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Hypoxic preconditioning induces neuroprotection against transient global ischemia in adult rats via preserving the activity of Na^+/K^+-ATPase

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ABSTRACT

We demonstrated previously that 30 min of hypoxic preconditioning (HPC) applied 1 day before 10 min of transient global cerebral ischemia (tGCI) reduced neuronal loss in the hippocampal CA1 subregion in adult rats. The aim of the present study was to investigate the role of Na^+/K^+-ATPase and protein kinase Mζ (PKMζ) in the protective effect of HPC against tGCI in adult rats. We found that the activity of Na^+/K^+-ATPase decreased in the hippocampal CA1 subregion after 10 min of tGCI. This effect was not seen after 30 min of HPC in adult rats. Corresponding to the changes in Na^+/K^+-ATPase activity, the surface expression of Na^+/K^+-ATPase α1 subunit increased after HPC. Furthermore, HPC dramatically reduced the number of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells in the hippocampal CA1 subregion after tGCI. However, neither PKMζ nor phosphorylation of PKMζ was changed after tGCI or HPC. The results of the present study are consistent with the hypothesis that both enhanced recovery of Na^+/K^+-ATPase activity due to preserved the protein levels of Na^+/K^+-ATPase α1 subunit and reduced DNA fragmentation after tGCI contribute to the protection afforded by HPC. However, PKMζ activation does not appear to play a role in this neuroprotection.

1. Introduction

Sublethal pretreatments, according to collective evidence, can provide neuroprotective adaptation against subsequent severe ischemia in the brain (Kapinya, 2005; Ran et al., 2005). This process has been termed preconditioning or ischemic tolerance. Studying preconditioning is conducive to identification of the underlying endogenous protective signaling cascades, with the long-term goal to allowing therapeutic augmentation of the endogenous protective mechanisms in cerebral ischemia and possibly to induce a protected state of the brain in conditions in which ischemia can be anticipated, such as during surgery of the heart or cardiac arrest upon resuscitation. Diverse types of preconditioning can produce tolerance to ischemic injury either *in vivo* or *in vitro* (Kirino, 2002). Moderate hypoxia that does not cause neuronal death and may be safer to be applied in clinical practice becomes an attractive method in animal research for ischemic tolerance. We recently demonstrated that hypoxic preconditioning (HPC) for 30–120 min significantly reduced cell death in the CA1 subregion after 10 min of transient global cerebral ischemia (tGCI). HPC was effective only when applied 1–4 days before ischemia. The maximum protection was observed with 30 min of hypoxia and 1 day interval between hypoxia and tGCI (Zhan et al., 2010). The protection afforded by HPC against cell death secondary to transient ischemia is dramatic and observable in organs other than brain. However, although a large number of studies have identified different triggers and mediators of HPC, the precise mechanisms by which HPC protects the brain against cell death secondary to ischemia/reperfusion (I/R) remain poorly understood.

The Na^+/K^+-ATPase, or Na^+/K^+-pump, is a transmembrane heterodimer protein composed of α and β subunits. The α-subunit is a multispanning membrane protein that is responsible for the catalytic and transport properties of the enzyme. Neurons express the α1 and α3 subunits. The β-subunit is a polypeptide that is essential for the normal activity of the enzyme (Blanco and Mercer, 1998). Na^+/K^+-ATPase is a vital player in maintaining ionic homeostasis. Blocking the Na^+/K^+-pump concomitantly reduces intracellular K^+ and increases Ca^{2+} and Na^+. Excessive Ca^{2+} and Na^+ influx and their accumulation in the intracellular space are most likely to be responsible for necrotic death (Choi, 1988), while excessive K^+ efflux and intracellular K^+ depletion may play key roles in apoptotic death (Dallaporta et al., 1998). During cerebral ischemia, the substrates required for energy production are rapidly depleted and mitochondrial dysfunction is observed. Mitochondrial adenosine triphosphate (ATP) depletion is a crucial factor in determining

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neuronal cell death. The production of ATP is critical for the maintenance of Na^+/K^+ pump activity, which is decreased during cerebral ischemia (Lees, 1991). Several studies have demonstrated that ischemic preconditioning (IPC) can modify intracellular Na^+ kinetics by preserving Na^+ efflux through the Na^+/K^+ pump, protecting against reperfusion-induced cell death in the brain and heart (Imahashi et al., 2001; Yorozuya et al., 2004; de Souza Wyse et al., 2000). However, whether the activation of Na^+/K^+-ATPase is involved in the mechanism of ischemic tolerance induced by HPC remains unknown. Similarly, the mechanism by which HPC enhances the recovery of Na^+/K^+-ATPase activity during reperfusion has not yet been elucidated.

Protein kinase Mζ (PKMζ), a brain-specific and atypical PKC isoform, lacks the autoinhibitory regulatory domain in PKCζ and other PKC isoforms. The lack of the autoinhibitory domain explains the constitutive kinase activity of PKMζ in the absence of second messengers that are needed to activate other PKC isoforms (Hernandez et al., 2003). Protein levels of atypical PKCs, including PKMζ, were maintained following anoxic insult in the rat hippocampal slice model while other PKC isoforms were downregulated (Libien et al., 2005). Moreover, atypical PKCs also bind ATP with a 2–3-fold higher affinity than conventional or novel PKCs (Spitaler et al., 2000), suggesting that the activity of PKMζ may be preserved at low ATP levels and, thus, substrates of PKMζ may remain phosphorylated during ischemia. The crucial role of PKCs in ischemic preconditioning of the heart has been supported by numerous studies (Kuno et al., 2007; Yang et al., 2010). In the brain, however, the neuroprotective role of PKCs during ischemic tolerance is not completely understood. Previous studies have indicated that PKCζ positively regulates Na^+/K^+-ATPase activity in rat thyroid cells (Marsigliante et al., 2003). However, whether PKMζ regulates Na^+/K^+-ATPase activity in the brain remains unclear. Considering that PKMζ and PKCζ share identical catalytic domains, we examined whether PKMζ is needed for the regulation of rat brain Na^+/K^+-ATPase activity during ischemia or ischemic tolerance.

