INTRODUCTION

Lithium is one of the primary drugs used for treating bipolar mood disorder, although its therapeutic mechanism remain unclear (Schou, 2001). There is growing evidence that lithium may represent an effective neuroprotective agent in regulating intracellular pH (pHi) and Na$^{+}$ concentration (Song et al., 2008). pHi and Na$^{+}$ concentration have an important role in the maintenance of normal cell function, and hence this parameter has to be tightly controlled within a narrow range, largely through the activity of sodium transporters located at the neuronal plasma membrane (Lagadic-Gossmann et al., 2004). Sodium transporters can be modulated by endogenous or exogenous molecules as well as, in some pathological situations, leading to pHi and Na$^{+}$ concentration changes that have been implicated in both neurogenesis and anti-apoptotic actions (Pedersen, 2006), the precise role of pHi and Na$^{+}$ concentration in neurogenesis and anti-apoptosis is still unclear.

Regulation of Neurogenesis and Apoptosis through the NHE1 in Lithium-treated Rat Dentate gyrus

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ABSTRACT
Lithium-induced neuroprotection are complex and may include the neurogenesis and anti-apoptotic events. Recently, several studies have suggested the possibility that replacement of external Na$^{+}$ with lithium which induced activations of sodium transporters such as the Na$^{+}$/H$^{+}$ exchanger 1 (NHE1) and electrogenic Na$^{+}$/HCO$_3$- cotransporter (eNBC). Thus, alteration of sodium transporters could be associated with neurogenesis and anti-apoptotic actions of lithium even though the drug’s therapeutic mechanisms remain obscure. The present study was undertaken to examine the changes of protein of NHE1 and eNBC after lithium treatment in normal and ischemic rats can regulate neurogenesis and/or apoptosis in dentate gyrus (DG). Lithium treatment was produced by pellet of standard diet containing 60 mmol/dL lithium for 25 days. The serum concentrations of lithium were found to be 0.76±0.2 mEq/L which is therapeutic dose of clinical practice. Immunoblotting analyses revealed that the NHE1 was significantly increased (259 ±8% of controls, n=4, P<0.01) whereas eNBC was unchanged (103±8%) compared with controls (n=4) after lithium treatment. Immunohistochemical studies demonstrated that bromodeoxyuridine (BrdU)-positive cells in the lithium-treated DG (n=3) were significantly higher compared with those in controls (n=3). In ischemia-reperfusion rats (n=6), terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining revealed apoptotic granule cells with ischemia-reperfusion rats while no apoptotic granule cells were showed with pretreatment of lithium. These findings suggest that significant increase of NHE1 after lithium treatment may at least partly contribute the neurogenesis and anti-apoptosis of DG via increased intracellular pH and volume increase. Therefore, lithium treatment may have therapeutic potential for ischemic stroke via neurogenesis and anti-apoptotic actions.

Key words : Lithium, Dentate gyrus, Neurogenesis, Anti-apoptosis

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muscle fibers (Kobayashi, 2000). Moreover, enhanced activity of NHE1 during cerebral ischemia could produce a marked exchange of Na\(^+\) for H\(^+\) in the cells, leading to intracellular alkalinization and volume increase (Nagafuji et al., 1992; Putney & Barber, 2003).

Electrogenic Na\(^+\)/HCO\(_3\)^\(-\) cotransporter (eNBC) occurs in a wide range of organisms and tissues (Boron & Boulpaep, 1989), including the CNS (Chesler, 1990). Munsch & Deitmer (1994) reported that lithium can make the inward currents of leech glial cells in the presence of eNBC. eNBC moves Na\(^+\) and HCO\(_3\)^\(-\) ions together across plasma membranes, depending on the isoform either as electrogentic cotransporters with Na\(^+\) : HCO\(_3\)^\(-\) stoichiometries of 1 : 3 and 1 : 2 or as electroneutral cotransporters with a stoichiometry of 1 : 1 (Boron et al., 1997). Therefore, activation of eNBC resulted in the extracellular pH is acidified and the intracellular pH of the neuronal cell is alkalinized (O’Connor et al., 1994).

The present study was undertaken to explore the long-term effects of therapeutic concentrations of lithium on the neurogenesis and anti-apoptotic actions of the dentate gyrus (DG) in normal and ischemic rat brain via alterations of NHE1 and/or eNBC proteins.

