Chronic Lithium Treatment Increased Intracellular S100ß Levels in Rat Primary Neuronal Culture

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Abstract- S100ß a neurotrophic factor mainly released by astrocytes, has been implicated in the pathogenesis of bipolar disorder. Thus, lithium may exert its neuroprotective effects to some extent through S100ß. Furthermore, the possible effects of lithium on astrocytes as well as on interactions between neurons and astrocytes as a part of its mechanisms of actions are unknown. This study was undertaken to determine the effect of lithium on S100β in neurons, astrocytes and a mixture of neurons and astrocytes. Rat primary astrocyte, neuronal and mixed neuro-astroglia cultures were prepared from cortices of 18-day's embryos. Cell cultures were exposed to lithium (1mM) or vehicle for 1day (acute) or 7 days (chronic). RT-PCR and ELISA determined S100β mRNA and intra- and extracellular protein levels. Chronic lithium treatment significantly increased intracellular S100β in neuronal and neuro-astroglia cultures in comparison to control cultures (P<0.05). Acute and chronic lithium treatments exerted no significant effects on intracellular S100β protein levels in astrocytes, and extracellular S100β protein levels in three studied cultures as compared to control cultures. Acute and chronic lithium treatments did not significantly alter S100β mRNA levels in three studied cultures, compared to control cultures. Chronic lithium treatment increased intracellular S100ß protein levels in a cell-type specific manner which may favor its neuroprotective action. The findings of this study suggest that lithium may exert its neuroprotective action, at least partly, by increasing neuronal S100ß level, with no effect on astrocytes or interaction between neurons and astrocytes.

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Introduction

Recent findings provide direct evidence for reductions in number and density as well as changes in cell body size and shape of neurons and glia in bipolar disorder (BD)(1). Biochemical and molecular data have related these cellular pathologies in BD to deficits in neurotrophic factors (1,2). Accordingly, it has been proposed that mood stabilizers including lithium exert their therapeutic effects by regulating synthesis and/or release of neurotrophic factors (2).

S100ß, a neurotrophic factor mainly released by astrocytes, has intracellular and extracellular regulatory activities (3). Extracellular S100ß produces a neuroprotective effect in normal physiological levels (nanomolar range) through receptor for advanced glycation end products (RAGE); and can induce neuroinflammation, apoptosis and neurodegeneration (3) in a micromolar range. Intracellular S100β is involved in cell proliferation, inhibition of apoptosis, regulation of calcium homeostasis, energy metabolism and enzyme functions (4).

Several lines of evidence suggest that S100ß might be involved in the pathogenesis of BD. Thus, transgenic mice with increased expression of S100ß were hyperactive, a behavior observed in manic states (5). In addition, increased S100ß protein levels have been reported in cerebrospinal fluid (CSF) of rats in an animal model of mania (6). Increased serum S100ß levels have been shown in depressive and manic episodes of BD (7-9). On the other hand, a decreased S100ß protein level was observed in postmortem dorso-lateral prefrontal

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cortex of BD patients in comparison to healthy controls (10). Moreover, a genetic linkage with BD was found at locus of S100ß gene (11,12), and an association between S100ß gene polymorphism and susceptibility to BD was reported (11,13).

Taken together, the recent neurotrophic deficit hypothesis of BD, the involvement of S100ß in the pathophysiology of BD and the proposed neuroprotective effect of lithium for its therapeutic action raised the question whether lithium exerts its neuroprotective effects, at least partly, through its effect on S100ß. In addition, no studies have yet determined to what extent the effect of lithium on astrocytes and/or an interaction between neurons and astrocytes contributes to its mechanism of action. Thus, this study was undertaken to evaluate the acute and chronic effects of lithium on the intracellular and extracellular levels of S100ß in the rat primary neuron, astrocyte and neuro-astroglia cultures.

Materials and Methods

The European Committee guidelines for the use of experimental animals were followed throughout the study. The ethics committee of Shiraz University of Medical Sciences for animal care approved the protocol.

