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Original article

Isoflurane post-conditioning down-regulates expression of aquaporin 4 in rats with cerebral ischemia/reperfusion injury and is possibly related to bone morphogenetic protein 4/Smad1/5/8 signaling pathway

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ABSTRACT

Aim: Aquaporins (AQPs) are water-channels that play important roles in brain water homeostasis and cerebral edema induced by brain injury. This study aimed to investigate the relationship between AQP4, bone morphogenetic protein 4 (BMP4)/Smad1/5/8 signaling pathway and isoflurane post-conditiong, which has effects on brain edema in rats with cerebral ischemia/reperfusion (I/R) injury.

Methods: Cerebral I/R injury was induced in rats by using the middle cerebral artery occlusion (MCAO) model for 90 min, followed by 24 h of reperfusion. Isoflurane post-conditioning (ISO) group received 90 min ischemia and underwent 1.5% isoflurane post-conditioning for 60 min after initiating reperfusion. Neurobehavior, brain water content, thionine staining and 2, 3, 5-triphenyl tetrazolium chloride staining were evaluated to measure levels of brain edema and damage. Expressions of AQP4, BMP4, Smad1/5/8 and phosphorylated Smad1/5/8 were detected by using Western blot, quantitative real-time polymerase chain reaction (qRT-PCR), and immunofluorescence (IF) staining.

Results: Compared with the Sham group, neurological behavior score, brain infarct volume and water content of MCAO model rats increased with reperfusion injury. However, in the ISO group, cell edema and damage of brain was significantly ameliorated ($P < 0.01$). qRT-PCR showed less AQP4 mRNA expression in the hippocampal tissue of the ISO group than in the I/R group ($P < 0.01$). Western blot and immunofluorescence results showed similar changes in protein levels of both groups. Related protein expressions showed expressions of BMP4 and Smad1/5/8 increased in the ISO group ($P < 0.01$), whereas total Smad1/5/8 expression didn't change in all groups. When BMP4 inhibitor (LDN193189) was injected, expression levels of AQP4 increased and neuronal density decreased ($P < 0.05$). By contrast, expression levels of BMP4 did not change significantly after pre-injection of AQP4 inhibitor (TGN020) ($P > 0.05$), but neuronal density increased ($P < 0.05$).

Conclusion: Isoflurane post-conditioning may inhibit occurrence of brain edema and reduce cerebral I/R injury through down-regulating expression of AQP4, This process may be related to the activation of BMP4/Smad1/5/8 signaling pathway.

1. Introduction

Stroke is a growing cerebrovascular condition which occurs upon interruption of blood flow to brain areas; this condition is also associated with high rate of severe disabilities and multiple functional impairments [1]. In recent years, mortality rate of stroke has decreased, whereas its incidence still increases, with ischemic cerebrovascular disease accounting for 80% of cases [2]. The following cerebral ischemia/reperfusion (I/R) injury will aggravate damage to neurocyte through reperfusion due to occlusion of cerebral artery. I/R injury, which includes primary injury of ischemia period and secondary injury during reperfusion, may form brain edema and aggravate brain injury that results from various factors, including cell membrane damage, excitatory amino acid toxicity, and neutrophil activation [3,4]. Brain edema is a deleterious neuropathological condition that contributes to severe disabilities and death during ischemic stroke, which follows

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blood-brain barrier (BBB) leakage [5]. High degree of edema is directly proportional to poor prognosis in patients. Therefore, more effective treatments that target underlying mechanisms of damage due to stroke are needed to reduce injury caused by brain edema and to improve patient outcomes.

Aquaporins (AQPs), a family of water-channel proteins, play an important role in water movement and homeostasis. AQP4, which is highly localized in perivascular end feet, is the most abundant and enriched AQP in the brain. Vascular endothelial cell astrocytes and perivascular end feet have been known as cellular components of BBB, which plays a pivotal role in brain edema after injury. Therefore, AQP4 is presumed to play an important role in maintaining water balance across the BBB [6]. A host of literature stated that AQP4 poses different effects on various types of brain edema: low expression of AQP4 can ameliorate brain edema and neurological deficits in cytotoxic brain edema model, which including focal cerebral ischemia model and water intoxication [7,8], whereas in vasogenic brain edema model such as brain tumors and focal frostbite damage, AQP4 deficiency may cause more serious outcome [9]. Thus, AQP4 can be presented as a potential and measurable therapeutic target for brain edema treatment because it regulates water movement during edema formation and resolution.

Bone morphogenetic proteins (BMPs) are a group of signaling molecules, which belongs to the transforming growth factor-β superfamily of proteins, with at least 20 structurally distinct members [10]. BMPs have been discovered for their capability to induce bone formation [11], beyond that, they are also found in different organ systems, specifically the nervous system [12]. Several studies have shown that BMP4 emerges as a vital factor regulating neuron survival and axon growth and stimulating neuronal differentiation of neuronal stem cells during adulthood and after injury of the central nervous system (CNS) [13–15]. BMP4/Smad1/5/8 signaling pathway exerts its effects by binding to transmembrane serine/threonine kinase receptors. Related complexes comprise BMP receptor IA(BMPRIA)/B and BMPRII phosphorylate receptor-associated Smad1, Smad5, and Smad8 (known as R-Smads), which can form heteromeric complexes with Co-Smad4 and translocate to the nucleus and control gene expression as transcription factors [10].

