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Inhaled Methane Protects Rats Against Neurological Dysfunction Induced by Cerebral Ischemia and Reperfusion Injury: PI3K/Akt/HO-1 Pathway Involved

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Background and Aims. Cerebral ischemia and reperfusion (I/R) could produce excess reactive oxygen species (ROS), which in turn induce neurological dysfunction and inflammation in cerebral tissues. This study was designed to study the effect of methane on cerebral I/R injury.

Methods. Fifty Sprague-Dawley (SD) rats were used to induce an animal model of cerebral I/R injury. Methane was mixed with air to achieve a final concentration of 2.2%. Rats started to inhale methane-air mixture after ischemia and continued it during the reperfusion. The neurological deficits, malondialdehyde (MDA) and tumor necrosis factor-α (TNF-α) in the brain tissue were examined. The protein kinase B (Akt) phosphorylation and heme oxygenase-1 (HO-1) expression was measured by Western Blot. The neurological deficits were re-measured after rats were treated with the HO-1 inhibitor Zinc protoporphyrin IX (ZnPP-IX), phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 and Akt inhibitor triciribine.

Results. Cerebral I/R induced neurological deficit, which was significantly decreased by methane. MDA and TNF-α levels were significantly enhanced by cerebral I/R, while methane caused significant reduction of MDA and TNF-α levels. Methane significantly increased Akt phosphorylation and HO-1 expression. The HO-1 inhibitor ZnPP-IX, PI3K inhibitor LY294002 and Akt inhibitor triciribine all significantly abolished the effect of methane on neurological deficit.

Conclusions. This finding suggests the possible application of methane for cerebral I/R injury and PI3K/Akt/HO-1 dependent antioxidant pathway may be involved.

Key Words: Cerebral ischemia and reperfusion injury, Methane, PI3K/Akt pathway, Heme oxygenase-1.

Introduction

Despite the fast development of medicine, stroke is still a leading disease that causes death in the modern world. As brain consumes most oxygen in the body, any blockade in the cerebral blood artery may cause ischemia in the brain (1). Restoration of blood flow is a most effective approach to treat the ischemia, but it may cause another consequence, ischemia-reperfusion (I/R) injury. The cerebral I/R induces a cytotoxic cascade as it may produce over amount of reactive oxygen species (ROS) and leads to neurological impairment, inflammation and/or apoptosis in the brain tissues (2,3). Although some medicine were reported to reduce neurological impairment or tissue injury, clinical tests show that they are not capable of controlling clinical cerebral I/R injury (4), which makes the discovery of treatments and interventions urgently needed.

For many years, methane is considered to be biologically inactive. However, accumulating evidence in recent years showed that low concentration of methane may produce protective effects. A study showed that methane can recover

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blood supply and reduce inflammatory reaction caused by intestinal I/R (5), which suggests an anti-inflammatory potential for methane. In this study, we first tested the impacts of inhaled low concentration methane on cerebral I/R. We measured the neurological deficits to evaluate the effect of methane. The anti-oxidative and anti- inflammatory ability of methane were examined through oxidative products and pro-inflammatory factors measurement.

Next, we investigated the role of PI3K/Akt pathway and HO-1 to study the mechanism. A study of Zhang X, et al. has reported that methane could inhibit NF-κB/MAPKs signal and inflammatory reaction through PI3K/Akt/GSK-3β pathway (6). Methane-rich saline resulted in Akt activation and the inhibition of PI3K-attenuated IL-10 production. Other studies indicated that HO-1 activation might require PI3K/Akt pathway (7−9). HO-1 is reported to have anti-inflammatory and anti-oxidant abilities (10). The activation of HO-1 signaling could protect cells from oxidative stress (11,12). Thus, in this study, to explore the roles of HO-1 and the PI3K/Akt pathway, the PI3K/Akt phosphorylation and HO-1 expression were examined with Western Blot. The neurological deficits were re-evaluated in the presence of inhibitors of PI3K/Akt pathway and HO-1 to examine their involvement.

