Objective: Brain edema is one of the characteristic features of patients with severe traumatic brain injury. The aim of this study was to examine the effects of Na\(^+\)-K\(^+\)-2Cl\(^-\) co-transporter on traumatic brain injury-induced brain edema and neuronal damage and to elucidate the relationship between Na\(^+\)-K\(^+\)-2Cl\(^-\) co-transporter and mitogen-activated protein kinase (MAPK) cascade.

Design: Laboratory investigation.

Setting: University research laboratory.

Subjects: Male Wistar rats weighing 350–400 g.

Interventions: Anesthetized animals were subjected to a weight-drop device (450-g weight, 1.8-m height) to induce traumatic brain injury.

Measurements and Main Results: The expression of Na\(^+\)-K\(^+\)-2Cl\(^-\) co-transporter and phosphorylation of MAPK cascade were determined by Western blot test. We also analyzed the degree of brain edema and neuronal damage in this study. We found that the messenger RNA and protein of Na\(^+\)-K\(^+\)-2Cl\(^-\) co-transporter were up-regulated mainly in hippocampus neurons from 2 to 24 hrs after traumatic brain injury. After traumatic brain injury, animals displayed severe brain edema and neuronal damage. The phosphorylation of extracellular signal-regulated kinase, MAPK kinase, and Raf also was significantly elevated after traumatic brain injury. Dexamethasone (15.2 mg/kg), a specific Na\(^+\)-K\(^+\)-2Cl\(^-\) co-transporter inhibitor, significantly attenuated the neuronal damage and brain edema after traumatic brain injury by decreasing the phosphorylation of Raf/MEK/ERK cascade proteins.

Conclusions: The present study suggests that Na\(^+\)-K\(^+\)-2Cl\(^-\) co-transporter plays an important role in TBI-induced brain edema and neuronal damage via activation of MAPK cascade.

Key Words: rats; traumatic brain injury; Na\(^+\)-K\(^+\)-2Cl\(^-\) co-transporter isoform 1; mitogen-activated protein kinase

Traumatic brain injury (TBI) causes neurologic dysfunction and death through primary or secondary mechanisms. The mortality of TBI equals about 27% (1). Primary TBI damages axons, blood vessels, and glial cells directly in a focal or diffuse pattern. The pathophysiology is amplified by secondary events that include hypoxia, hypotension, ischemia, edema, and increased intracranial pressure (2, 3). Brain edema is classified as vasogenic edema and cytotoxic edema. Vasogenic edema contributes to the swelling process and the subsequent increase in intracranial pressure. Cytotoxic edema consists of intracellular fluid accumulation that occurs during water intoxication and anoxia-generating conditions, such as trauma and stroke. There is evidence that cytotoxic edema is induced by disruption of ionic homeostasis (4, 5).

The Na\(^+\)-K\(^+\)-2Cl\(^-\) co-transporter plays an important role in cellular ionic homeostasis. Na\(^+\)-K\(^+\)-2Cl\(^-\) co-transporter isoform 1 (NKCC1) is expressed in neurons and astrocytes throughout the brain (6, 7) and plays an important role in regulating neuronal volume and ion homeostasis (8–10). Na\(^+\)-K\(^+\)-2Cl\(^-\) co-transporter isoform 2 (NKCC2) is expressed only on the apical surface of epithelial cells lining the thick ascending limb of Henle (11, 12). There is evidence that blockade of NKCC1 significantly protects animals from focal ischemia (13, 14), reduces ischemia-induced damage of cortical neurons, and limits acidosis-induced glial swelling (15).

Mitogen-activated protein kinase (MAPK) is a family of serine/threonine protein kinases containing extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK) and extracellular-regulated kinase-5 (ERK5) (16). The arrangement of MAPK cascades includes a G-protein upstream of a core module consisting of three kinases: a MAPK kinase kinase (Raf), MAPK kinase (MEK), and ERK. There are two isoforms of ERK: ERK1 and ERK2 (p44 and p42, respectively). ERK is crucial for many physiologic and pathologic responses (17). In our previous studies, we demonstrated that ERK is critical to TBI-induced neuronal damage (18, 19). Based on these findings, we hypothesized that NKCC1 is involved in TBI-induced brain edema and neuronal damage through MAPK cascade. In this study, we quantified hippocampal NKCC1 expression, MAPK cascade protein phosphorylation, brain edema level, and neuronal damage after TBI to test this hypothesis.

