Neurobiology of Disease 95 (2016) 12–21

Contents lists available at ScienceDirect

Neurobiology of Disease

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

Zinc contributes to acute cerebral ischemia-induced blood–brain barrier disruption

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ARTICLE INFO

Article history:
Received 10 March 2016
Revised 12 June 2016
Accepted 3 July 2016
Available online 5 July 2016

Keywords:
Blood–brain barrier
Brain ischemia
Zinc
Matrix metalloproteinases
Tight junction proteins
Cell death
Microvessel

ABSTRACT

Zinc ions are stored in synaptic vesicles and cerebral ischemia triggers their release from the terminals of neurons. Zinc accumulation in neurons has been shown to play an important role in neuronal death following ischemia. However, almost nothing is known about whether zinc is involved in ischemia-induced blood–brain barrier (BBB) disruption. Herein, we investigated the contribution of zinc to ischemia-induced acute BBB disruption and the possible molecular mechanisms using both cellular and animal models of cerebral ischemia. Zinc greatly increased BBB permeability and exacerbated the loss of tight junction proteins (Occludin and Claudin-5) in the endothelial monolayer under oxygen glucose deprivation conditions. In cerebral ischemic rats, a dramatically elevated level of zinc accumulation in microvessels themselves was observed in isolated microvessels and *in situ*, showing the direct interaction of zinc on ischemic microvessels. Treatment with a specific zinc chelator *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN), even at 60-min post-ischemia onset, could greatly attenuate BBB permeability in the ischemic rats as measured by Evan's Blue extravasation, edema volume and magnetic resonance imaging. Furthermore, zinc accumulation in microvessels activated the superoxide/matrix metalloproteinase-9/-2 pathway, which leads to the loss of tight junction proteins (Occludin and Claudin-5) and death of endothelial cells in microvessels themselves. Our findings reveal a novel mechanism of cerebral ischemia-induced BBB damage, and implicate zinc as an effective and viable new target for reducing acute BBB damage following ischemic stroke.

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1. Introduction

Zinc is essential for normal cellular functions and serves a signaling role in the brain, but research during the last two decades has demonstrated the important role of zinc in the pathogenesis of cerebral ischemia (Galasso and Dyck, 2007; Shuttleworth and Weiss, 2011). It was reported that zinc vesicles were released from a subset of glutamatergic terminals of neurons under pathological conditions (Assaf and Chung, 1984; Koh et al., 1996). Microdialysis studies confirmed the increase of extracellular zinc in cerebral ischemia models, suggesting that zinc may be a critical mediator of ischemic brain injury (Frederickson et al., 2006; Kitamura et al., 2006). Our recent studies also showed that cytosolic zinc dramatically accumulated in neurons in the first a few hours of stroke, contributing to brain damage through promotion of neuronal cell death (Dong et al., 2015; Zhao et al., 2014). Until now, most studies of zinc in brain focused on its neuronal functions, while almost nothing is known about the role of zinc in blood–brain barrier (BBB) disruption after ischemic stroke.

BBB permeability in the early stage of ischemic stroke is regarded as a crucial event for the safety and efficacy of thrombolytic therapy (Kastrup et al., 2008; Liu et al., 2012b). BBB is formed by the endothelium of brain microvessels, which is surrounded by extracellular matrix and several associated cell types, including astrocytes, pericytes, and neurons. It is increasingly appreciated that the connections and interactions within the neurovascular unit play integrative roles in maintaining BBB integrity and in responding to ischemic insult (del Zoppo, 2010). Neurons, which are very sensitive and vulnerable to ischemia due to their high demands for oxygen and nutrition, are recognized as the first cells to respond to ischemia (Mabuchi et al., 2005). Thus, impaired signals, such as glutamate and reactive oxidative species from neurons, are likely to induce early microvasculature damages following cerebral ischemia. Therefore, we hypothesized that release of high concentration of zinc, as an impaired signal from neurons, may contribute to acute BBB disruption following ischemic stroke.

