004).

As illustrated in Fig. 1A, brain tissue in black rectangle was collected for isolating mitochondria and then mitochondria-related assay. The images from small orange rectangle were selected as the representatives of the penumbra in NG or RhodZin-3 staining.

Cytosolic zinc staining

Brain sections (20 μm) were stained with the zinc-selective membrane-impermeable fluorescent indicator Newport Green (NG, Invitrogen) to detect cytosolic free zinc. Sections were washed in PBS and incubated with NG (10 μM) and DAPI (a nuclear specific marker) in the dark for 3 min. After washing in PBS, images were acquired with a fluorescence microscope (Nikon 80i, Japan) and analyzed with Leica Qwin software (Zhao et al., 2014). Three different areas in the penumbra from each rat were selected to count NG-positive cells and analyze fluorescent intensity. The fluorescent intensity was assessed after subtraction of background fluorescent intensity.

Isolation of brain mitochondria

Highly purified mitochondria were isolated from non-ischemic and ischemic brain tissues, according to the manufacturer's protocol of a Qproteome mitochondria isolation kit (Qiagen). Brain tissues (200 mg) were cut into pieces and incubated in 500 μL ice-cold Lysis Buffer with protease inhibitors. Homogenization of the sample was performed using the Tissue Ruptor rotor-stator homogenizer for 10s. The homogenate was centrifuged at 1000×*g* for 10 min at 4 °C, and the cell pellets were resuspended in 1.5 mL ice-cold Disruption Buffer by pipetting up and down using a 1 mL pipette tip. The lysate was centrifuged at 1000×*g* for 10 min at 4 °C. The supernatant was transferred to a new tube and centrifuged at 6000×*g* for 10 min at 4 °C. The pellet containing full active intact mitochondria was collected and resuspended in Storage Buffer and stored at −80 °C for further analysis. To avoid the excess injury, the mitochondria were pipetted gently and they appear aggregated in the images shown in figures.

Measurements of zinc levels in mitochondria

Free zinc in mitochondria was measured using a mitochondrial-specific fluorescent zinc indicator RhodZin-3 (Invitrogen)(Bonanni et al., 2006). Two methods were used in this study to evaluate zinc level in mitochondria within the penumbra.

(i) Quantification of zinc levels in isolated mitochondria. Isolated mitochondria were co-stained with RhodZin-3(10 μM, Invitrogen) and mitochondrial membrane potential-independent probe Mito Tracker Green (MTG, 100nM, Invitrogen) for 45 min at room temperature. Mitochondrial zinc fluorescence was detected using a fluorescence microplate reader in a spectrophotometer (Thermo fisher) at 550/575 nm and 490/516 nm as excitation/emission wavelengths, respectively. The fluorescence of MTG here was used as a loading control for the amount of mitochondria.

(ii) Co-staining of zinc with neuronal cells in brain sections of ischemic rats. Brain sections were co-stained with RhodZin-3 and NeuN (a neuronal specific marker, Millipore). Brain sections (20 μm) were fixed in cold acetone for 10 min and blocked in 3% bovine serum

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