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INHIBITION OF TRANSFORMING GROWTH FACTOR BETA-ACTIVATED KINASE 1 CONFERS NEUROPROTECTION AFTER TRAUMATIC BRAIN INJURY IN RATS

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Abstract—The transforming growth factor beta-activated kinase 1 (TAK1), a member of the Mitogen-activated protein kinase kinase kinase family, is characterized as a key regulator in inflammatory and apoptosis signaling pathways. The aim of the present study was to evaluate the role of the TAK1 pathway in experimental traumatic brain injury (TBI) in rats. Adult male Sprague–Dawley rats were subjected to TBI using a modified Feeney's weight-drop model. The time course showed that a significant increase of TAK1 and p-TAK1 expression in the cortex after TBI. Moreover, TBI induced TAK1 redistribution both in neurons and astrocytes of the lesion boundary zone. The effects of specific inhibition of the TAK1 pathway by 5Z-7-oxozeaenol (OZ, intracerebroventricular injection at 10 min post-trauma) on histopathological and behavioral outcomes in rats were assessed at 24 h post injury. The number of TUNEL-positive stained cells was diminished and neuronal survival and neurological function were improved with OZ treatment. Biochemically, the high dose of OZ significantly reduced the levels of TAK1 and p-TAK1, further decreased nuclear factor-κB and activator protein 1 activities and the release of inflammatory cytokines. In addition, we found that both 10 min and 3 h post-trauma OZ therapies could markedly improve neurological function and neuronal survival after long-term survival. These results revealed that the TAK1 pathway is activated after experimental TBI and the inhibitor OZ affords significant neuro- protection and amelioration of neurobehavioral deficits after experimental TBI, suggesting a potential rationale for manipulating this pathway in clinical practice.

Key words: TAK1, traumatic brain injury, 5Z-7-oxozeaenol, NF-κB, AP-1, inflammation.

INTRODUCTION

Traumatic brain injury (TBI) is acknowledged as one of the leading causes of morbidity and mortality, which constitutes a major healthy and socioeconomic problem throughout the world (Maas et al., 2008). The development of the lesion after TBI consists of the primary injury and the secondary injury cascade. The secondary injury leads to ongoing neuronal degeneration within minutes to months after the injury (Morganti-Kossmann et al., 2007). A variety of biochemical and molecular mechanisms are responsible for secondary brain damage including excessive glutamate release, production of free oxygen radicals, lipid peroxidation, and endogenous neuroinflammation mechanisms (McIntosh et al., 1998). Despite intense research, the pathogenesis of the secondary injury following TBI is still not well understood, and no specific pharmacological treatment is available.

Transforming growth factor beta-activated kinase 1 (TAK1), a member of the mitogen-activated protein kinase kinase kinase family, plays an essential role in tumor necrosis factor (TNF), interleukin 1 (IL-1), and Toll-like receptor (TLR) signaling pathways (Ninomiya-Tsuji et al., 1999b; Sato et al., 2005; Shim et al., 2005). TAK1 was recently shown to be activated in rodents after neonatal hypoxic-ischemic injury (Nijboer et al., 2009), in a rodent model of middle cerebral artery occlusion (MCAO), and *in vitro* after oxygen glucose deprivation (Neubert et al., 2011). Importantly, recent evidences from animal experiments indicated that the inhibition of TAK1 activation decreased infarct volume after focal cerebral ischemia (Neubert et al., 2011). However, to the best of our knowledge, the role of TAK1 in conditions associated with experimental TBI has not yet been elucidated. In the present study, we investigated the time-course of TAK1 activation after TBI. To investigate the role of the TAK1 pathway after TBI, we assessed histologic and behavioral parameters

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Abbreviations: AP-1, active protein 1; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; HRP, horseradish peroxidase; ICV, intracerbroventricular; IF, immunofluorescence; IHC, immunohistochemistry; IL, interleukin; MMP, matrix metalloproteinase; NeuN, mouse anti-neuronal nuclei; NF-κB, nuclear factor-kappaB; OZ, 5Z-7-oxozeaenol; PBS, phosphate-buffered saline; TAK1, transforming growth factor beta-activated kinase 1; TBI, traumatic brain injury; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

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after post-traumatic injection of the selective TAK1 inhibitor 5Z-7-oxozeaenol (OZ).

EXPERIMENTAL PROCEDURES

Animals and trauma model

The male Sprague–Dawley rats weighing 250–300 g were used in this study. Rats were housed in a reversed 12-h light/12-h dark cycle controlled environment with free access to food and water. All procedures were approved by the Nanjing University Animal Care and Use Committee and in accordance with the Guide for the Care and Use of Laboratory Animals by the National Institute of Health (NIH).

Experimental TBI was performed with modification of Feeney's weight-drop model as previously described in our laboratory (Hang et al., 2005). Briefly, under intraperitoneal anesthesia with Phenobarbital sodium (50 mg/kg) and spontaneous breathing, a 20-mm midline incision was made over the skull, the skin and fascia were reflected, and a bone window of right parietal was opened by dental drill, 5 mm in diameter, with the center positioned 1.5 mm posterior and 2.5 mm lateral to the bregma. The dura was intact during the operation. A steel weight (40 g with a flat-end) fell on a little pillar (4 mm in diameter, 5 mm long) on the dura from a height of 25 cm along a stainless steel rod. The pillar was allowed to compress the cortex in a maximum depth of 5 mm. The sham operated animals were also anesthetized and the right parietal was made a bone window without cortex contusion. Rectal and temporalis muscle thermistors are used to maintain core and brain temperatures at 36.8–37.2 °C using feedback warming lamps. Arterial blood samples were analyzed intermittently to maintain these parameters within normal physiological ranges. The rats were placed on the heating pad to maintain their temperature around 37.0 °C until they could move independently. After recovering from anesthesia, rats were returned to their cages with food and water provided *ad libitum*.