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Available online on ScienceDirect (www.sciencedirect.com).

http://dx.doi.org/10.1016/j.nbd.2016.07.003
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Matrix metalloproteinases (MMPs) comprise a large family of zinc endopeptidases that play an important role in mediating BBB vasculature disruption in ischemic stroke. Free radicals have been shown to regulate the activation of MMPs during ischemic stroke (Duansak and Schmid-Schonbein, 2013; Fu et al., 2014; Gu et al., 2012). Among all the MMP family members, gelatinases MMP-9 and MMP-2 have been the focus of many studies because of their substrate specificity for tight junction proteins (TJPs), which are structural components of the BBB (Jian and Rosenberg, 2005; Rosell et al., 2006). The MMP-mediated degradation of TJPs is significantly increased and contributes to BBB disruption after cerebral ischemia (Yang and Rosenberg, 2011). Given this premise, we speculated that the free radicals-MMPs pathway may be involved in the mechanism of zinc-mediated BBB disruption following acute cerebral ischemia.

In the present study, using *in vitro* and *in vivo* cerebral ischemic BBB models, we investigated the contribution of zinc to acute BBB disruption, and explored the possible mechanism of zinc-mediated BBB damage during acute cerebral ischemia.

2. Materials & methods

2.1. Preparation of in vitro BBB model with endothelial cell line

Brain microvascular endothelial cell (EC) was grown on a cell culture insert and prepared for as an *in vitro* BBB model, as we previously described (Li et al., 2014). The EC cell line (bEnd3) and cell culture mediums (Dulbecco's modified Eagle medium, DMEM) were obtained from American Type Culture Collection (Manassas, VA, USA). Briefly, ECs were cultured in DMEM supplemented with 10% FBS (Life technologies, CA, USA) and Antibiotic-Antimycotic (Life technologies, CA, USA) at a humidified atmosphere of 5% CO_2/95% air at 37 °C until they reached monolayer.

2.2. Exposure to $ZnCl_2$ and oxygen/glucose deprivation (OGD)

After ECs reached monolayer, varying concentrations of zinc chloride (0, 50, 100 and 150 μmol/L) were added in a double blind manner to the glucose free medium [DMEM-G(-)] or complete culture medium (DMEM) to mimic the ischemia-mediated increase of extracellular zinc. These concentrations were selected based on our previous study (Pan et al., 2013). DMEM-G(-) was pre-equilibrated with 95% N_2/5% CO_2 in a humidified airtight chamber (Billups-Rothberg Inc., Del Mar, CA, USA) equipped with an air lock and flushed with 95% N_2/5% CO_2 for 15 min. The EC monolayer was exposed to OGD to mimic ischemic conditions, as we described previously (Li et al., 2014). The chamber was then sealed and kept at 37 °C for 90 min. The oxygen concentration was below 0.2% as monitored by an oxygen analyzer (Sable Systems, Las Vegas, NV, USA). Control ECs were incubated with DMEM with 10% FBS at 37 °C in 5% CO_2/95% air. OGD was terminated by removing cells from the hypoxic chamber and returning to DMEM. Then the cell was incubated in 5% CO_2/95% air at 37 °C for 16 h.

2.3. EC monolayer permeability assay

EC monolayer permeability was analyzed by measuring the fluorescence of FITC-BSA across the monolayer, as we previously described (Li et al., 2014). Seven hundred and fifty microliter of assay medium was added to each lower chamber and 150 μL assay medium containing 100 μg/mL FITC-BSA was added to each insert. Incubation continued for 1 h at 37 °C in 5% CO_2/95% air. After the inserts were carefully removed, the medium in each lower chamber was thoroughly mixed. Aliquots of 150 μL conditioned media was collected from the lower chambers, FITC-BSA assay medium (total fluorescence added to inserts), and the assay medium itself (background) was used for measuring fluorescence intensity with a fluorescence plate reader. Endothelial monolayer permeability was quantified by using the following equation: Clearance (%) = (Fluorescence in lower chamber/Total fluorescence added in upper chamber) × 100.

