Tacrolimus Postconditioning Alleviates Apoptotic Cell Death in Rats after Spinal Cord Ischemia-reperfusion Injury via Up-regulating Protein-Serine-Threonine Kinases Phosphorylation

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Summary: The effects of tacrolimus postconditioning on protein-serine-threonine kinases (Akt) phosphorylation and apoptotic cell death in rats after spinal cord ischemia-reperfusion injury were investigated. Ninety male SD rats were randomly divided into sham operation group, ischemia-reperfusion group and tacrolimus postconditioning group. The model of spinal cord ischemia was established by means of catheterization through femoral artery and balloon dilatation. The spinal cord was reperfused 20 min after ischemia via removing saline out of balloon. The corresponding spinal cord segments were excised and determined for Akt activity in spinal cord tissue by using Western blotting at 5, 15, and 60 min after reperfusion respectively. Spinal cord tissue sections were stained immunohistochemically for detection of the phosphorylated Akt expression at 15 min after reperfusion. Flow cytometry was applied to assess apoptosis of neural cells, and dry-wet weights method was employed to measure water content in spinal cord tissue at 24 h after reperfusion. The results showed that the activities of Akt in tacrolimus postconditioning group were significantly higher than those in ischemia-reperfusion group at 5, 15, and 60 min after reperfusion (P<0.05, P<0.01). The Akt activities reached the peak at 15 min after reperfusion in ischemia-reperfusion group and tacrolimus postconditioning group. The percentage of apoptotic cells and water content in spinal cord tissue were significantly reduced (P<0.01) in tacrolimus postconditioning group as compared with those in ischemia-reperfusion group at 24 h after reperfusion. It is concluded that tacrolimus postconditioning can increase Akt activity in spinal cord tissue of rats, inhibit apoptosis of neural cells as well as tissue edema, and thereby alleviate spinal cord ischemia-reperfusion injury.

Key words: protein-serine-threonine kinases; reperfusion injury; spinal cord ischemia; tacrolimus postconditioning

Spinal cord ischemia-reperfusion injury (SCIRI) may be provoked by spine fracture and dislocation, vascular lesions of spinal cord, various types of spine surgery, aortic aneurysm repair, and so on. SCIRI tends to cause paraplegia or even death. For instance, the incidence of paraplegia is up to 16% following thoracoabdominal aortic repair1, which seriously spoils life quality of the patients. Our group recently confirmed that tacrolimus postconditioning could efficiently reduce lipid peroxidation damage, and promote hindlimb motor function recovery after SCIRI2. The signal transduction pathways underlying these phenomena, however, remain unresolved. In this study, we further explored possible mechanisms for the neuroprotective effect of tacrolimus postconditioning, with purpose to elucidate the changing pattern of serine-threonine protein kinase (Akt) since the onset of SCIRI and its role in tacrolimus postconditioning-induced tolerance of spinal cord to ischemia-reperfusion injury in rats.

1 MATERIALS AND METHODS

1.1 Experimental Subjects and Grouping

Ninety adult male Sprague-Dawley rats weighing 270 to 320 g were purchased from the Experimental Animal Center of Wuhan University (China). The animal experiment was approved by the Animal Ethics Committee of Wuhan University, China. Rats were randomly divided into three groups: ischemia-reperfusion (IR) group, tacrolimus postconditioning (TP) group, and sham operation (SO) group with 30 rats in each group. IR group underwent reperfusion by drawing saline out of balloon 20 min after spinal cord ischemia. TP group experienced a single injection of tacrolimus (0.5 mg/kg) through the left common carotid artery immediately at the onset of reperfusion 20 min after spinal cord ischemia. TP group experienced a single injection of tacrolimus (0.5 mg/kg) through the left common carotid artery immediately at the onset of reperfusion 20 min after spinal cord ischemia. TP group experienced a single injection of tacrolimus (0.5 mg/kg) through the left common carotid artery immediately at the onset of reperfusion 20 min after spinal cord ischemia. TP group experienced a single injection of tacrolimus (0.5 mg/kg) through the left common carotid artery immediately at the onset of reperfusion 20 min after spinal cord ischemia.
ischemia-reperfusion and tacrolimus postconditioning.

1.2 Preparation of SCIRI Model

On the basis of the report issued by Taira et al,[3] with some modifications, a rat model of SCIRI was established by means of catheterization through femoral artery and balloon dilatation as described previously[2].

