Lithium-induced inhibition of Src tyrosine kinase in rat cerebral cortical neurons: a role in neuroprotection against *N*-methyl-D-aspartate receptor-mediated excitotoxicity

Ryota Hashimoto, Koichiro Fujimaki, Mi Ra Jeong, Lori Christ, De-Maw Chuang*

Molecular Neurobiology Section, Mood and Anxiety Disorders Program, National Institute of Mental Health, National Institutes of Health, Bldg. 10, Rm. 4C-206, 10 Center Dr MSC 1363, Bethesda, MD 20892-1363, USA

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Abstract The neuroprotective effects of lithium, a mood stabilizer, against glutamate-induced excitotoxicity in rat cortical neurons were associated with a decrease in Tyr1472 phosphorylation of the *N*-methyl-D-aspartate (NMDA) receptor NR2B subunit and a loss of receptor activity. Since this receptor tyrosine phosphorylation is mediated by the Src-family tyrosine kinases, we investigated the effects of lithium on the Src kinase activity. Levels of phosphorylated Src kinase at Tyr416, an index of Src activation, were reduced after treatment with LiCl (1 mM) for more than 3 days. Protein levels of Src-family kinases such as Src, Fyn, and Yes were unchanged by lithium treatment. The activities of cytosolic protein tyrosine kinase and protein phosphatase were also unchanged by lithium treatment, indicating the selectivity and the modulation. Moreover, the levels of postsynaptic densities (PSD) and SynGAP, the scaffolding proteins of the NMDA receptor complex, were unaltered by lithium. A Src kinase inhibitor, SU6656, and an NR2B antagonist, ifenprodil, partially blocked glutamate excitotoxicity. Our results suggest that lithium-induced inactivation of Src kinase contributes to this drug-induced NMDA receptor inhibition and neuroprotection against excitotoxicity.

Key words: Lithium; Src kinase; Tyrosine phosphorylation; Cerebral cortical neuron; Bipolar disorder

1. Introduction

Protein phosphorylation by the Src-family tyrosine kinases has been implicated in the regulation of a variety of cellular functions [1]. One of the prominent substrates of the Src-family kinases is the *N*-methyl-D-aspartate (NMDA) receptor, which plays a central role in neuronal development, synaptic plasticity and excitotoxicity. Src-family kinases bind to scaffolding proteins in the NMDA receptor complex [2]. Tyrosine phosphorylation of the NMDA receptor subunits, NR2A and NR2B, by Src-family kinases such as Src and Fyn, results in enhancement of receptor activity [3,4]. NR2B Tyr1472 is one of the sites phosphorylated by Src and Fyn kinases [5,6] and its tyrosine mutation causes a loss of stimulus-induced calcium influx through the NMDA receptor channel [7]. Although the function of Src-family kinases in the central nervous system is becoming clear, their regulation of activity is still largely elusive.

Lithium has long been a primary drug used for the treatment and prophylaxis of bipolar mood disorder; however, its therapeutic mechanisms remain unclear. Recently, this drug was found to have robust neuroprotective effects against a variety of insults including excitotoxicity in vitro and in vivo [8,9]. We have reported that long-term, but not acute lithium treatment induces a selective decrease in the level of NR2B Tyr1472 phosphorylation in rat cerebral cortical neurons [10]. This lithium-induced effect is correlated with a loss of glutamate-stimulated NMDA receptor activity and with its neuroprotection against NMDA receptor-mediated excitotoxicity [10,11]. Since phospho-tyrosine levels associated with NMDA receptors could be regulated by both tyrosine protein kinase and tyrosine protein phosphatase, this study was undertaken to examine the effects of lithium on the activities of these enzymes with a particular emphasis on the Src-family kinases.

2. Materials and methods

2.1. Animals and chemicals

All procedures employing experimental rats were performed in compliance with the National Institutes of Health guidelines for the care and use of laboratory animals.

2.2. Primary cultures of rat cerebral cortical neurons and drug treatments

Primary cultures of rat cerebral cortical neurons were prepared from 17-day-old embryonic rats cultured as described previously [12]. Cells were plated at a density of 4.2×10^5 cells/cm^2 on polyethyleneimine-precoated 96-well plates or 6-well plates. Cultures were maintained in serum-free B27/Neurobasal medium (Life Technologies, Rockville, MD, USA). Over 95% of the cells present on day 5 in vitro were differentiated into neurons, as characterized by the appearance of long neurites expressing neurofilament protein [13]. Routinely, cortical neurons on day 9 in vitro were treated with 1 mM LiCl and maintained for an additional 5 days in culture. Glutamate (8 μM) was then added to the culture medium on day 15 in vitro and cell viability was determined 24 h later.

