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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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 (pO₂) 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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Keywords

zinc; mitochondria; oxygen; ischemia; brain; penumbra

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 & van Zijl 2011, Song & 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 & 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 & Dyck 2007). Intracellular compartments and zinc binding proteins are involved in keeping zinc homeostasis (Sensi *et al.* 2009, Shuttleworth & 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, Sensi *et al.* 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 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 (280-300g) were subjected to middle cerebral artery occlusion (MCAO) surgery as described 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 90min of MCAO and then were reperused for 22.5h 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 min after the onset of MCAO, the animals were put into an anesthesia box, which delivered 100% oxygen (NBO, 5L/min) and lasted until the start of reperfusion (Liu *et al.* 2006). The normoxic rats underwent similar treatment except for ventilation with air (21% O₂, 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 (15mg/kg) was administered by intraperitoneal injection at 30min prior to MCAO onset. In our previous study, we found that 15 mg/kg of TPEN by intraperitoneal injection 30min before MCAO were able to reduce infarct volume and block zinc activities following ischemia (Zhao *et al.* 2014). Therefore, intraperitoneal injection TPEN (15mg/kg) 30min 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 5mm away from the tip of the frontal lobe. The first and third slice 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 20min. 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 μ m) 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 μ M) and DAPI (a nuclear specific marker) in the dark for 3min. 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 (200mg) were cut into pieces and incubated in 500 μ L 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 $\times$ g for 10 min at 4°C and the cell pellets were resuspended in 1.5mL ice-cold Disruption Buffer by pipetting up and down using a 1mL pipette tip. The lysate was centrifuged at 1000 $\times$ g for 10 min at 4°C. The supernatant was transferred to a new tube and centrifuged at 6000 $\times$ 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 μ M, 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 550/575nm and 490/516nm 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 μ m) were fixed in cold acetone for 10 min and blocked in 3% bovine serum albumin (BSA) for 1h. Sections were incubated with anti-NeuN (1: 500) as the primary antibody, followed by AlexaFluor488 anti-mouse IgG (Invitrogen) as the secondary antibody. The sections were then stained with RhodZin-3 (150nM) for 45min. Images were performed on a fluorescence microscope.

Analysis of mitochondrial membrane potential

To detect variations of mitochondrial membrane potential, we utilized a lipophilic cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) kit (Genmed), according to the protocol of manufacturer. JC-1 is capable of entering into mitochondria and exhibits potential-dependent accumulation in mitochondria. Mitochondrial depolarization is indicated by a decrease in the red/green fluorescence of JC-1. Briefly, isolated mitochondria (10 μ L, about 5 μ g mitochondrial proteins) were incubated in JC-1 staining buffer for 10 min at room temperature in the dark. Images were then performed on a fluorescence microscope. The fluorescence was assessed using a fluorescence microplate reader (Thermo fisher) at 490/516nm (green) and 490/590nm (red) as excitation/emission wavelengths, respectively. The ratio of red/green intensity was measured to evaluate the membrane potential of isolated mitochondria from each group.

Western blot assay for mitochondrial cytochrome C

Isolated mitochondria were incubated with lysis buffer (50mM Tris-HCl, pH 7.6, 150mM NaCl, 5mM CaCl₂, 0.05% Brij-35, 1% Triton X-100) for 30min for SDA-PAGE. All blots were probed with cytochrome C antibody (1:500, BD Biosciences). The membranes were re-probed with anti-COX4 (1:500, Proteintech) as loading control.

Statistical analysis

Data were presented as mean $\pm$ SD. Statistical analysis was performed using student *t*-tests or ANOVA. A value of $p < 0.05$ was considered statistical significance.

Results

Intracellular zinc accumulates in the ischemic penumbra and core of ischemia stroke rats

Cytosolic free zinc in ischemic or contralateral hemisphere was double stained with zinc-specific NG staining and DAPI. Representative merged images in contralateral area, the ischemic penumbra and the core were shown in Fig. 1B-D. NG-positive cells were hardly

visible in contralateral tissue (Fig. 1B). On the contrary, there were numerous NG-positive cells in the penumbra (Fig. 1C) and core (Fig. 1D) in rats with 90-min MCAO plus 22.5-h reperfusion, suggesting intracellular zinc accumulation may be involved in cerebral ischemia/reperfusion injury.