**MATERIALS AND METHODS**

1. **In vivo treatment of rats with lithium**

The studies were performed on 28 male Sprague Dawley rats initially weighing 270 ± 8 g. The control rats were maintained on a standard rodent diet without lithium (n=14). The lithium containing diet containing 60 mmol/dL was prepared by adding 1 M lithium and water to powdered standard diet to give a wet mash, which was subsequently oven dried at 60°C. Lithium treated rats (n=14) was given pelleted standard diet containing 60 mmol/dL lithium for 25 days.

For labeling proliferating cells, bromodeoxy uridine (BrdU) (100 mg/kg; Sigma, St, louis, Mo) was daily injected intraaperitoneally for 3 days into rats starting 21 days after lithium treatment (n=6). After completing the lithium treatment protocols, rats were anesthetized with isoflurane and venous blood was taken for assessment of serum lithium concentration, The brains of rats were fixed with a transcardiac infusion of 4% paraformaldehyde following perfusion with isotonic saline to remove blood from the cerebral vasculature.

2. **Ischemia-reperfusion injury**

Ischemia-reperfusion injury (n=6) was initiated by occlusion of the left middle cerebral artery as described previously (Hasegawa et al., 1994). Anesthesia was induced with 3% isoflurane in a mixture of oxygen/nitrous oxide (30:70) and the rats were maintained with 1% isoflurane in the oxygen/nitrous oxide gas mixture. A catheter was inserted and positioned in the femoral artery. The arterial blood pressure was measured and recorded continuously until the procedures were ended. Temperature was monitored before, during, and after ischemia using thermometer probe placed in the rectum. Temperature control was accomplished with the aid of a heating pad and kept at 37°C. Under dissecting microscope, the left common carotid artery was exposed through blunt dissection and a ventral midline neck incision. The occipital and thyroid artery branches of the external carotid artery (ECA) were ligated and dissected using loop tip surgical cauterizer. The pterygopalatine artery was also isolated and ligated close to its origin and the ECA was ligated approximately 3 to 5 mm distal to its origin. The common and internal carotid arteries were then temporarily clamped using microvascular clips to allow insertion of a 4-0 nylon filament (3 cm in length) with blunt tip into the internal carotid artery by way of the ECA. The ECA was dissected and the nylon filament was loosely secured inside the ECA using 5-0 suture. This allowed gentle movement of the nylon filament into the internal carotid artery and the eventual occlusion of the middle cerebral artery for 2 h. For reperfusion, the nylon filament was withdrawn 2 h after middle cerebral artery occlusion (MCAO) and then perfused for 14 days. To examine whether treatment with lithium decreases the apoptosis of dentate granule cells in ischemic-reperfusion brain, lithium at a dose of 60 mmol/dL for 25 days starting before ischemic-reperfusion injury (n=3). The experimental procedures used were reviewed and approved by the animal care and use Committee of Dongguk University. Animal care and use were in accordance with the guidelines of the National Institutes of Health (Bethesda, MD).

3. **SDS-PAGE and immunoblotting**

DG and surrounding area was removed from the lithium treated animals (n=8) and control animals (n=8) and then snap-frozen at −70°C for western analysis. Tissue samples were homogenized in ten volumes of homogenizing buffer (0.32 mol/L sucrose, 25 mmol/L imidazole, 1 mmol/L EDTA, pH 7.2) containing 8.5 mmol/L leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride for 10 s with polytron. Aliquots were stored at −70°C. Samples of homogenate were run on 9 ~ 15% polyacrylamide mini gels (Bio-rad Mini Protein) in duplicates in which one gel was run in parallel and subjected to Coomassie (Coomassie brilliant blue 0.3 g, 2-propanol 200 mL, acetic acid 80 mL, H\(_2\)O 640 mL) staining to assure identical loading. The other gel was subjected to immunoblotting. After electrophoresis, the protein was transferred to nitrocellulose paper for 2 h at 400 mA, 120 V in a Bio-Rad trans-blot system using a trans-
fer buffer solution (50 mmol/L Tris-base, 380 mmol/L glycine, 20% methanol). After transfer, the membranes were visualized by Ponceau S (0.1% (w/v) Ponceau S, 0.1% acetic acid) to assess completion of the protein transfer and destained with distilled water. The membrane was washed four times (10 min each) in PBST (80 mmol/L Na2HPO4, 20 mmol/L NaH2PO4, 100 mmol/L NaCl, 0.1% Tween-20, pH 7.5) and incubated with rabbit anti-NHE1 affinity purified polyclonal antibody (Chemicon, Temecula CA, 1: 200), rabbit anti-eNBC affinity purified polyclonal antibody (Chemicon, Temecula CA, 1: 1,000) overnight at 4°C, and then washed twice (10 min each) in PBST. After washing with PBST twice, the membrane was incubated with HRP-conjugated secondary antibodies (DAKO, P448; Santa Cruz Biotechnology, SC 2031, 1: 3,000). After washing with PBST twice, the membrane was incubated with streptavidin conjugated to horseradish peroxidase (HRP) in PBS for 30 min. After washing in PBS three times, the sections were incubated with streptavidin conjugated to horseradish peroxidase in PBS for 30 min, washed three times in PBS, and reacted with a solution containing diaminobenzidine (DAB) and hydrogen peroxide (0.001%). The sections were counter-stained with Mayer’s hematoxylin to visualize cell nuclei. All counting of BrdU-positive cells were performed in a blinded and randomized manner. Five fields (0.1 mm²/field) per hippocampus were used for BrdU-positive cell counting. Control and experimental animal group consisted of three rats. Comparisons between groups were made by unpaired t-test. P values < 0.05 were considered significant.

7. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining for apoptotic cells

TUNEL staining (ApopTag kit, Intergen) was employed for the detection of DNA fragmentation and apoptotic bodies in the brain tissue. Briefly, after deparaffinizing brain sections, digesting protein using proteinase K, and quenching endogenous peroxidase activity with 3.0% H2O2 in PBS, slides were placed in equilibration buffer and then in working terminal deoxynucleotidyl transferase (TdT) enzyme, followed by stop/wash buffer. After two drops of anti-digoxigenin-peroxidase were applied to the slides, peroxidase was detected with DAB and examined under microscope (Olympus BX 20). Apoptotic, TUNEL-positive cells (brown-stained) were confirmed in three equidistant hippocampal sections of three each rat. Negative controls were performed using distilled water or PBS for TdT enzyme in the preparation of working TdT.

RESULTS

1. Serum lithium concentrations in rats after treatment with lithium

In the present study, the serum lithium concentrations in 60 mmol/dL lithium treated rats for 25 days was 0.76 ± 0.2 mEq/L, which required therapeutic dosage for the treatment of manic depression in humans (0.5 to 0.9 mmol/L).

2. Altered expressions of NHE1 and eNBC in rats after treatment with lithium

To evaluate the effect of lithium treatment on the protein expressions of NHE1 and eNBC in the DG, immunoblotting analyses were performed. In all control and lithium-treated rats, immunoblotting for NHE1 and eNBC revealed a major strong band at 130 kDa and 110 kDa (Fig. 1A). The expression
of NHE1 after lithium treatment was significantly increased (259±8% of controls, n=4, P<0.01). In contrast, the expression of eNBC (103±8%) in lithium-treated rats was unchanged (Fig. 1B).

3. Neurogenesis in the lithium-treated rat DG

As shown in Fig. 2A, the data of immunohistochemical studies demonstrated that most of the BrdU-positive cells in the normal DG were detected in the subgranular zone (SGZ) of DG (13±2.5, n=3, marked in arrows). In addition to that, as seen in Fig. 2B, there was a statistically significant increase of BrdU-positive cells in lithium treated rats compared with that of the controls (18±2.0, n=3, marked in arrows, P<0.05).

4. Anti-apoptosis in the lithium-pretreated rat DG

In the ischemic-reperfusion rats, brown TUNEL-positive apoptotic cells indicating a high degree DNA fragmentation were increased in the granular layer of DG 2 weeks after the onset of reperfusion (Fig. 2C, I-R). In contrast, there was no brown TUNEL-positive apoptotic cell in the DG of lithium pre-treated ischemic-reperfusion rats (n=3) (Fig. 2C, Li60+I-R).

DISCUSSION

In this study, we show that therapeutic dose of lithium treatment enhanced the expression of NHE1 and incorporation of BrdU into developing rat stem cells in the DG. The expression of eNBC was not changed after lithium treatment. NHE1 was reported to be a major carrier involved in the regulation of both cytosolic pH and cell volume after lithium treatment although underlying mechanisms of these effects are unclear (Kobayashi et al., 2000; Putney et al., 2002).

Activation of NHE1 has been shown to play an important role in the proliferation process. Recent reports supported the notion that pharmacological inhibition of NHE1 inhibits (Delvaux et al., 1990; Karmazyn et al., 2001), and exogenous expression of NHE1 accelerates proliferation (Kapus et al., 1994). Moreover, the expression of NHE1 is increased by long-term exposure to mitogens (Besson et al., 1998), and NHE1 activity is modulated during the cell cycle (Putney & Barber, 2003). Therefore, it appears that neurogenesis in the DG after lithium treatment may contributed by NHE1-mediated modulation of cell proliferation. Furthermore, an NHE1-mediated increase in pHi appears to play at least a permissive role in cell-cycle progression (Putney & Barber, 2003). It is also well known that increases in pHi may accelerates cell proliferation by stimulating the synthesis of protein, RNA, and DNA. Thus, lithium-induced alkaline shift of pHi by increase of NHE1 could be an important role in the neurogenesis of the DG.