2.3. Measurement of neurotoxicity

Cortical neuronal cultures were plated on 96-well plates and maintained for 16 days. Viability of cortical neurons was determined by the

*Corresponding author. Fax: (1)-301-480 9290.
E-mail address: chuang@helix.nih.gov (D.-M. Chuang).

Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMDA, *N*-methyl-D-aspartate; PSD, post synaptic densities; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase

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3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which detects mitochondrial dehydrogenase activity, as described previously [14]. Neuroprotection is expressed as a percentage of maximal neuroprotection, using values derived from glutamate-treated cells and the untreated control as a reference. One-way ANOVA was used for the statistical analysis, and significant differences in cell viability were determined by post-hoc comparisons of means using Bonferroni's post-hoc test.

2.4. Western blotting

Cells were lysed with sodium dodecyl sulfate (SDS) buffer (50 mM Tris–HCl, pH 7.5, 0.5% SDS, 1 mM dithiothreitol). The lysates were sonicated at 30 amplitudes (Sonic&Materials, Danbury, CT, USA) in an ice-bath four times, for 10 s each time, and then an aliquot of 50 μg protein was dissolved in the sample buffer for SDS–polyacrylamide gel electrophoresis and Western blotting as described previously [15]. Separated proteins were transferred onto a polyvinylidene difluoride membrane, which was incubated with a primary antibody against Src (monoclonal; Oncogene, San Diego, CA, USA), Fyn (polyclonal; Santa Cruz, Santa Cruz, CA, USA), c-Yes (monoclonal; Wako Chemicals, Richmond, VA, USA), phospho-Src (polyclonal against phospho-Tyr416; Cell Signaling Technology, Beverly, MA, USA), postsynaptic density (PSD)-95 (monoclonal; Upstate, Lake Placid, NY, USA) or SynGAP (polyclonal; Upstate). Membranes were washed and then exposed to the secondary antibody, horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Amersham Pharmacia Biotech, Arlington Heights, IL, USA). Immunoreactive protein bands were visualized by ECL detection and quantified using a CCD camera (Sierra Scientific, Sunnyvale, CA, USA) and Macintosh NIH Image 1.6 software. Statistical differences between untreated and lithium-treated samples were analyzed by Student's *t*-test. The detection limits were 10 μg protein of lysates from samples of untreated cells for phospho-Src, total Fyn and Yes, and 2 μg protein for total Src, PSD-95 and SynGAP. The coefficients of variation (determined by standard deviation/mean) in untreated controls and lithium-treated samples were 9.3% and 11.5% for phospho-Src, 8.3% and 6.2% for total Src, 9.5% and 9.9% for total Fyn, 10.2% and 11.2% for total Yes, 7.6% and 7.3% for PSD-95, and 8.1% and 9.7% for SynGAP, respectively.

2.5. Protein tyrosine kinase activity assay

The tyrosine kinase activity of cortical primary culture cells was measured using the SignaTECT Protein Tyrosine Kinase Assay System (Promega, Madison, WI, USA), according to the manufacturer's instructions. Briefly, cultured cells were solubilized on ice using protein tyrosine kinase (PTK) extraction buffer. The lysates were centrifuged at 10 000 × *g* and the supernatants were collected. An aliquot of 5 μl containing 2 μg cytosolic protein from each sample was then incubated for 15 min at 30°C in 20 μl of PTK reaction buffer containing 8 mM imidazole hydrochloride (pH 7.3), 8 mM β-glycerophosphate, 0.2 mM ethylene glycol-bis(2-aminoethyl-ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 20 mM $MgCl_2$, 1 mM $MnCl_2$, 0.1 mg/ml bovine serum albumin, 1 mM Na_3VO_4, 25 μM ATP, 25 μCi/ml [γ-^{32}P]ATP (3000 Ci/mmol) (NEN, Boston, MA, USA), and 0.3 mM PTK biotinylated peptide substrate as a substrate. Several concentration-dependent experiments were performed to ensure that this amount of cytosolic protein (2 μg) was within the linear range of the PTK assay. Increasing or decreasing the pH value from 7.3 did not enhance the activities of PTK. The reaction was terminated by addition of 12.5 μl of 7.5 M guanidine hydrochloride. An aliquot of 12.5 μl of each sample was spotted onto a SAM^2 biotin capture membrane. These membranes were washed with 2 M NaCl, then with 2 M NaCl in 1% H_3PO_4. The incorporated ^{32}P radioactivity was then quantified and the enzyme-specific activity was determined.