In the present study, we investigate the role of Na^+/K^+-ATPase and PKMζ in hypoxic preconditioning by using a model of HPC to protect againt tGCI in adult rats. We also explore the potential mechanisms by which HPC enhances the recovery of Na^+/K^+-ATPase activity during reperfusion.

2. Materials and methods

2.1. Animal model

Experiments were performed on adult male Wistar rats weighing 250–300 g (Southern Medical University, Guangdong, China). Animals used in this study were treated in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, Revised 1996). The Guangzhou Medical College Committee on Use and Care of Animals closely monitored the experiments to ensure compliance with the NIH regulations. All efforts were made to minimize animal suffering, to reduce the number of animals used.

Rats were preconditioned by expositing them to a 30 min period of systemic hypoxia 24 h before a tGCI. Hypoxia induced as described previously (Zhan et al., 2010). Briefly, the rats were placed in a sealed plastic chamber of 9000 cm^3 through which air containing 8% O_2 and 92% N_2 flowed continuously at room temperature. Total gas flow was 200 mL/min and no more than three animals were placed in the chamber at any given time.

Transient global ischemia was induced by applying the four-vessel occlusion method (Pulsinelli and Brierley, 1979). Briefly, the animals were anesthetized with chloral hydrate (350 mg/kg, i.p.). Vertebral arteries were electrocauterized, and common carotid arteries were isolated. A teflon/silastic occluding device was placed loosely around each carotid artery without interrupting carotid blood flow. Forebrain ischemia was induced in the awake rats 24 h after the surgery by occluding both common carotid arteries for 10 min. After occlusion, rats who had lost their righting reflex within 1 min and whose pupils were dilated were selected for experiments. Rectal temperature was maintained at 37–38 °C throughout the procedure.

Sham-operated and Sham-operated, hypoxia-treated rats were performed with the same surgical procedures except that the arteries were not occluded. Sham-operated, hypoxia-treated rats were exposed to 30 min hypoxia 24 h before described above procedures.

2.2. Histology

Seven days after ischemia, animals were perfused intracardially with normal saline, followed by 4% paraformaldehyde in PBS under anesthesia. Brains were removed quickly and further fixed with the same solution at 4 °C overnight. Post-fixed brains, dehydrated with 30%, 50%, 70%, 80%, 90%, 95%, and 100% ethanol were embedded with paraffin. The coronal sections selected from the dorsal hippocampus (between AP 4.8 and 5.8 mm, interaural or AP −3.3 to 3.4 mm, Bregma) were prepared in 5 μm thick sections by using a microtome followed by deparaffinizing with xylene, redehydrating with ethanol at graded concentrations of 30–100% (v/v) and washing with water. Sections were stained with Cresyl violet and examined under a light microscope (×660). The surviving cells in a 1-mm length of the CA1 pyramidal layer were counted. All of the data from cell counting were collected from two specific regions in the CA1 layer. Cell damage was quantified bilaterally in sections from each brain and assessed double-blindedly. Four sections for each animal were evaluated.

2.3. Measurement of Na^+/K^+-ATPase activity

With minor modifications, Na^+/K^+-ATPase activity from the hippocampal CA1 subregion was assayed according to the manufacturer's instruction (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Briefly, the tissues from the CA1 subregion of rats subjected to tGCI with or without HPC after 0, 24 and 48 h ($n = 6$ in each group) of reperfusion were homogenized in 10 v/wt. of buffer (0.32 mM sucrose, 5 mM HEPES, and 1 mM EDTA, pH 7.5). Membrane fractions were prepared using a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1 mM. After centrifugation at 69,000*g* for 2 h, the fraction at the 0.8–1 mM sucrose interface was taken as the membrane enzyme preparation. The reaction mixture for Na^+/K^+-ATPase assay contained 5 mM $MgCl_2$, 80 mM NaCl, 20 mM KCl, 40 mM Tris–HCl buffer, pH 7.4, in a final volume of 200 μl. The reaction was initiated by the addition of ATP to a final concentration of 3 mM. Control assays were carried out under the same conditions with addition of 1.0 mM ouabain. Na^+/K^+-ATPase activity was calculated by the difference between the two assays. Released inorganic phosphate (Pi) was measured as described previously (de Souza Wyse et al., 2000). All samples were run in duplicate. Specific activity of the enzyme is expressed as nmol Pi released per min per mg of protein. For the final calculation, the Na^+/K^+-ATPase activity of each group was expressed as a percentage of the Sham-operated control.

2.4. Western blotting

The rat was anesthetized at 0, 24 and 48 h ($n = 6$ in each group) after reperfusion or at 24 h after hypoxia, and the brain was rapidly removed and chilled in ice-cold PBS for 3 min. The brain tissue was cut into 2 mm coronal slices using a brain matrix (Shuolinyuan