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Inhibition of Na^+-K^+-$2Cl^-$ Cotransporter-1 attenuates traumatic brain injury-induced neuronal apoptosis *via* regulation of Erk signaling

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ABSTRACT

Traumatic brain injury (TBI) is the leading cause of mortality and morbidity worldwide and is characterized by immediate brain damage and secondary injuries, such as brain edema and ischemia. However, the exact pathological mechanisms that comprise these associated secondary injuries have not been fully elucidated. This study aimed to investigate the role of the Na^+-K^+-$2Cl^-$ cotransporter-1 (NKCC1) in the disruption of ion homeostasis and neuronal apoptosis in TBI. Using a traumatic neuron injury (TNI) model *in vitro* and a controlled cortex injury (CCI) model *in vivo*, the present study investigated changes in the expression and effects of NKCC1 in TBI using western blot, RNA interference, a lactate dehydrogenase (LDH) release assay, TdT-mediated dUTP Nick end-labeling (TUNEL) analysis, sodium imaging, brain water content, and neurological severity scoring. TBI induced the expression of NKCC1 to be significantly upregulated in the cortex, both *in vitro* and *in vivo*. Pharmacological inhibitor bumetanide (Bume) or NKCC1 RNA interference significantly attenuated TBI-induced intracellular Na^+ increase, inhibited neuronal apoptosis, and improved brain edema and neurological function. Furthermore, NKCC1 inhibition also significantly inhibited TBI-induced extracellular signal-regulated kinase (Erk) activation. Erk inhibition significantly protected neurons from TBI injury; however, Erk inhibition had no effect on NKCC1 expression or the neuroprotective effect of NKCC1 inhibition against TBI. This study demonstrates the role of NKCC1 in TBI-induced brain cortex injury, establishing that NKCC1 may play a neurotoxic role in TBI and that the inhibition of NKCC1 may protect neurons from TBI *via* the regulation of Erk signaling.

1. Introduction

Traumatic brain injury (TBI) is one of the most prevalent causes of neurological dysfunction and death, which leads to substantial economic and social burdens worldwide (Algattas and Huang, 2014). However, the mechanisms of TBI remain elusive, especially regarding secondary brain injuries, such as cerebral ischemia, hypoxia, and brain edema. Brain edema, which causes an increase in intracranial pressure (ICP), results in decreased cerebral vascular perfusion, cerebral ischemia, and even death; moreover, it is the pathological hallmark of TBI (Donkin and Vink, 2010; Marmarou and et al., 2006). Two types of brain edema are involved in TBI: cytotoxic and vascular brain edema (Kahle and et al., 2009; Klatzo, 1967; Papadopoulos et al., 2002). Vascular edema has long been considered the major contributor to the process of brain edema. However, increasing evidence indicates that cytotoxic brain edema is the chief cause of mortality in TBI and acts by disturbing ion homeostasis, increasing ICP, and impairing extracellular neurotransmitter clearance (Rungta and et al., 2015; Kimelberg, 2004). Therefore, it is critical to investigate the mechanisms of cytotoxic brain edema in TBI.

NKCC1, an important member of ion transporting systems in

Abbreviations: Anterior-posterior, AP; Apoptotic index, AI; Bumetanide, Bume; Controlled cortex injury, CCI; Dorsal-ventral, DV; Extracellular signal-regulated kinase, Erk; Intracellular Na^+, $[Na^+]_i$; Intracranial pressure, ICP; Lactate dehydrogenase, LDH; Medial-lateral, ML; Na^+/Ca^{2+} exchanger, NCX; Na^+-K^+-$2Cl^-$ cotransporter-1, NKCC1; Neurological severity score, NSS; Scramble-shRNA, Scr-shRNA; Tdt-Mediated dUTP Nick End-Labeling, TUNEL; Traumatic brain injury, TBI; Traumatic neuron injury, TNI.

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neuronal cells, plays a critical role in the maintenance of cell volume homeostasis *via* the regulation of inward Na^+-K^+-$2Cl^-$ cotransport (Haas, 1994). The inappropriate activation of NKCC1 would result to cell swelling and tissue edema (Jayakumar and Norenberg, 2010). The role of NKCC1 had been extensively investigated in neurological diseases, including cerebral ischemia (Luo and et al., 2008; Chen and et al., 2005), epilepsy (Brandt and et al., 2010), glioma (Garzon-Muvdi and et al., 2012), neuropathic pain (Hasbargen and et al., 2010) and spinal cord injury (Cote and et al., 2014). The inhibition of NKCC1 significantly protects neurons from ischemic injury, reduces ischemia-induced damage of cortical neurons, and limits acidosis-induced glial swelling. Furthermore, limited studies have identified the emerging role of NKCC1 in TBI. For example, Jayakumar et al. demonstrated that NKCC1 inhibition reduced fluid percussion injury-induced astrocyte swelling (Jayakumar and et al., 2011). Lu et al. established that NKCC1 inhibition in the choroid plexus attenuated TBI-induced brain edema and neuronal damage (Lu and et al., 2006). However, whether and how NKCC1 affects cortical neuronal injury induced by TBI remains elusive.

In the present study, a traumatic neuron injury (TNI) model *in vitro* and a controlled cortex injury (CCI) model *in vivo* were used to mimic TBI. Both an NKCC1 inhibitor and NKCC1 RNA interference were applied to determine the role of NKCC1 in TBI and investigate the related mechanisms involved in neuroprotection by NKCC1 inhibition. The identification of the role of NKCC1 in TBI may lead to novel targets or therapies for TBI treatment.