2.6. Protein tyrosine phosphatase activity assay

The tyrosine phosphatase activity of cortical primary culture cells was measured using the SignaTECT Protein Tyrosine Phosphatase Assay System (Promega), according to the manufacturer's instructions. Briefly, cultured cells were solubilized on ice using protein tyrosine phosphatase (PTP) extraction buffer. The endogenous phosphate was removed from the 10 000 × *g* supernatant using a Sephadex G-25 column. An aliquot of 5 μl containing 2 μg cytosolic protein from each sample was then incubated for 15 min at 30°C in 50 μl of reaction buffer containing 10 mM Tris–HCl, pH 7.3, and 100 μM of tyrosine-phosphopeptide as a substrate in 96-well microtiter plates. Several concentration-dependent experiments were performed to ensure that this amount of cytosolic protein (2 μg) was within the linear range of the PTP assay. Increasing or decreasing the pH value from 7.3 did not enhance the activities of PTP. The reaction was terminated by addition of 50 μl of molybdate dye/additive mixture. After the mixture was incubated at room temperature for 15 min, optical density of the samples was measured using a plate reader at a wavelength of 630 nm, and the PTP activity was calculated.

2.7. Protein determination and statistical analysis

Protein concentrations were determined by the bicinchoninic acid kit (Pierce, New York, NY, USA), using bovine serum albumin as the standard. One-way ANOVA was used for the statistical analysis, and significant differences in cell viability were determined by post-hoc comparisons of means using Bonferroni's post-hoc test.

3. Results

Cerebral cortical neurons were treated with LiCl (1 mM) on day 9 in vitro and maintained for 5 additional days in culture. Levels of phosphorylated Src kinase at Tyr416, an index of Src activation, were found to be decreased by lithium treatment (Fig. 1). Quantification of immunoblots revealed that levels of phospho-Tyr416 in Src kinase were reduced to 57.3 ± 9.5% of the untreated control ($P < 0.05$). In contrast, levels of total Src, Fyn and Yes, another member of the Src family of kinases, were unaffected by lithium treatment. Moreover, levels of two NMDA receptor scaffolding proteins, PSD-95 [16] and SynGAP [17], were unchanged by lithium. The lithium-induced decrease in levels of phospho-Tyr416 in Src was time-dependent, with little or no change after treatment for 10 min to 1 day, but rather showed a marked loss at day 3 and day 5 (Fig. 2). Cytosolic tyrosine kinase activity and tyrosine phosphatase activity, measured using PTK biotinylated peptide and tyrosine-phosphopeptide as the respective substrates, were not significantly changed by pretreatment with lithium in the concentration range of 0.2–1.0 mM (Fig. 3A,B), thus indicating the selectivity of lithium-induced Src kinase inactivation.

Since tyrosine phosphorylation of NMDA receptors has been linked to the activation of this receptor, we assessed its role in glutamate-induced, NMDA receptor-mediated excito-

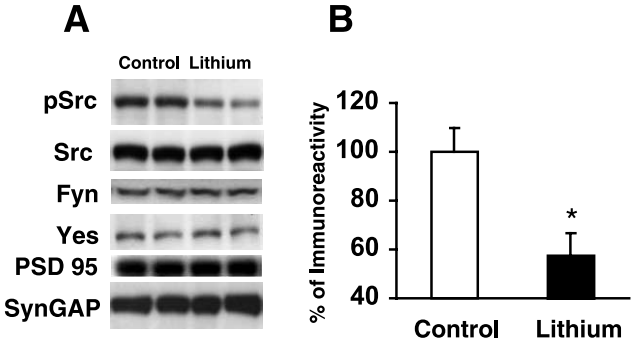

Fig. 1. Long-term lithium treatment decreases levels of Tyr416-phosphorylated Src kinase. A: Cortical cultures after 9 days in vitro were treated with 1 mM LiCl or its vehicle for 5 days and then harvested for Western blotting for Tyr416-phosphorylated Src kinase, and total protein levels of Src, Fyn, and Yes kinases as well as of PSD-95 and SynGAP. The immunoblots done in duplicate are representative of four independent experiments. B: Relative phospho-Src kinase levels were obtained by normalizing to total Src kinase levels and were expressed as percent of untreated control. Quantified data represent means ± S.E.M. from four independent experiments. *$P < 0.05$ compared with control.