Isoflurane is one of the inhalation anesthetics widely used in clinical practice, Neuroprotective effect of isoflurane have been confirmed. Existing studies show that low concentrations of isoflurane pretreatment in a relatively short period confers protection on ischemia injury. Treatment after injury bears importance because of unpredictable cerebrovascular events. Therefore, the interest in isoflurane post-conditioning as an effective therapeutic method has been increasing. In this study, we detected the role of AQP4 in cerebral I/R injury through measuring infarct volume and brain edema and assessed its effects on neuroprotection by isoflurane post-conditioning and potential underlying mechanisms.

2. Measurement of brain water content

2.1. Animal preparation

All animals received humane care in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications number 80–23, revised in 1996). Experimental protocol was approved by the Animal Care and Use Committee of the First Affiliated Hospital of the Medical College, Shihezi University.

Male adult Sprague–Dawley rats (6–8 weeks old and weighing ~243.1 ± 35 g) were provided by the experimental animal center of Shihezi University. Rats were housed in rooms with controlled temperature, humidity, and 12 h light/12 h dark cycle, with food and water provided ad libitum. Rats were randomly divided into eight groups: (1) Sham group; (2) I/R group; (3) Isoflurane post-conditioning (ISO) group, rats received 90 min ischemic treatment and underwent 1.5% ISO post-conditioning for 60 min after an immediate reperfusion; (4) AQP4 inhibitor (TGN020) group, in which rats received an intraperitoneal injection of TGN020, a pharmacologic AQP4 inhibitor [16], at a dose of 100 mg/kg (Sigma-Aldrich, SML0136, St. Louis, MO, USA) before any ischemic treatment; (5) ISO + TGN020 group; (6) BMP4 inhibitor (LDN193189) group, in which rats received intraperitoneal injection of LDN193189, a pharmacologic BMP4 inhibitor [17], at a dose of 6 mg/kg (Selleck Chemicals, S1076, USA), before any ischemic treatment; (7) ISO + LDN193189 group; and (8) dimethyl sulfoxide(ISO + DMSO) group, in which rats were injected with DMSO before any ischemic treatment.

2.2. Surgical procedure and animal treatment

All surgical procedures were performed after intraperitoneal injection of chloral hydrate anesthesia (0.3 mL/100 g). After anesthetization, rats were placed in supine position, and a midline skin incision was made to expose the right common carotid artery, internal carotid artery and external carotid artery (ECA). Middle cerebral artery occlusion (MCAO) was performed as previously described [18]. A 3-0 monofilament nylon thread with a rounded tip was inserted into the ECA and gently advanced to a distance of 18.5 ± 0.5 mm to block blood flow of the right middle cerebral artery. Rectal temperatures were maintained at 37 °C during surgery using an incandescent lamp. After 90 min, the 3-0 filament was pulled out and reperfusion occurred. After the animals awaken, reperfusion was considered successful when rats incurred neurological behavior score of more than 1. Rats were excluded when the nylon filament did not completely block or penetrated blood vessels. Meanwhile, rats in the Sham group were subjected to the same procedure without any vessel ligation or occlusion. For the ISO group, rats received 90 min ischemic and underwent 1.5% ISO post-conditioning for 60 min after an immediate reperfusion. Subsequently, rats were sacrificed at appropriate time points for the following studies.

2.3. Neurobehavioral evaluation

Animals were examined for neurological function at 24 h after reperfusion. Neurological behavior of rats was assessed on a five-point scale according to the methods of Longa et al. [19]. A score of 0 indicated no neurological deficit. A score of 1 indicated mild focal neurological deficit, with rats failing to fully extend their left forepaw. A score of 2 indicated moderate focal neurological deficit, with rats circling to the left. A score of 3 indicated severe focal deficit, with rats slumping to the left when walking. Rats with a score of 4 failed to walk independently and exhibited depressed level of consciousness. Animals that died because of pulmonary insufficiency and suffocation were excluded.

2.4. Brain water content measurement

Rats were sacrificed at 24 h after reperfusion and isoflurane post-conditioning. Rats without ischemic treatment were treated as 0 h group. Brain tissues were immediately weighed (wet weight) and dehydrated at 100 °C for 24 h. Samples were then reweighed to obtain dry weights. Brain water content was calculated using the following formula: [(wet weight − dry weight)/wet weight] × 100% and used as index for brain edema.

2.5. Infarct volume measurement

Animals were selected randomly from each group and were anesthetized by intraperitoneal injection of 10% chloral hydrate at 24 h after reperfusion. After sacrificing rats by decapitation, brains were quickly isolated and sectioned into five coronal slices of 2 mm thickness. Slices were then stained in pre-heated with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich, St. Louis, MO, USA) for