1.3 Detection of Expression of Total Akt (T-Akt) and Phosphorylated Akt (P-Akt) by Western Blotting

Five rats from each group at 5, 15, and 60 min after reperfusion respectively were anesthetized intraperitoneally with 10% chloral hydrate (400 mg/kg) and assumed a prone position. The segments of lumbar spinal cord tissue (L2–L5) were excised as a whole by using a scalpel and rinsed in ice saline. After removal of fibrous connective tissues, the spinal cord segments were stored in a –80°C refrigerator or immediately used for detection. The segments were incubated on ice with protein lysates and thoroughly homogenized. Homogenates were centrifuged at 12 000 r/min for 60 min at 4°C and supernatants were collected for testing. Protein concentrations were determined through Coomassie Brilliant Blue G-250 assay. After heating for 3 min at 100°C, proteins were separated via SDS-polyacrylamide gel electrophoresis, and transferred via electroblotting from gels to PVDF membranes. The blotted membranes were blocked with 5% skim milk for 2 h at room temperature and incubated overnight at 4°C with anti-T-Akt and anti-P-Akt primary antibodies (1:1000). Subsequently, blots were incubated for 60 min at room temperature with horseradish peroxidase (HRP)-labeled secondary antibody (1:5000). Reactions were visualized with enhanced chemiluminescence (ECL) and exposure. Bands were imaged and preserved by using automated imaging system. The Akt activities were represented by the ratios of the absorbance (A) values of P-Akt band to T-Akt band in the same sample.

1.4 Immunohistochemical Staining for Expression of P-Akt

On the basis of the P-Akt expression profiles derived from Western blotting, the observation time point for immunohistochemical staining was set at 15 min after spinal cord reperfusion. Five rats from each group were anesthetized by intraperitoneal injection of 10% chloral hydrate (400 mg/kg) and received thoracotomy. Aortic perfusion was carried out via the left ventricle cannula with 250 mL saline until effluent became clear, and then followed by perfusion of 4% paraformaldehyde for 30 min. The L2–L5 spinal cord segments were excised as described above, and postfixed in 4% paraformaldehyde for 24 h. The segments were embedded in paraffin, and cut into 5-μm thick continuous transverse sections. After being dewaxed and rehydrated, the sections were incubated with 3% hydrogen peroxide solution for 10 min at room temperature in order to inactivate endogenous peroxidase, and then blocked with normal goat serum for 20 min at room temperature. Phosphate buffer solution (PBS) instead of the primary antibody was used as a negative control, and the remaining steps unchanged. After incubation with anti-P-Akt primary antibody overnight at 4°C, the sections were incubated chronologically at 37°C with biotinylated secondary antibody and reagents SABC for 20 min each. Finally, reactions were visualized with diaminobenzidine (DAB). The sections were counterstained with hematoxylin, then dehydrated and made transparent with xylene. After sealing with neutral resin, the sections were observed under a light microscope. Cells with nucleus and cytoplasm stained brown were regarded as positive cells.

1.5 Examination of Apoptosis by Flow Cytometry

Five rats from each group at 24 h after reperfusion were anesthetized as described above. The lumbar spinal cord segments (L2–L5) were excised as a whole by using a scalpel, and sheared in PBS at 4°C. After blown gently, the samples were filtered through 200-mesh and 400-mesh screens in order to remove cell aggregates and disperse cells. Suspensions were centrifuged at 300 r/min for 10 min at 4°C and supernatants were discarded. Precipitates were diluted with PBS and cell-counted to prepare single cell suspensions with concentration of 1×10⁶/mL. Single cell suspensions of 1 mL were centrifuged at 1000 r/min for 5 min at 4°C. Supernatants were discarded and precipitates were dissolved with 500 μL binding buffer (1×), followed by mixture with 5 μL Annexin V-FITC and 10 μL propidium iodide. The specimens were incubated away from light for 10 min at room temperature, and mounted on flow cytometre to detect apoptosis within 1 h.

1.6 Determination of Water Content in Spinal Cord Tissue

Five rats from each group at 24 h after reperfusion were anesthetized as described above. The segments of lumbar spinal cord tissue (L2–L5) were excised as a whole by using a scalpel and rinsed in ice saline. After removal of fibrous connective tissues, the segments were dried with filter paper and then put into a beaker. The wet weights were obtained by using an electronic scale. Subsequently, the specimens were dried in an electronic thermostat for 48 h at 96±2°C to a constant weight (namely, the difference between two weight values is no more than 0.2 mg). The weights were documented as dry weights. Water content in spinal cord tissue was calculated according to the following formula: Water content=(Wet weight–Dry weight)/Wet weight×100%.

1.7 Statistical Analysis

Statistical analyses were performed by using SPSS software package (version 19.0) and all values were expressed as x±s. One-way analysis of variance (one-way ANOVA) was used to test inter-group variations. Least significant difference (LSD) method was applied to test inter-mean variations by pairwise comparison. A P<0.05 was considered to be statistically significant.

2 RESULTS

2.1 Western Blot Analysis

The activities of Akt were remarkable in spinal cord tissue of rats in the SO group. In the IR group, the Akt activities in spinal cord tissue were dropped to a minimum at 5 min, went up at 15 min, and then decreased again at 60 min after reperfusion. There was a significant difference in Akt activities between the IR group and the SO group at each time point after reperfusion (P<0.01). The Akt activities in spinal cord tissue of rats in the TP group were still significantly lower than those in the SO group at 5, 15, and 60 min after reperfusion (P<0.01), and the peak of Akt activities was also observed at 15