NBO reduces zinc accumulation in the penumbra and shows similar protective effect as a zinc chelator, TPEN

We next investigated whether NBO treatment could reduce ischemia-induced zinc accumulation. Typical zinc staining images in the ischemic penumbra and core from NBO or normoxic groups are shown in Fig. 2A. Statistical analysis demonstrated that the number of NG-positive cells in the penumbra was markedly reduced to 164 ± 34 in the NBO group, compared to 330 ± 46 in the normoxic group (Fig. 2B). In contrast, there was no significant change in positively zinc-stained cells in the ischemic core between NBO and the normoxic group (Fig. 2B). Quantitative analysis also showed that NBO significantly reduced cytosolic zinc fluorescence intensity in NG-positive cells in the penumbra to 35 ± 8 , when compared with 77 ± 21 in normoxic group (Fig. 2C). These results indicate that NBO can reduce zinc accumulation in the ischemic penumbra, but not in the core region.

TTC staining results demonstrated that NBO treatment significantly reduced the infarct size of ischemic rats, as compared with the normoxic group (Fig. 2D). To address whether the decreased infarct volume by NBO in ischemic rats is related to reduction of zinc levels, we measured the infarct volume in ischemic rats injected with selective zinc chelator, TPEN, which has been shown to reduce zinc accumulation and lead to decreased brain injury following ischemia (Zhao *et al.* 2014). As shown in Fig. 2E, compared with the DMSO group, TPEN treatment reversed MCAO-induced infarction. These data suggest that NBO has the same neuroprotective effect as zinc chelator, TPEN, and may share the same neuroprotective mechanism as TPEN by blocking zinc toxicity in the penumbra.

NBO treatment decreases zinc accumulation in mitochondria isolated from penumbral tissue of ischemic rats

As zinc overload in mitochondria is associated with mitochondrial injury under ischemic conditions, we investigated whether NBO could reduce zinc accumulation in mitochondria in the penumbra. Mitochondria were isolated from the penumbra of ischemic hemisphere and contralateral region and co-stained with mitochondria-specific zinc indicator RhodZin-3, and MTG, a specific potential-independent mitochondrial probe, a loading control for the amount of mitochondria. Quantitative analysis, calculating the ratio of RhodZin-3/MTG, showed that 90-min ischemia with 22.5-h of reperfusion triggered more zinc accumulation in mitochondria from the penumbra than those from non-ischemic hemisphere. NBO treatment significantly reduced ischemia-triggered zinc overload in mitochondria from the penumbral area, as compared to normoxic rats (Fig. 3A). We also used TPEN administration as a positive control for reducing zinc in the mitochondria. We found that chelating zinc with TPEN significantly suppressed zinc accumulation in mitochondria in penumbral area, as compared with the DMSO group (Fig. 3B). These findings suggest that NBO treatment may prevent penumbral injury by reducing ischemia-induced zinc accumulation in mitochondria.

NBO treatment suppresses mitochondrial zinc in neuronal cells of the penumbra

Whereas the survival of neurons in the penumbra is closely correlated with good outcomes for stroke animals, mitochondrial zinc accumulation is closely related to the fate of neurons. To clarify whether the increased mitochondrial zinc (Fig.3) is located in neurons, the brain sections were co-stained with RhodZin-3 and NeuN (a specific marker of neuronal cells). Typical images in the penumbral region showed that mitochondrial-selective zinc staining was co-localized with NeuN-positive cells in normoxic rats. NBO treatment significantly decreased the mitochondrial-specific zinc accumulation in NeuN-positive cells in the penumbra (Fig. 4A). Similarly, chelating zinc by TPEN significantly suppressed zinc accumulation in mitochondria of penumbral neuronal cells, as compared with the DMSO control group (Fig. 4B). These data suggest that ischemia induces mitochondrial zinc accumulation in neurons, which could be rescued by NBO treatment. NBO treatment may rescue the neuronal cells in the penumbra from ischemic injury by reducing zinc overload in mitochondria.