Embryonic cortices were obtained from 18-day embryos of Sprague-Dawley rats (14). Cortices were dissected and triturated in cold Hank's Balances Salt solution followed by centrifugation at 800 x g for 10 min. Precipitated cells were resuspended in Hank's Balances Salt solution and used for different primary cultures as previously described (14). Briefly, the neuronal cells ($3.5x10^6$) were seeded in 60mm polyethylene imine (PEI)-coated dishes in neurobasal media (Gibco, USA) supplemented with 2% B27, 2mM l-glutamine, 50 unit/ml penicillin and 50 µg/ml streptomycin. For preparing astrocyte culture, astrocytes were separated by standard shaking procedure for 72 hrs (14). The purified astrocytes were detached by trypsin–EDTA (0.05%) and seeded in 10cm PEI-coated dishes containing Dulbecco's Modified Eagle's Medium (DMEM), horse serum (10%), l-glutamine (2mM), penicillin (50 units/ml) and streptomycin (50 µg/ml). Horse serum was replaced with 1% G5, a serum-free supplement, before exposing the cells to lithium. Mixed neuron-astrocyte culture was prepared as previously described (14). Briefly, dissociated cells (5x106) were seeded in PEI-coated dishes in DMEM medium with 10% horse serum, 2 mM l-glutamine, 50 units/ml penicillin and 50 µg/ml streptomycin. After 72h, B27 (1%) was added to the culture medium. On the 8th day, horse serum was replaced with 1% G5 supplement, before exposing the cells to lithium.

Cultures were kept at 37°C in a 5% CO_2, 95% O_2 humidified incubator. The cells were exposed to lithium (1mM) or vehicle for 24 h (acute treatment) or seven days (chronic treatment) (15-17). The Cultures' media were replenished every other day during the seven days of lithium exposure.

The purity of cell cultures was confirmed by immunocytochemistry as previously described (14). Briefly, neurons and astrocytes were incubated with microtubule-associated protein 2 (MAP-2) (Abcam, USA) (1:100 dilution) and glial fibrillary acidic protein (GFAP) antibodies (Abcam, USA) (1:100 dilution), respectively, for 2hrs at room temperature. Cells were then exposed to secondary antibodies at 1:400 dilutions (Alexa Flour 596 for MAP-2 and Alexa Flour 488 for GFAP) (Abcam, USA) and incubated for 1.5hrs at room temperature. Finally, cell nuclei were stained with DAPI. Cells were visualized and counted by fluorescence microscopy at a magnification of 100 x. At the time of lithium treatment, the enrichment of neuronal and astrocyte cultures was more than 90%. The mixed neuron-astrocyte cultures were comprised of approximately 55% astrocytes and 44% neurons.

S100β mRNA level was measured by real time PCR. Total RNA was extracted by phenol-chloroform method using TriPure Isolation reagent in accordance with manufacturer instruction (18). cDNA was synthesized from 1µg total RNA using RevertAid H Minus first strand cDNA synthesis kit according to manufacturer guidelines. The relative levels of S100β [refSeq: NM013191.1] and GAPDH [RefSeq: NM017008.3] mRNAs were determined by a quantitative real time PCR assay (Applied Biosystem, PRISM 7500, USA). Specific primers were: for S100β, forward primer 5'- TCA CTG AGG GAC GAA ATC AAC AC -3', and reverse primer 5'- GGT GCT ATT GGT AGT CTG CCT TG -3'; and for GAPDH: forward primer 5'- CGT GAT CGA GGG CTG TTG G-3', and reverse primer 5'-CTG CTT CAG TTG GCC TTT CG-3'. Primers were designed to span exon-exon junction in order to preclude the amplification of genomic DNA. The size of target amplicon was 108bp for S100ß and 97bp for GAPDH. The threshold cycles (Ct) of samples were used to calculate the ratio of expressions between lithium treated and untreated samples using Pfaffl method (19).

ELISA determined S100β protein level. For measuring intracellular S100β protein levels, cells were lysed in NP40 buffer. To determine extracellular S100β

protein levels, media were collected after 1 day of acute and every other day of chronic lithium treatment. Samples stored at -70°C until use. Total protein was measured by Bradford method (20). Rat S100ß ELISA kit (MyBiosource, USA) was used according to manufacturer guidelines to quantify S100β protein levels. Briefly, media or cell lysates were added to wells pre-coated with polyclonal anti-S100β antibody. Then, HRP conjugate was added to each well and incubated for one hour at 37°C. Wells were washed and then incubated with tetramethylbenzidine, as an HRP substrate, at room temperature for 15 min. After adding stop solution, the absorbance was measured at 450nm in a micro-plate reader (Micura, England). The S100ß concentrations were interpolated from the standard curves using samples with known S100ß concentrations. The intra-assay and inter-assay coefficient of variance were 6% and 10%, respectively.