MATERIALS AND METHODS

Experimental Animals. Adult male Wistar rats weighing 350–400 g were used in the study. Animals were housed individually in hanging wire cages in a temperature-controlled animal colony at 24°C with a normal 12-hr/12-hr light/dark cycle, with lights turned on at 6 am. The animals had free access to food and water and were allowed to acclimate to the light/dark cycle at room temperature for 1 wk before beginning of the experiments. The protocols were approved by the Institute of Biotechnology, National Chia-Yi University, Chia-Yi, Taiwan (KTL); and the Institute of Biotechnology, National Chia-Yi University, Chia-Yi, Taiwan (NCC, CYW, YLY).

Supported, in part, by grants NSC 93-2320-B-415-001 and 94-2320-B-005-025 from the National Science Council, Taiwan.

The authors have not disclosed any potential conflicts of interest.

For information regarding this article, E-mail: ylyang@mail.ncyu.edu.tw.

Copyright © 2008 by the Society of Critical Care Medicine and Lippincott Williams & Wilkins.

DOI: 10.1097/CCM.0B013E31816590C4

Crit Care Med 2008 Vol. 36, No. 3

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.
the Animal Care and Use Committee of National Chia-Yi University. A special weight-drop device (20) was used to deliver standard traumatic impact to the animals. While animals were under pentobarbital anesthesia (40 mg/kg, intraperitoneally), a midline incision was made in the scalp and the adjacent skin flaps were spread laterally. A metal helmet was sewn to the skull to prevent its fracture by the trauma-inducing impact. Rats were then placed in the prone position on the bottom plate of the weight-drop device, and a 450-g weight was allowed to fall freely from a height of 1.8 m onto the metal helmet to induce TBI. A right femoral arterial catheter was inserted to collect blood for measurement of blood pH, P-O2, and P- CO2 (RapidLab 248, Bayer). To elucidate the role of NKCC1 in TBI-induced brain edema, the NKCC1-specific inhibitor bumetanide was administered intravenously (15.2 mg/kg, Sigma, St. Louis, MO) 20 mins before traumatic brain injury. The dose of bumetanide used here followed that used by O’Donnell et al. (14) with some modification. Based on previous studies, we administered PD98059 (500 ng/kg, Sigma, St. Louis, MO) 20 mins before traumatic brain injury. The dose of PD98059 was determined by comparing the control group and the TBI-bumetanide group.

NKCC1 Messenger RNA Assay. After dissection of the brain, the total hippocampal RNA was extracted with Trizol reagent (GIBCO BRL, Grand Island, NY), and complementary DNA was synthesized by reverse transcription in 14 μL of reaction buffer (Perkin Elmer, Foster City, CA). The reaction was run at 42°C for 30 mins and terminated at 99°C for 5 mins. Diethylpyrocarbonate-treated aqueous solution (75 μL) was then added to dilute the complementary DNA to 100 μL, and the diluted samples were stored at −20°C until use. Polymerase chain reaction was performed with 15 μL of complementary DNA-containing reaction mixture (Perkin Elmer) and the primers 5'-GACCTCCTGTGCTCTATGGCC-3' (forward), 3'-CGTAATCGTAGGGATTTGGTCGTAT-3' (reverse) for GAPDH as well as 5'-AGGACTCAACAGGATTTGCTGAT-3' (forward) and 3'-CAGAAGTGGTGGTACCTCTTCCGA-5' (reverse) for glyceraldehyde phosphate dehydrogenase as an internal control (22). Semi-quantitative densitometry in conjunction with AlphaEase software (Alpha Innotech) was used for the quantification.