NBO treatment improves membrane potential of mitochondria from penumbral tissues through removing zinc accumulation in mitochondria

Isolated mitochondria from penumbral or non-ischemic tissues were incubated with JC-1, a fluorescence probe of mitochondrial membrane potential. As is shown in Fig. 5A, healthy mitochondria from non-ischemic tissues had high membrane potential and showed high red fluorescence. In contrast, damaged mitochondria from an ischemic hemisphere exhibited low potential and showed low red fluorescence. Statistical analysis in Fig. 5B indicated that JC-1 fluorescence intensity was drastically decreased in the penumbra of the ischemic hemisphere, when compared with contralateral tissue in normoxic rats. NBO treatment significantly rescued ischemic-induced mitochondrial membrane potential dissipation in penumbral tissues. Similar effects were obtained in our positive control experiments. Mitochondria from TPEN-administrated rats preserved mitochondrial membrane potential in comparison to the DMSO group (Fig. 5C and 5D). These results suggest that NBO treatment plays a positive effect on maintaining the mitochondrial membrane potential through decreasing zinc levels in mitochondria.

NBO treatment attenuates ischemia-induced cytochrome C release from penumbral mitochondria through reducing zinc accumulation in mitochondria

Cytochrome C release from mitochondria is a crucial event that is associated with the mitochondria-dependent cell death pathway. We investigated the effect of NBO on the level of cytochrome C in mitochondria fraction in the penumbra of ischemic rats. As shown in Fig. 6A and 6B, a significant reduction of cytochrome C level in mitochondrial fraction was observed in penumbral tissues of normoxic rats, when compared with that in Non-I hemisphere. NBO treatment partially rescued ischemia-induced cytochrome C loss in mitochondrial fraction. As the positive control of NBO treatment (Fig 6C and 6D), TPEN treatment noticeably restored the cytochrome C expression in ischemic mitochondria, compared with the DMSO group. These data indicate that NBO treatment reduces cytochrome C release from mitochondria and thus inhibits the mitochondria-mediated cell death pathway through reducing zinc accumulation in mitochondria.

Discussion

Normobaric hyperoxia is a potential treatment for ischemic stroke (Henninger & Fisher 2006, Deng *et al.* 2014, Chen *et al.* 2014). Excessive zinc released during ischemia is considered to be a critical factor in ischemic brain injury. In this study, we have focused on the investigation of a new mechanism and the signaling pathway that is related to zinc accumulation in NBO-afforded neuroprotective effect in transient ischemic rats followed by 90min ischemia and 22.5h reperfusion. Our results demonstrate that zinc plays a critical role in mitochondria-dependent cell death pathway through accumulation in mitochondria follow transient ischemic stroke (Fig. 3-6). NBO treatment was found to reduce cytosolic free zinc accumulation in the penumbra and suppress mitochondrial zinc overload in ischemic stroke rats. Therefore, NBO might protect against ischemic stroke through regulating mitochondrial zinc levels. Since elevation of intracellular zinc occurs soon after ischemia onset (Zhao *et al.* 2014), it would be very interesting to investigate the time-dependent events at earlier time points. Further study is warranted.