2. Materials and methods

2.1. Animals and groups

Ninety-six mature (male, 12 weeks old, 24–26 g) and 16 embryonic (E14–15 days) C57BL/6 mice were obtained from the Experimental Center of the Fourth Military Medical University. Mice were maintained at 24 °C in an air-conditioned room and exposed to a 12-h light/dark cycle (lights on at 7:00 A M. and lights off at 7:00 P M.). All experiments adhered to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Fourth Military Medical University Committee on Animal Care.

Two experiments were performed in this study. In experiment 1, to investigate the changes in NKCC1 in TBI *in vivo*, mice were divided into two groups: the sham group ($n = 13$) and the CCI group ($n = 40$). The CCI group was further divided into six subgroups based on corresponding time points (1 h ($n = 4$), 6 h ($n = 4$), 12 h ($n = 10$), 24 h ($n = 14$), three days ($n = 4$) and five days ($n = 4$) after CCI). Changes in protein expression ($n = 4$ per group), the apoptotic index ($n = 4$ for the sham group and 24 h after CCI groups), neurological function and brain water content (sham, $n = 5$; 12 h after CCI, $n = 6$; 24 h after CCI, $n = 6$) were identified. In experiment 2, to investigate the effects of NKCC1 in TBI *in vivo*, mice were divided into two groups: the sham group ($n = 9$) and the CCI group ($n = 34$). The CCI group was further divided into four subgroups: CCI ($n = 9$), Scr-shRNA + CCI ($n = 7$), NKCC1-shRNA + CCI ($n = 8$), and Bume + CCI ($n = 10$) groups. The apoptotic index ($n = 3$ per group), neurological function and brain water content (sham, $n = 6$; CCI, $n = 6$; Scr-shRNA + CCI, $n = 4$; NKCC1-shRNA + CCI, $n = 5$; and Bume + CCI group, $n = 7$) were determined.

2.2. Mouse cortical neuron culture

Primary neuronal cultures of mouse cortex were prepared, as previously described (Ma and et al., 2011). Briefly, the cortices of mouse embryos were minced and subsequently incubated in papain solution (Worthington, Lakewood, NJ, USA) and DNase I (Beyotime, Haimen, China) for 30 min at 37 °C. Cells were resuspended in a Neurobasal medium supplemented with 2% B27 supplement and plated onto poly-D-lysine (Sigma Aldrich, Saint Louis, MO, USA) coated dishes. After 7–10 days of culture, cells were used for the following experiments.

2.3. TNI model in cultured neurons

A TNI model was established according to Mukhin's method (Mukhin and et al., 1997), with some modifications. This model and its variants are highly reproducible and induce severe TNI (Margulies and Hicks, 2009; Rao and et al., 2015; Morrison and et al., 2011). Briefly, TNI was performed using a rotating scribe injury device with ten holes. This device enabled ten steel needles to freely cross through these holes. After one turn of this device, ten concentric circular scratches were produced in the neuronal layer with equal distances (1.5 mm) between the scratches.

2.4. Animal model preparation

TBI *in vivo* was induced in mice using a Precision Cortical Impactor (PCI3000; Hatteras Instruments, Cary, NC, USA), according to previous methods (Bilgen, 2005). Briefly, following anesthetization with isoflurane in oxygen (4% for induction and 2.5% for maintenance), mice were placed in a stereotaxic frame. A midline longitudinal scalp incision was performed, and the skull was exposed. A craniotomy was subsequently performed using a dental drill with a trephine bit (ø = 3 mm) on the left motor cortex (anterior-posterior (AP) −2 mm, medial-lateral (ML) 2.0 mm from bregma). Next, a metal tip (ø = 2.5 mm) was angled vertical to the cortex surface at the center of the craniotomy. The tip was subsequently lowered to touch the cortex surface, as determined by an electronic continuity sensor. TBI was induced by retracting the tip upwards by 2 cm, followed by a down stroke (velocity: 1.5 m/s, deformation depth: 1.5 mm, duration: 120 ms). Immediately after the injury, the wound was closed using standard suture material (3-0 Ethilon, Johnson & Johnson, New Brunswick, NJ, USA), and the wound area was treated with lidocaine cream.

For lentivirus stereotaxic cortical injection, three cortical injections were performed in the right hemisphere (ipsilateral to the lesion) as follows: 0 mm AP, 2 mm ML from the bregma, and 1.5 mm dorsal-ventral (DV) from the skull (point 1); -2 mm AP, 3 mm ML from the bregma, and 1.5 mm DV from the skull (point 2); -4 mm AP, 2 mm ML from the bregma, and 1.5 mm DV from the skull (point 3), as previously described (Rao and et al., 2015; Cetin and et al., 2006; Lowery and Majewska, 2010). Each injection contained 1.5 μl of EGFP-labeled lentivirus suspension (1×10^9 TU/ml) at a rate of 0.2 μl/min, with an additional needle retaining time of 10 min. Seven days after lentivirus injection, mice were subjected to the CCI method. For drug treatment, Bume was intravenously administered (15 mg/kg; Sigma, St. Louis, MO, USA) 20 min prior to CCI (O'Donnell and et al., 2004).

2.5. Western blot analysis

Western blot analysis was performed according to routine laboratory methods in our institution, as previously described (Luo and et al., 2012). Proteins extracted from the neurons and cortexes of mice were used for western blot. Membranes were incubated overnight at 4 °C with the appropriate primary antibody: NKCC1 (1:1000; Santa Cruz Biotechnology, Santa Cruz, USA), p-ERK (1:1000), ERK1/2 (1:500), cleaved-Caspase-3 (1:1000), Caspase-3 (1:1000), and β-actin (1:2000; Cell Signaling Technology, Danvers, MA, USA). The immunoreactivity of each band was detected