Western Blot Analysis. In a different experiment, rats were decapitated and the brains were rapidly removed after the induction of TBI. Following dissection, the hippocampus was weighed and rapidly homogenized in six volumes of ice-cold homogenizing buffer. Following electrophoretic separation on 8% sodium dodecylsulfate gel, the resolved proteins were transferred electrophoretically to polyvinylidene difluoride membrane. Monoclonal anti-NKCC1 (1:2000 dilution), anti-phosphorylated ERK (1:5000 dilution), anti-phosphorylated MEK (1:1000 dilution), and anti-phosphorylated Raf antibodies (1:1000 dilution) (Cell Signaling) were used for detection, and bound antibody was visualized with an enhanced chemiluminescence assay (RPN 2108; Amersham International, Amersham, UK). Semi-quantitative densitometry in conjunction with AlphaEase software (Alpha Innotech) was used for quantification.

Immunofluorescence Double Labeling. Rats were anesthetized and subjected to intracardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.5). The brains were removed and kept in 4% paraformaldehyde for 6 hrs for fixation and were then immersed in 25% sucrose for 3–4 days at 4°C. The brains were then immersed in embedding compound and sectioned into 20-μm-thick slices on a cryostat. The sections were incubated with phosphate-buffered saline containing 10% normal goat serum at room temperature to eliminate nonspecific binding. For immunofluorescence double labeling, rabbit anti-human antibody specific to neuron-specific enolase (1:100 dilution; Chemicon), mouse antibody to glial fibrillary acidic protein (1:100 dilution), and mouse antibody to NKCC1 (1:200 dilution) were used as primary antibodies. Rhodamine-conjugated goat anti-rabbit antibody (1:400 dilution) and fluorescein isothiocyanate-labeled horse anti-mouse antibody (1:400 dilution) were used as secondary antibodies.

Evaluation of Neuron Damage. Forty-eight male Wistar rats were randomly divided into sham, TBI induced, and TBI with bumetanide groups (n = 16 for each group). Eight hours after TBI, rats were killed with an overdose of pentobarbital (100 mg/kg, intraperitoneally) and then were perfused transcardially with 0.9% NaCl and 10% neutral formalin. After formalin perfusion, the rats were decapitated, the brains were removed and kept in 4% paraformaldehyde for 24 hrs. The brains were then immersed in 25% sucrose for 3–4 days at 4°C. The brains were then cut in 50-μm-thick sections on a vibratome (Fisher, Pittsburgh, PA) and were then mounted on gelatin-coated slides. Immunohistochemistry was done on 5-μm-thick sections using the following antibodies: rhodamine-labeled goat anti-mouse antibody (1:400 dilution) and fluorescein isothiocyanate-labeled rabbit anti-mouse antibody (1:400 dilution) were used as secondary antibodies. The hippocampus was used for quantification, and the data are presented as ratios to control values. Bars represent mean ± SEM of ten rats per group.
Mean arterial blood pressure, pH, PCO2, and PO2 in different rat treatment group. Table 1 summarizes the physiologic variables before and after TBI induction in the vehicle-treated group, vehicle-treated animals and bumetanide-treated group, which significantly increased from 2 hrs (about two-fold compared with the sham-operated group, peaked at 8 hrs after TBI (about 2.5-fold), and lasted for 24 hrs. We found that NKCC1 RNA increased significantly by 2 hrs after trauma. We also evaluated the role of NKCC1 up-regulation after TBI. Western blot analysis showed that NKCC1 protein in hippocampus after traumatic brain injury (TBI). A, top panel, NKCC1 expression during TBI; bottom panel, α-tubulin as an internal control. Times are indicated in numerals and represent hours after injury. B, quantification of NKCC1 protein expression. Semi-quantitative densitometry in conjunction with AlphaEase software was used for the quantification, and the data are presented as ratios to control values. Bars represent mean ± SEM values (n = 8). *p < .05 was considered significantly different from sham values by Mann-Whitney U test.