Experimental design

First, we determined time course of TAK1 and p-TAK1 expression in the cortex after TBI in rats. Adult male rats were randomly divided into the control group and TBI groups. After the induction of TBI the animals were randomly assigned into five groups: animals surviving brain trauma for 1, 3, 6, 12 (each $n = 6$), and 24 h ($n = 10$). Sham-operated rats were used as control. Because there was no significant difference of all detected variables between control groups from 1 to 24 h in the preliminary study, animals of the control group were killed for sample collection at 6 h after being sham operated.

Then, effect of OZ after experimental brain trauma was investigated. This group of animals went through the same procedure as the above-mentioned TBI animals. After induction of TBI the rats were randomly assigned into four groups: rats treated with vehicle (dimethylsulfoxide – DMSO) or OZ (2, 10, or 20 μg; Tocris Bioscience) 10 min after TBI (each $n = 9$). Rats in the sham group were subjected to the surgical procedures as described above and to these rats were administered equal volumes of DMSO without OZ. After neurologic scoring at 24 h post injury, brains were harvested. To determine the possible long-term benefits of acute TAK1 inhibition, we subjected a separate cohort of rats to determine the beam-walking score at days 1, 3, and 7 post OZ administration. In order to investigate the clinical utility of TAK1 inhibition, we also injected OZ 3 h post injury. The functional outcome was assessed at days 1, 3, and 7 after TBI. Nissl stain was used to measure the neuronal survival at day 7 post-injury.

OZ treatment protocol

The TAK1 inhibitor OZ was purchased from Tocris Bioscience. OZ was dissolved in pure DMSO just before use (Bersudsky et al., 1997). OZ solution (5 μl) was injected into the left lateral ventricle 10 min post-injury, using a 10 μl Hamilton microsyringe. For the intracerbroventricular (ICV) injections, each animal was positioned in a stereotactic frame under phenobarbital sodium anesthesia. Coordinates for the injection placement were 1.0 mm posterior to bregma, 1.4 mm lateral to midline, and 4.4 mm below the skull surface (Paxinos and Watson, 2007) and the injection duration was 10 min.

Beam-walking score

The behavioral functional tests were performed in animals by an observer who was blinded to the experimental conditions. This score evaluates motor coordination of animals. The test was performed as described earlier (Su et al., 2011). The beam-walking performance was scored in a blinded fashion using a grading scale from 1 to 7 as follows: 1, the rat is unable to place the affected hind limb on the horizontal surface of the beam; 2, it places the affected hind limb on the horizontal surface of the beam and maintains balance but is unable to traverse the beam; 3, the rat traverses the beam dragging the affected hind limb; 4, it traverses the beam and once places the affected hind limb on the horizontal surface of the beam; 5, the rat crosses the beam and places the affected hind limb on the horizontal surface of the beam to aid less than half its steps; 6, the rat uses the affected hind limb to aid more than half its steps, and 7, the rat traverses the beam with no more than two foot slips. All rats were trained to traverse the beam 24 h before and on the day of injury immediately before anesthesia. The maximum was seven for non-operated rats. Beam-walking score was assessed after injury in vehicle- and OZ-treated rats at 24 h, 72 h and 7 days by an investigator who was blinded with regard to the study groups.

Biochemical assays

The surrounding brain tissue of the injured cortex was dissected from the region that was less than 3 mm from the margin of the contusion site on ice as described in our previous study (Wang et al., 2012), which were stored at −80 °C until homogenization. Brain tissue segment was homogenized on ice in 10 mM Tris–HCl buffer (pH 7.4), 10 mM EDTA, containing 3% Triton-100, 1% SDS and 200 mM NaCl using a homogenizer. The homogenized samples were then centrifuged at 13,000*g* for 10 min at 4 °C. Following centrifugation, protein concentration in each sample was assayed with BCA reagent (Sigma). Samples were then diluted (1:1) in a sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 5 mM β-mercaptoethanol, 10% glycerol), boiled for 5 min, and stored at −20 °C. Proteins were fractionated by 10% SDS–PAGE, transferred to a polyvinylidene difluoride (PVDF; Bio-Rad, Shanghai, China) membrane, and incubated for 1 h in blocking solution (3% milk, 150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 0.05% Tween 20) at room temperature. Blots were incubated with specific antibodies overnight at 4 °C. The primary antibodies, diluted according to manufacturer's recommendations, were directed against TAK1, phosphorylated TAK1 (p-TAK1, Cell Signaling, Danvers, MA, USA). Antibody against β-actin (1: 1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was incubated for 1 h at room temperature. Blots were washed extensively in TBST (150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 0.05% Tween 20), then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. The blotted protein bands were visualized by enhanced chemiluminescence (ECL) Western blot detection reagents (Amersham, Arlington Heights, IL, USA) and were exposed to X-ray film. Developed films were digitized using an