Generally, cell division is at least partially dependent on an increase in cell volume and inorganic ion uptake (Rouzaire-Dubois et al., 2005). Conversely, cell-cycle progression is inhibited in shrunken cells (Dmitrieva et al., 2001). These findings sug-
suggest that NHE1-mediated intracellular sodium overload and subsequent intracellular volume increase after lithium treatment may at least in part contribute to the neurogenesis in the DG. Evidences from this study suggest that NHE1-mediated neurogenesis after lithium treatment results not only from NHE1-mediated alkaline shift of pHi, but also from the NHE1-mediated volume increase.

It has been recognized that ischemia results in a marked reduction of tissue pH (Rehncrona, 1985) and cerebral ischemia has been demonstrated to produce an intracellular acidosis in the ischemic core as well as in the ischemic penumbra (Yao et al., 1995). Acidification of tissue is the consequence of oxygen depletion, which necessitates a switch from aerobic metabolism to anaerobic glycolysis, leading to generation of lactic acid as well as protons. The first observations of intracellular acidification in mammalian cells undergoing apoptosis were made about a decade ago (Barry & Eastman, 1992), since then several reports have supported the view that this phenomenon might be a more generalized apoptotic feature than previously thought. Indeed, such an acidification has been detected following exposure of cells to apoptotic stimuli such as UV irradiation, staurosporine, etoposide, arsenic, anti-Fas antibodies, growth factor deprivation and somatostatin (Matsuyama & Reed, 2000). Substantial evidence testifies that activation of NHE1 is a pivotal event in the cell damage induced by ischemia/hypoxia and/or subsequent reperfusion in the heart (Wang et al., 2003) and brain (Phillis et al., 1999; Wu et al., 2003), and in other tissues, such as liver (Gores et al., 1989) and lung (Rios et al., 2005). In this study, we demonstrated that pretreatment of lithium in the ischemia-reperfusion rats abolished a significant up-regulation of apoptosis in the granule cells of DG after ischemia-reperfusion. With respect to the role of NHE1 modulates apoptosis, the present experiment indicates that increased H+ ion outward transport by enhanced NHE1 is involved for counter-

Fig. 2. Immunohistochemical identification of BrdU-positive cells in the DG of the lithium-treated rat brain (A and B). A: BrdU-positive cells (marked in arrows) in the control DG were shown in the subgranular zone (SGZ) of DG (13 ± 2.5, n = 3). B: There are statistically significant increase of BrdU-positive cells in the DG of lithium treated rat (18 ± 2.0, n = 3, P < 0.05). C: control, Li60: rats with diet containing 60 mmol/dL lithium for 25 days. C: TUNEL identification of apoptotic cells in the DG of ischemia-reperfusion injury without (I-R, n = 3) and with lithium-pre-treated rats (Li60+I-R, n = 3). In the ischemic-reperfusion rats, brown TUNEL-positive apoptotic granule cells (marked in arrows) were increased 2 weeks after the onset of reperfusion. In contrast, there was no brown TUNEL-positive apoptotic cell in the lithium pre-treated DG. I-R: Rats were occluded the middle cerebral artery for 2 h and then perfused for 14 days (ischemia-reperfusion), Li60+I-R: Rats were maintained with diet containing 60 mmol/dL lithium for 25 days and then started the ischemia-reperfusion. Scale bar, 50 μm.
acting apoptosis by limiting the intracellular acidification. A major reason for the neuronal apoptosis appears to be that the optimum pH for death effectors, such as endonucleases, caspses, and cathepsins, is in the acidic range (Matsuyama et al., 2000). Thus, in this study, pretreatment of lithium in ischemia-reperfusion rats could further increase of NHE1 by intracellular acidification from the ischemic injury and subsequently enhanced NHE1-mediated exchange of Na⁺ for H⁺ across the plasma membrane which results in intracellular alkalization and volume increase. Finally, evidences from this study suggest that the lithium-mediated increased of NHE1 in normal and ischemic rat brain is directly responsible for the neurogenesis and anti-apoptotic action of DG. Taken together, the effectiveness of lithium treatment at therapeutic dose in increasing neural progenitors and decreasing apoptotic granule cells in normal and ischemic rat DG raises the possibility that lithium may be used for the prevention or treatment of acute brain injuries such as stroke.

REFERENCES