RESULTS

NKCC1 Up-Regulation After TBI. Table 1 summarizes the physiologic variables in different rat treatment group. Mean arterial blood pressure, pH, PCO2, and PO2 were not significantly different before and after TBI induction in the vehicle-treated animals and bumetanide-treated animals. It appeared that systematic administration of bumetanide elicited no significant effects on mean arterial blood pressure, pH, PCO2, and PO2. In a second experiment, we examined the expression of NKCC1 in hippocampus after trauma. We found that NKCC1 RNA increased significantly by 2 hrs after trauma (about two-fold compared with the sham-operated group, p < .05) and peaked at 8 hrs after TBI (about 2.5-fold) and that the expression lasted for 24 hrs (Fig. 1). Western blot analysis showed that NKCC1 protein in hippocampus significantly increased from 2 hrs (about 1.5-fold compared with the sham-operated group, p < .05), peaked at 8 hrs after TBI (about 1.8-fold), and lasted for 24 hrs after trauma (Fig. 2).

The sites of NKCC1 expression were identified by immunocytochemical double-labeling. NeuN was used as a marker of neurons (Fig. 3B), and glial fibrillary acidic protein, indicating that NKCC1 was mainly expressed in neurons (Fig. 3C) but not in astrocytes (Fig. 3F).

Figure 2. Western blot analysis of Na⁺-K⁺-2Cl⁻ co-transporter isoform 1 (NKCC1) protein in hippocampus after traumatic brain injury (TBI). A, top panel, NKCC1 expression during TBI; bottom panel, α-tubulin as an internal control. Times are indicated in numerals and represent hours after injury. B, quantification of NKCC1 protein expression. Semi-quantitative densitometry in conjunction with AlphaEase software was used for the quantification, and the data are presented as ratios to control values. Bars represent mean ± SEM values (n = 8). *p < .05 was considered significantly different from sham values by Mann-Whitney U test.

Three hours after TBI, animals revealed neuronal swelling and shrinkage followed by neuronal loss. Compared with the sham group (Fig. 4A), neuronal damage reached a peak at 8 hrs after the induction of TBI (Fig. 4B), neuronal damage score = 2.7 ± 0.5, p < .05. Administration of bumetanide (Fig. 4C) and the ERK inhibitor PD98059 (500 ng/μL; intracerebroventricular route) (21) (Fig. 4D) significantly attenuated the TBI-induced neuronal loss (neuronal damage scores = 1.7 ± 0.8, p < .05, and 1.6 ± 0.7, p < .05, respectively). This finding clearly showed that NKCC1 and ERK were essential for the neuronal loss resulting from TBI. These data are summarized in Table 2.

We also evaluated the role of NKCC1 on brain edema formation during TBI.
Our results demonstrated that the water content averaged 75.36% ± 0.57% in the sham-operated rats and significantly increased after 24 hrs of trauma to 82.35% ± 0.42%, which induced severe brain edema (Fig. 5 groups: sham, 24 hrs after TBI [TBI-24h]). Administration of bumetanide (15.2 mg/kg, intravenously) and PD98059 (500 ng/μL, intracerebroventricular route) significantly decreased the brain edema to water content 78.65% ± 0.37% and 77.97% ± 0.75% after TBI 8 hrs (p < .05). This result suggests that NKCC1 is involved in TBI-induced brain edema via MAPK cascade activation.

To provide direct evidence that NKCC1 activated the MAPK cascade after TBI, we analyzed the effect of bumetanide on TBI-induced MAPK cascade phosphorylation. Compared with sham groups (Fig. 6A, first panel, right lane), TBI significantly increased phosphorylation of Raf (Fig. 6A, second panel, middle lane), MEK (Fig. 6A, third panel, middle lane), and ERK (Fig. 6A, third panel, right lane). Bumetanide administration significantly inhibited the TBI-induced phosphorylation of Raf to 45% (Fig. 6A, first panel, right lane), MEK to 52% (Fig. 6A, second panel, right lane), and ERK to 48% (Fig. 6A, third panel, right lane). These results suggest that NKCC1 is involved in MAPK cascade activation after TBI.