Materials and Methods

Animals and Drugs

Sprague-Dawley (SD) rats (230−250 g) were purchased from Fudan university animal center. They were housed under 25°C and a 12 h d/night cycle in the Animal Center of Jinshan Hospital afflitated to Fudan University. All animals were allowed free access to water. The animals were fasted for 1 d prior to experiments. All the procedure of the study was approved by the animal care committee in Jinshan Hospital afflitated to Fudan University. Methane (99.99%) was brought from Taiyu Gas Company (Chengdu, China) and mixed with air to achieve a final concentration of 2.2%. Zinc protoporphyrin IX (ZnPP-IX) was used as a HO-1 inhibitor. LY294002 and triciribine were used to inhibit PI3K/Akt pathway. All these drugs were bought from Sigma Aldrich (CA, USA).

Experimental Protocol

Fifty rats were randomly grouped into Control group, Sham group, Cerebral I/R group, Cerebral I/R + Methane group and Methane group, 10 rats in each group. Rats in the Control group had no treatment at all; Rats in the Sham group had a fake cerebral I/R surgery and breathed air in a chamber; Rats in Cerebral I/R group received a cerebral I/R surgery and breathed air in a chamber; Rats in Cerebral I/R + Methane had a cerebral I/R surgery and breathed Methane/air mixture in a chamber; Rats in Methane received no cerebral I/R surgery and breathed Methane/air mixture in a chamber. Similarly to Strifler G, et al. (13), the inhalation with air or air mixed with 2.2% methane started after ischemia and continued during the reperfusion. ZnPP-IX, LY294002 or triciribine was given to rats at 30 min before cerebral I/R surgery started (with a dosage of 3 mg/kg, 100 mg/kg or 2 mg/kg of body weight, i.p.).

Cerebral I/R Surgery

Before the cerebral I/R surgery, animals were given 100 mg/kg sodium pentobarbital (i.p.) for complete anaesthesia. Afterwards, an incision was made at the middle line of the neck, and the right common carotid artery was then dissected. Next, a special nylon suture was put into the anterior cerebral artery to perform the middle cerebral artery occlusion procedure (MCAO). Two hours later, the blood flow was resumed by pulling out the suture in the middle cerebral artery and the ‘‘reperfusion’’ started. When performing the surgery on rats in Sham group, the nylon suture was not actually put into the cerebral artery.

Evaluation of Neurological Deficits

Neurological deficits were measured similarly to the method of Jia D, (14). No nerve injury symptom: 0 points; contralateral papillary flexion: 1 point; reduced contralateral forepaw clenched grip: 2 points; spontaneously move in all directions and circle to the opposite side when pulling their tails: 3 points; loss of consciousness: 4 points. The water maze study was performed with the method of de Bruin JP, (15).

Measurement of MDA and TNF-α in the Brain Tissue

For measurement of MDA, first of all, 100 mg of brain tissue was homogenized in phosphate buffer (10 mM, pH = 7.4). Next, they were centrifuged at 12,000 g for 30 min and the supernatant was collected. The MDA level in the supernatant was using a MDA assay kit (Nanjing Jiancheng, Nanjing, China), similarly to Weng D, et al. (16). The protein level was measured with a protein assay kit (Nanjing Jiancheng, Nanjing, China) with BCA method.

For measurement of TNF-α, firstly, brain tissue was washed and homogenized in ice cold saline. Afterwards, it was centrifuged at 3000 g at 4°C for 15 min. The concentration of TNF-α was examined with enzyme-linked immunosorbent commercial assay kits (Sigma, St. Louis, USA), according to the manufacturer manuals. The concentration was determined based on the absorbance read on a microplate reader (Azure Biosystems, Inc, Dublin, CA, USA) and shown as pg/mg protein.

Western Blotting Analysis

Protein was transferred to polyvinylidene fluoride membranes using Mini-Protean Tetra Electrophoresis System