2.4. Focal cerebral ischemia/reperfusion model of rats and drug administration

The Institutional Animal Care and Use Committee of Capital Medical University (Beijing, China) and University of New Mexico (NM, USA) approved all animal experiments. The animals were used in compliance with the principles set forth in the NIH Guide for Care and Use of Laboratory Animals. Male Sprague-Dawley rats (290–320 g) were anesthetized with 2% isoflurane and subjected to 90-min middle cerebral artery occlusion (MCAO) followed by 4-h or 22.5-h reperfusion using the suture occlusion model, as we previously described (Qi et al., 2012). Fifty rats that displayed circling to non-ischemic side prior to reperfusion were considered to have a successful MCAO and included in this study: 8 rats with 22.5-h reperfusion for magnetic resonance imaging (MRI) measurement, and 42 rats with 4-h reperfusion for the rest of experiments. Two rats without circling and one with subarachnoid hemorrhage were excluded.

Our previous study showed that treatment of ischemic rats with a zinc specific chelator, *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN), significantly decreased infarct volume and neuronal cell death at a dose of 15 mg/kg (Zhao et al., 2014). In this study, we chose the same TPEN dose (15 mg/kg) to investigate the contribution of zinc to BBB disruption after ischemia. TPEN (Sigma-Aldrich, MO, USA) was dissolved in 10% dimethyl sulfoxide (DMSO) to a final concentration of 5 mmol/L. DMSO or TPEN was injected intraperitoneally in a double-blind manner. Considering the possible applications in clinic, we administrated TPEN at 60 min post MCAO onset, instead of pretreatment before ischemia, to mimic the intervention on ischemic stroke patients.

2.5. Measurement of BBB permeability in ischemic rats by Evan's Blue extravasation and MRI

BBB disruption was quantitatively assessed by measuring Evan's Blue (EB) extravasation to ischemic hemispheric tissue, as we previously reported (Liu et al., 2009b). EB was intravenously administered *via* tail vein at 2 h of reperfusion. After 2-h circulation (at the end of 4-h reperfusion), intravascular EB were removed by transcardial perfusion with saline. The brains were sectioned into five 2-mm-thick coronal slices from a 10-mm-thick region 3 mm away from the tip of the frontal lobe. Brain slices were photographed and non-ischemic and ischemic hemispheric tissues were weighed and homogenized in trichloroacetic acid. The supernatant was diluted four fold with ethanol, and fluorescence intensity was measured on a microplate fluorescence reader.

As cerebral hemorrhage during acute ischemic stroke often occurs around 24 h post ischemia, we evaluated BBB permeability after 90-min ischemia/22.5-h reperfusion using dynamic contrast-enhanced (DCE)-MRI in live rats, mimicking the evaluation of BBB integrity in stroke patients. Animals were anesthetized with 2% isoflurane and DCE-MRI was performed as we previously reported (Yang et al., 2015). Briefly, the contrast agent Gd-DTPA (Magnevist, Bayer Healthcare Pharmaceuticals Inc., NJ, USA) at a dose of 0.1 mmol/kg was injected into the femoral vein. DCE-MRI was conducted using a 4.7 T Biospec dedicated research MR scanner (Bruker-Biospin; Billerica, MA, USA), equipped with 500 mT/m (rise time 80–120 μs) gradient set (for performing small animal imaging) and a small bore linear radio frequency coil (internal diameter 72 mm). The details of pulse sequence T1_EPI for T1 mapping are: FOV = 4 cm × 4 cm, slice thickness = 1.5 mm, slice gap = 0, matrix size = 128 × 128, TR/TE = 10,000 ms/8.3 ms, number of segments = 4, number of average = 1, total scan time = 2 m 40 s. T1 map was reconstructed with the t1epia fitting function in the Bruker ParaVision Image Sequence Analysis (ISA) tool. The Ki map was constructed from repeated estimates of $\Delta(1/T1(t))$. An in-

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