DISCUSSION

The present study demonstrated that the expression of NKCC1 RNA and protein was significantly elevated in hippocampus 2–24 hrs after TBI and that NKCC1 overexpression directly contributed to brain edema formation. Bumetanide administration significantly decreased the neuronal damage and brain edema via NKCC1 inhibition. In this study, we also found that bumetanide injection significantly attenuated the TBI-induced Raf/MEK/ERK phosphorylation. This suggests that stimulation of NKCC1 by TBI drives the activation of MAPK cascade, resulting in brain edema and neuronal damage.

The contribution of vasogenic and cytotoxic brain edema to brain swelling and neuronal damage remains a critical problem. Many studies have shown that cellular edema is the major contributor to TBI-induced brain swelling, while the blood-brain barrier opening only plays a permissive role in brain edema formation (24, 25). The present study demonstrated the importance of NKCC1 in cytotoxic brain edema formation. We found that NKCC1 mainly expressed in neurons is up-regulated after TBI. NKCC1 is essential for ion homeostasis and volume regulation in neuron and astrocytes and is regulated by various neurotransmitters and hormones (26). The NKCC1 activity is stimulated by glutamate through activation of N-methyl-d-aspartate, a-aminoo-3-hydroxy-5-methylisoxazole-4-propionate, and metabotropic glutamate receptors (27, 28). However, NKCC1 also stimulates glutamate release (29). During ischemia or TBI, the K⁺ concentration is elevated and induces glutamate release, which is mediated by volume-sensitive NKCC1. Blockade of NKCC1 activity by bumetanide significantly decreases the glutamate release (29, 31). The interaction between NKCC1 and glutamate exacerbates the NKCC1 overexpression and brain edema formation during TBI.

Cytokines also up-regulate NKCC1 expression. NKCC1 is induced about 10- to 20-fold at the messenger RNA level and 8- to 10-fold at the protein level by both recombinant human interleukin-1β and...
Our previous studies indicated the observed TBI-induced significant increases in interleukin-1 (18, 19). In the present study, we found that NKCC1 was mainly expressed in neurons after TBI. These results suggest that interleukin-1β may be involved in NKCC1 induction and related to the modulation of blood-brain barrier function during TBI. Not only the cytokine responsiveness but also NKCC1 genes are regulated by fluid mechanical forces. Tropper et al. showed (33) that both the nature of the fluid shear stress (laminar vs. nonlaminar) and the magnitude of the applied shear stress appear to be important in the regulation of NKCC1 expression. Mechanical force is capable of inducing a number of acute events, such as the stimulation of an inward rectifying potassium current, an increase in nitric oxide and intracellular calcium level, and even sustained morphologic changes (34). Our model also produces a large amount of mechanical force, which we believe up-regulated NKCC1 level and consequent effects.

In this study, we demonstrated that NKCC1 activated the ERK-mediated signal transduction pathway after TBI, which is also consistent with other studies (35, 36). The most important question emanating from this study relates to the mechanism by which the stimulation of NKCC1 controls the MAPK cascade phosphorylation. NKCC1 stimulation increases intracellular free [Ca2+] and is proposed to affect the signal transduction pathway either by a direct effect on one of its proteins or by Ca2+ activation of certain isoforms of the protein kinase family (35, 37, 38). Further studies are necessary to elucidate the mechanism in detail.

CONCLUSIONS

The results presented here indicated that NKCC1 plays an important role in TBI-induced brain edema and neuronal damage via the Raf/MEK/ERK cascade and that systemic delivery of an NKCC1-specific inhibitor significantly ameliorated brain edema and neuronal damage.

ACKNOWLEDGMENTS

We thank Dr. Hsu-Huei Weng for statistical assistance and Dr. Jeng-Hsiung F. Peng for editorial assistance.

REFERENCES

3. Wilcockson DC, Campbell SJ, Anthony DC, et al: The systemic and local acute phase re-