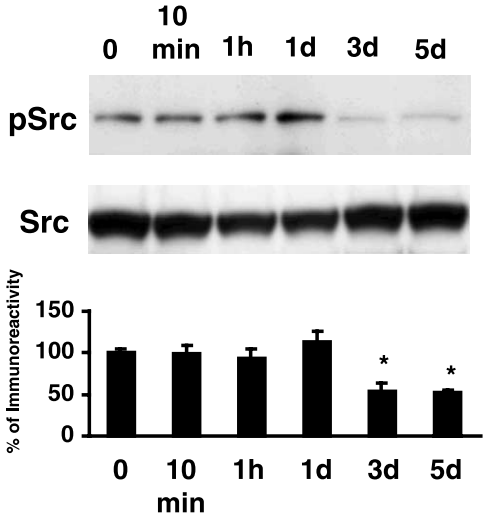

Fig. 2. Lithium-induced decrease of Tyr416-phosphorylated Src levels is time-dependent. A: Cortical cultures were treated with LiCl (1 mM) or its vehicle for the indicated times. All cultures were harvested on day 14 in vitro for Western blotting for phospho-Src (Tyr416) kinase and total Src kinase protein levels. Immunoblots shown are representative of three independent experiments. B: Phospho-Src kinase levels relative to total Src kinase protein levels. Quantified data represent means ± S.E.M. of percent of untreated control from three independent experiments. $*P < 0.05$ compared with control.

toxicity in cortical cultures. Pretreatment with SU6656 [18], a Src kinase inhibitor, for 1 h reduced glutamate-induced excitotoxicity by 46.8% (Fig. 4). As expected, long-term LiCl (1 mM) pretreatment completely blocked glutamate excitotoxicity. Ifenprodil, an antagonist of NR2B subunits of NMDA receptors [19], suppressed glutamate excitotoxicity by 62.3% and MK801, a non-competitive NMDA receptor antagonist, completely suppressed excitotoxicity (Fig. 4), indicating a prominent role of this receptor subunit in mediating excitotoxicity. Neither SU6656, ifenprodil, MK801 nor lithium alone significantly affected cell viability (data not shown).

4. Discussion

The present study demonstrated that long-term lithium treatment of rat cortical neurons caused a decrease in the activation of Src kinase, with no effect on the protein levels of Src, Fyn and Yes kinases as well as PSD-95 and SynGAP. Moreover, neither the activity of cytosolic PTK nor that of cytosolic PTP was affected by lithium treatment, indicating the selectivity of modulation by this drug. The relationship of lithium-induced down-regulation of Src kinase activation and the drug-induced decrease in NMDA receptor function and neuroprotection against excitotoxicity is supported by the observations that the time course of lithium-induced decrease in Src phospho-Tyr416 levels (Fig. 1) correlated well with this drug-induced decrease in NR2B Tyr1472 phosphorylation and loss in NMDA receptor-mediated Ca^{2+} influx [10], and that glutamate excitotoxicity was attenuated by the presence of ifenprodil, an NR2B antagonist, and SU6656, a Src kinase inhibitor (Fig. 4). To our knowledge, this is the first demonstration of the modulation of Src kinase activity by treatment with a mood stabilizer. NR2B tyrosine phosphorylation is mediated by both Src and Fyn kinases through binding to PSD via their SH2 domains [6]. The effects of lithium on levels of activated Fyn kinase have not yet been studied largely due to the unavailability of specific antibodies for activated, tyrosine-phosphorylated Fyn kinase. Recently, it was reported that short-term treatment with LiCl (3 mM) increases the activity of membrane-bound PTP in PC12 cells [20]. The discrepancy between their and our results may reflect differences in the treatment time (6 h vs. 6 days), PTP assay (membrane-bound vs. cytosolic activity) and cell types used in the studies.