Zinc is an important mediator of intracellular and intercellular neuronal signaling. Zinc has been found to be stored in specific synaptic vesicles in glutamatergic neurons and plays an important role in the pathological processes of ischemic stroke (Sensi *et al.* 2009). Recent studies from our lab and others have shown that high concentrations of zinc under ischemic conditions contribute to ischemia-induced neuronal death (Zhao *et al.* 2014, Yin *et al.* 2002). In the present study, we observed zinc accumulation in an ischemic hemisphere (both the penumbra and ischemic core) in 90-min MCAO/22.5-h reperfusion rats (Fig. 2). NBO reduced the numbers of NG-positive cells and NG fluorescence intensity in these NG-positive cells in the penumbra, which has been regarded as the ischemic tissue that could be rescued at the early stage of ischemic stroke. Interestingly, NBO could not reduce NG-positive cells in the ischemic core. Our previous data showed that NBO treatment could significantly increase pO₂ in the penumbra, but not in the core following ischemia (Liu *et al.* 2004). Moreover, administration of NBO during ischemia increased the penumbral blood flow, but did not affect the blood flow in core region (Liu *et al.* 2006). Data in this study showed that the increased pO₂ and improved blood flow in the penumbra may be involved in regulating zinc levels and contributes to the neuroprotective effect by NBO. Besides, the cerebral blood flow is associated with vascular remodeling, which can improve functional recovery in ischemic stroke rats (Liu *et al.* 2014). However, the relationship between zinc and vascular remodeling needs to be further investigated.

TPEN, a membrane-permeable chelator, has been shown to block both intracellular and extracellular free zinc (Cuajungco & Lees 1998) and reduces zinc-mediated cell apoptosis in neurons (Medvedeva *et al.* 2009). In this study, TPEN was administrated as a positive control and as a diagnostic tool to investigate the effect of zinc on NBO neuroprotection. Our findings showed that NBO presented similar protective effects as TPEN administration, suggesting that NBO and TPEN may share the same mechanism in protecting the penumbra by reducing cytosolic zinc accumulation in the penumbra.

Mitochondria are important in buffering zinc levels in neurons. However, little is known about how zinc level changes in mitochondria during transient focal ischemia. In this study,

we provide the first evidence that ischemia induced mitochondrial zinc accumulation in the penumbra (Fig.3). What's more, the abnormally high zinc levels were located in neurons in penumbral tissues (Fig.4). NBO treatment decreased mitochondrial zinc levels both in isolated mitochondria and neuronal mitochondria in the penumbra (Fig.3 and 4). Therefore, our data demonstrates that NBO decreases mitochondrial zinc accumulation in penumbral neurons following transient focal ischemia.

We next investigated the mechanisms related to zinc levels in NBO-afforded neuroprotection. Abnormal zinc accumulation in mitochondria can affect mitochondrial function and lead to mitochondria-mediated cell death in oxygen glucose deprivation (OGD) and global ischemia models (Calderone *et al.* 2004, Bonanni *et al.* 2006, Medvedeva *et al.* 2009). However, the role of mitochondrial zinc in transient ischemic stroke is still unknown. In the current study, we focused on zinc accumulation-induced mitochondrial membrane potential dissipation and cytochrome C release. Abnormal mitochondrial membrane potential causes mitochondrial dysfunction and eventual cell death. Our findings support this mechanism because mitochondria isolated from the brain penumbra following ischemia showed membrane potential disruption and cytochrome C release (Fig.5 and 6). More importantly, these events were reversed by NBO and TPEN, suggesting that mitochondrial zinc contributes to mitochondrial dysfunction, resulting in cell death. These findings suggest a possible mechanism of NBO in protecting the penumbra, thus reducing cerebral ischemic injury in ischemic stroke rats. A Ca^{2+} uniporter was reported to be involved in mediating zinc entry into mitochondria, which contributed to mitochondrial dysfunction in OGD treated neurons (Medvedeva & Weiss 2014). Further studies are needed to investigate how zinc accumulates in mitochondria during cerebral ischemia.

In conclusion, the data from this study suggest a novel mechanism of NBO treatment in rescuing penumbra through reducing zinc accumulation in penumbral tissues of cerebral ischemic rats. These findings provide further evidence that NBO treatment could be a viable neuroprotective treatment to salvage penumbral tissues during acute ischemic stroke.

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Abbreviations

NBO	normobaric hyperoxia
pO₂	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

Highlights

- NBO inhibits cytosolic zinc accumulation in the penumbra of ischemic stroke rats
- NBO prevents ischemic-induced zinc accumulation in mitochondria
- NBO improves mitochondrial functions through reducing zinc accumulation

Figure 1. Zinc accumulation in different brain regions during cerebral ischemia/reperfusion

A. The illustration of ischemic penumbra (P), core and contralateral (Contr) regions in a rat brain section. Representative co-stained images of zinc-specific NG staining (green) and nuclear specific indicator DAPI (blue) in the contralateral tissues (**B**), the penumbra (**C**) and the core (**D**). Scale bar=10µm.

