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Neuroprotective and axonal outgrowth-promoting effects of tetramethylpyrazine nitrone in chronic cerebral hypoperfusion rats and primary hippocampal neurons exposed to hypoxia

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ABSTRACT

Chronic cerebral hypoperfusion is an important risk factor for vascular dementia and other brain dysfunctions, for which there are currently no effective medications available. We investigated the neuroprotective and axonal outgrowth promoting effects of tetramethylpyrazine nitrone (TBN) in a permanent bilateral occlusion of the common carotid arteries (2VO) rat model and in primary hippocampal neurons exposed to oxygen glucose deprivation (OGD). At 6th week after 2VO, TBN increased the time spent in novel arms in the Y-maze test and improved the discrimination ratio in object reorganization task. TBN attenuated axonal damage, and reduced oxidative DNA injury and lipid peroxidation in white matter. TBN also attenuated the neuronal apoptosis and ameliorated accumulation of astrocytes in parietal cortex and CA1 region of hippocampus. Western blot analyses indicated that TBN increased Bcl-2 expression, decreased Bax and Caspase 3 expressions, and upregulated the phosphorylation levels of high-molecular weight neurofilament (p-NFH), Akt (p-Akt) and glycogen synthase kinase-3β (p-GSK3β) in hippocampus at 6th week after chronic hypoperfusion. *In vitro*, TBN rescued hippocampal neuronal viability and axonal elongation from OGD damage. The p-Akt and p-GSK3β upregulation by TBN was abolished by a specific phosphoinositide 3-kinase (PI3K) inhibitor LY294002, resulting in suppression of axonal outgrowth. Collectively, the results showed that TBN alleviated white matter lesion and impairment of cortex and hippocampus, attenuated oxidative damage and enhanced axonal outgrowth through the regulation of PI3K/Akt/GSK3β signaling pathway, leading to improved cognitive deficit in a rat chronic hypoperfusion model.

1. Introduction

The rat model of permanent occlusion of the bilateral common carotid arteries (2-vessel occlusion, 2VO) has been widely used to examine the role of cerebral hypoperfusion in neurodegenerative processes including vascular dementia and Alzheimer's disease (AD) (Farkas et al., 2007). Experiments found that chronic cerebral hypoperfusion plays an important role in neural damage to the hippocampus, cerebral cortex, whiter matter areas and visual system (Lee et al., 2013; Peng et al., 2007; Zhang et al., 2011). In addition, increased permeability of blood-brain barrier (BBB), oxidative stress, inflammation, trophic uncoupling, and demyelination are also involved in the pathologies of hypoperfusion impairment (Feng et al., 2012; Ueno et al., 2012, 2015). Axonal neurons are vulnerable to the insufficient supply of oxygen and glucose under chronic hypoperfusion. During brain repair after 2VO, axonal outgrowth and plasticity are critical processes related to improvements in the behavioral deficits (Ueno et al., 2015).

Oxidative stress plays an important role in brain injury during cerebral hypoperfusion through induction of damages to blood vessels and activation of astrocytes and oligodendrocyte precursor cells (Dong et al., 2011; Ergul, 2011). Furthermore, reactive oxygen species suppress the pro-survival action of endothelial cells on neurons by inducing trophic uncoupling (Xu et al., 2010).

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Accordingly, scavenging of free radicals might reduce brain damage and behavioral deficits in cerebral hyperfusion. For example, edaravone, a powerful free radical scavenger, showed protective effect on white matter lesions in a rat model of chronic hypopersusion (Ueno et al., 2009).

Tetramethylpyrazine (TMP), one of the active ingredients of the traditional Chinese medicine *Ligusticum wallichii* Franchant (Chuanxiong), has protective effect in rats subjected to chronic hypoxia *via* its antioxidative and antiapoptotic actions (Ding et al., 2013). To enhance the antioxidative and neuroprotective effects of TMP, we have previously designed and synthesized a novel dual-functional agent, 2-[[(1,1-dimethylethyl)oxidoimino]-methyl] -3,5,6-trimethylpyrazine (TBN), which is a derivative of TMP armed with a nitrone moiety (Sun et al., 2008). TBN readily penetrates the BBB, scavenges free radicals and inhibits Ca^{2+} influx. Therefore, TBN provides significant neuroprotection in models of transient and permanent stroke, Parkinson's disease (PD), and traumatic brain injury (TBI) (Guo et al., 2014; Sun et al., 2008, 2012; Zhang et al., 2016a; Zhang et al., 2016b). In the present study, we investigated the neuroprotective and axonal outgrowth-promoting effects of TBN in a rat model of chronic cerebral hypoperfusion and in a primary cultured neuronal injury model of oxygen-glucose deprivation (OGD) *in vitro*, and examined its molecular mechanism(s) of action promoting axonal outgrowth.