The NMDA receptor is the primary glutamate receptor subtype involved in excitotoxicity and neuronal death. The ability of lithium to down-regulate Src kinase activation and Src kinase family-mediated NR2B tyrosine phosphorylation suggests that this drug may have additional clinical uses in intervention of neurodegeneration. NR2B antagonists have been shown to effectively promote cell survival against excitotoxicity without causing complete receptor blockade, thus reducing side effects of classical NMDA receptor blockers [21]. In this context, many NMDA receptor antagonists were reported to partially block the devastating side effects of dyskinesia associated with L-DOPA therapy for Parkinson's disease; however, NR2B selective antagonists were found to be most efficacious [22]. These observations suggest that NR2B inhibition is a potential therapeutic strategy. Thus, chronic lithium treatment, through inhibition of Src tyrosine kinase-mediated NMDA receptor activation, may be benefi-

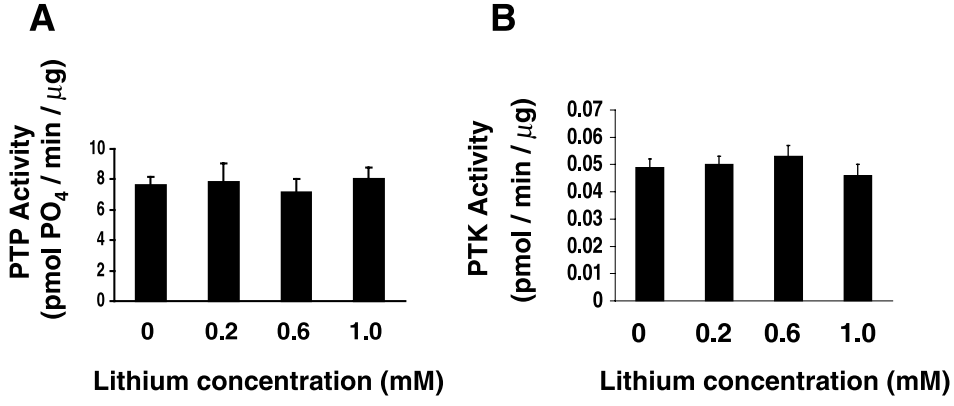

Fig. 3. Lithium does not affect levels of cytosolic PTP (A) and PTK (B) activities. Cortical cultures were treated with indicated concentrations of LiCl or vehicle starting from day 9 in vitro and harvested on day 15 in vitro. Cytosolic proteins were prepared and activities of cytosolic PTK and PTP were then measured. Data are means ± S.E.M. of kinase or phosphatase activities from three independent experiments.

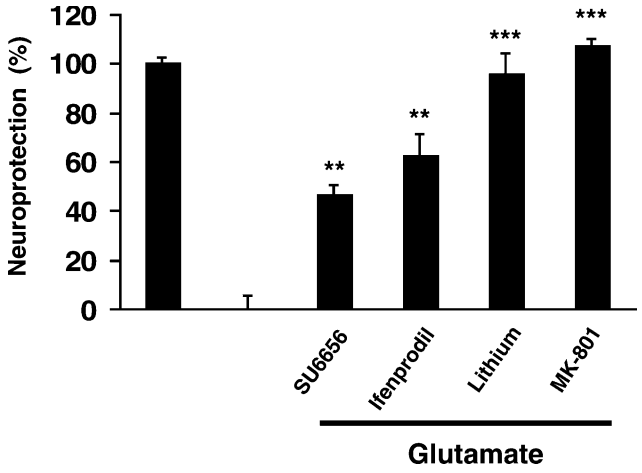

Fig. 4. A Src kinase inhibitor, an NR2B receptor antagonist, long-term lithium treatment and a non-competitive NMDA receptor antagonist diminish glutamate-induced excitotoxicity in cortical neurons. Cortical cultures were pretreated with SU6656 (0.5 μM), ifenprodil (5 μM) or MK801 (10 μM) for 1 h or LiCl (1 mM) for 6 days prior to exposure to glutamate (8 μM) for 24 h. Neuroprotection was determined using the MTT assay 24 h after glutamate addition, as described in Section 2. Results are means ± S.E.M. of viability measurements from five or six cultures. The viability value of glutamate-treated cells was 60.7 ± 5.3% of untreated control. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with glutamate alone.

cial for the treatment of excitotoxicity-related neurodegenerative diseases.

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