Figure 2. NBO reduces zinc accumulation in the penumbra and shows similar protective effect as a zinc chelator, TPEN

A. Typical images of NG staining in the penumbra and core in normoxic- or NBO-treated rats. Scale bar=50µm. **B.** Quantitative analysis of the number of NG-positive cells that were counted from three different fields of the penumbra or core in each rat. The images in small orange rectangle were selected as the representatives of the penumbra for NG staining quantification. **C.** Quantitative analysis of the fluorescence intensity in NG-positive cells in the penumbra. **D.** NBO reduced infarction volume in ischemic rats. NBO was initiated 10 min after the onset of MCAO and lasted until reperfusion. The control rats were ventilated with air. The infarction volume was assessed at the end of reperfusion. **E.** A zinc specific chelator, TPEN, decreased infarction volume in ischemic rats. TPEN or vehicle control (DMSO) was administrated 30 min before MCAO. Quantification of cerebral infarct volume was assessed at the end of reperfusion. Data are represented by means ± SD (n=5 for A, B and C; n=7 for D, E); * P <0.05 vs normoxic-treated or DMSO-treated MCAO group.

Figure 3. NBO treatment decreases zinc level in mitochondria isolated from the penumbra

A. Quantification of the ratio of fluorescence RhodZin-3/MTG in normoxic or NBO rats. Mitochondria were isolated from ischemic hemisphere (I) or contralateral region (Non-I), and were co-stained with RhodZin-3 and MTG, as a loading control. **B.** Quantification of the ratio of fluorescence RhodZin-3/MTG in TPEN or DMSO rats. TPEN treatment significantly reversed ischemia-induced mitochondrial zinc level in the penumbra. Data are expressed as means $\pm$ SD (n=4 for each group). * P <0.05 vs Non-I in normoxic group or DMSO group; # P <0.05 vs. I in normoxic or DMSO group.

Figure 4. NBO treatment reduces mitochondrial zinc level in neuronal cells from penumbral tissues in brain section

Brain sections were co-stained with RhodZin-3 (red) and neuro-specific marker (NeuN, green). **A.** Normoxic and NBO groups. **B.** DMSO and TPEN groups. Scale bar=25μm. Non-I: non-ischemic hemisphere; I: the penumbra in ischemic hemisphere.

Figure 5. NBO maintains membrane potential of mitochondria in the penumbra of ischemic rats
A. JC-1 staining was performed to measure membrane potential of mitochondria, which were isolated from the penumbra of ischemic hemisphere (I) or non-ischemic hemisphere (Non-I) of NBO/normoxic control rats. **B.** Quantitative analysis in NBO or normoxic rats. **C.** JC-1 staining in TPEN or DMSO group. **D.** Quantitative analysis in TPEN or DMSO rats. Data are expressed as means $\pm$ SD (n= 4 for each group). * P <0.05 vs Non-I in normoxic or DMSO group; # P <0.05 vs. I in normoxic or DMSO group. Scale bar=50 μ m.

Figure 6. NBO reduces cytochrome C release from mitochondria in the penumbra

A. Representative blots of cytochrome C in isolated mitochondria in normoxic- and NBO-treated rats. The level of COX-4 in mitochondria was used as loading control. **B.** Quantitative analysis of cytochrome C level in mitochondrial fractions in normoxic- and NBO-treated rats. **C.** Western Blots of cytochrome C in mitochondrial fraction in TPEN or DMSO rats. **D.** Quantitative analysis of cytochrome C level in TPEN or DMSO group. Data are expressed as means $\pm$ SD (n=4 for each group). * P <0.05 vs Non-I in normoxic or DMSO group; # P <0.05 vs. I in normoxic or DMSO group.