2. Materials and methods

2.1. Chemicals and reagents

TBN (CAS: 1083171-75-8) was synthesized by the Sundia Meditech Company (Lot.XQP1230C, Shanghai, China). The purity of TBN is 99.3%, which was confirmed by HPLC in our laboratory. Primary antibodies against Capase 3, Bcl-2, Bax, phospho-Akt (p-Akt), Akt, phospho-GSK3β (p-GSK3β), GSK3β, glia fibrillary acidic protein (GFAP) and β-actin, and HRP conjugated secondary antibodies were from Cell Signaling Technology (Boston, MA, U.S.). Primary antibodies against phospho-NFH (p-NFH) and 8-hydroxy-deoxyguanosine (8-OHdG, a marker of oxidative DNA damage) were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.). Primary antibodies against NeuN, 4-hydroxy-2-nonenal (4-HNE, a marker of lipid peroxidation) and Tau-1 were from Millipore (Billerica, MA, U.S.). Cresol violet was from J&K Chemistry (Shanghai, China). Mouse & Rabbit Universal Immunohistochemical Detection System was from Gene Technology (GTVision™ III,Shanghai, China). All other reagents were from Sigma-Aldrich (St. Louis., MO, U.S.) except where stated otherwise.

2.2. Animals

A total of 34 male Sprague-Dawley rats (250–300 g) were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China). All rats were housed under controlled temperature and humidity with a 12 h light/dark cycle and had free access to food and water throughout the experiment. The study was approved by the Research Ethics Committee of Jinan University. All animal experiments were performed in accordance with the guidelines and regulations for the use and care of animals of the Center for Laboratory Animal Care of Jinan University (Guangzhou, China) and conformed to internationally accepted ethical standards (Guide for the Care and Use of Laboratory Animals. US NIH Publication 86-23, revised 1985), which ensured humane and proper care of research animals. All efforts were made to minimize the numbers of animals used and ensure minimal suffering.

2.3. 2VO surgery

Before surgery, all animals were fasted for 8 h. Animals were anesthetized with 4% isoflurane in a mixture of air, and maintained sedated with a mixture of 1–1.5% isoflurane during the surgical period. Rectal temperature was maintained at 37.0 ± 0.5 °C with a heating pad. A midline incision was made to expose both the common carotid arteries, which were then tightly double ligated with 4-0 silk sutures. Seven rats in the sham group were subjected to a sham-operation consisting of same procedure without ligation of both the common carotid arteries.

2.4. Drug administration

The 2VO animals were randomly divided into three experiment groups: (1) TBN group (n = 8). The rats were treated via tail vein injection of TBN (30 mg/kg) at 3 h and 6 h after surgery and then twice daily for a total of seven consecutive days. (2) Memantine (Mem) group (n = 8). The rats were treated via tail vein injection of memantine (15 mg/kg) at 3 h and 6 h after surgery and twice daily for a total of seven consecutive days. (3) Vehicle group (n = 11). The rats were treated via tail vein injection of equal volume of saline. Rats in the sham-operated group (n = 7) were also administrated with equal volume of saline. One animal in the TBN treatment group died at the fifth week after surgery. The reason of death was confirmed not to be associated with TBN toxicity through post-mortem analysis.

2.5. Measurement of cerebral blood flow

Cerebral blood flow (CBF) were measured in a right temporal window using a laser Doppler flowmetry (Perimed, Stockholm, Sweden). The probes were positioned in the 5 mm left and 2 mm behind the bregma, which could reflect the change of blood flow in temporal cortex (Harada et al., 2005). CBF were monitored at pre-operation, 5 min, day 7, 14, 21, 28, 35 and 42 after operation.

2.6. Y-maze test

The Y-maze test was performed according to the protocols published previously (Sarnyai et al., 2000). Briefly, rat was placed into one of the arms (starting arm) and allowed to explore the maze with one of the arms closed for 10 min (training period). After 1 h of inter-trial interval, rats were allowed to explore freely all three arms of the maze for 5 min (testing period). Time spent in three arms was recorded. Discrimination ratio was calculated as Novel arms/(Novel arms + Other arms) for dwell time.

2.7. Object recognition task

The novel object recognition test was performed as previously reported (Camarasa et al., 2010). First, each rat was placed into a square box (50 × 50 × 50 cm, length × width × height) for 10 min per day for 2 days without any object to habituate the environment. Then, two identical objects, A1 and A2, were placed parallel near one wall of the square box. Rat was placed singly in the box and allowed to explore the objects for 10 min. The rat was then returned to cage. Object A2 was replaced with a novel object (TB). One h later, the rat was returned to the box and was allowed to explore the object for 5 min in the test phase. Exploratory behavior (reaching object) was defined as directing the nose at the object at a distance of less than 5 cm and/or touching the object with the nose. Digital camera recorded the latency of the first time reaching the novel object, and the times to explore the familiar object (TA) and the novel object (TB). Novel and familiar objects were alternated