Results were expressed as the mean± SEM. To compare S100ß gene expression between lithium- and vehicle-treated cells, Pfaffl method used by REST software (REST-384-beta) (19) due to different amplification efficiency of S100ß and GAPDH. Paired t-test assessed differences between lithium- and vehicle-treated cells in S100ß protein levels. To compare the effect of lithium on S100ß between three cultures, the percent changes from control were analyzed by one-way ANOVA followed by *post-hoc* LSD test. SPSS version 18 was used and a *P* value of <0.05 was considered as a significance level.

Results

Effects of acute and chronic lithium treatment on S100β mRNA

Gel electrophoresis of PCR product confirmed a specific single band at the 108bp for S100β (Figure 1).

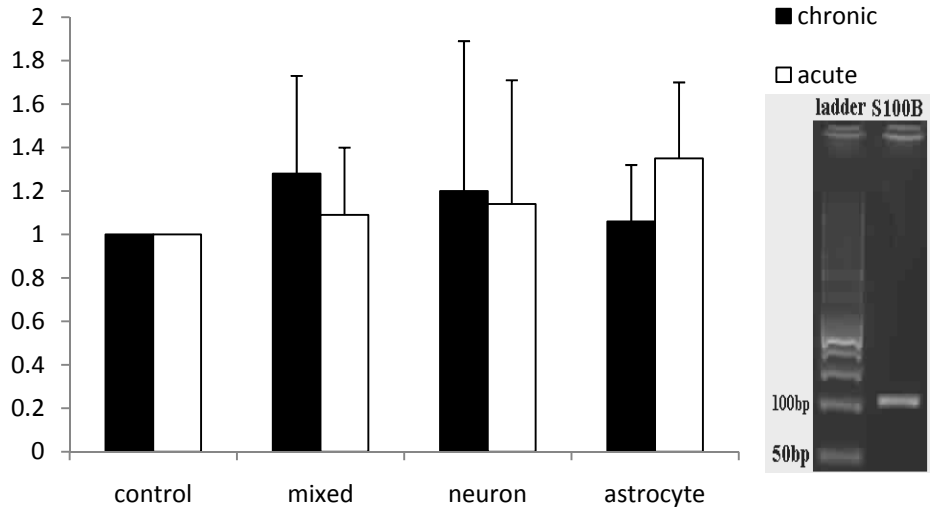

Figure 1. Effects of acute (1 day) and chronic (7days) lithium (1mM) treatment on mRNA level of S100β in rat primary mixed neuron-astrocyte, neuronal and astrocyte cultures in comparison to vehicle-treated cultures (control, 100µl sterile distilled water). Data presented as mean ± SEM

Chronic lithium treatment increased mRNA levels by 1.28 ± 0.69, 1.20 ± 0.45 and 1.06 ± 0.26 in rat primary mixed neuro-astroglia, neuron and astrocyte cultures, respectively, as compared with their relevant vehicle-treated cultures (Figure 1), but these changes were not statistically significant. Furthermore, there were no significant differences in the percent changes from control in S100ß mRNA levels between neuronal, astrocyte and neuro-astroglia cultures following chronic lithium treatment. Acute lithium treatment did not significantly change S100β mRNA levels in three studied cultures in comparison to control cultures.

Effects of acute and chronic lithium treatment on S100β protein levels

Chronic lithium treatment significantly increased the intracellular S100β protein level in neuronal (619%) and mixed neuro-astroglial (331%) cultures (*P*<0.05), but not in astrocyte culture (187%), as compared to the relevant controls (Figure 2). There were no significant differences in the basal level of S100β (in the absence of lithium) or percent change from basal intracellular

S100β protein levels between neuronal, astroglial and mixed neuro-astroglial cultures.

Extracellular S100β protein levels decreased in mixed neuro-astroglia (84%) and increased in neuronal (142%) and astrocyte (118%) cultures following chronic lithium treatment as compared to respective control cultures (Figure 3), but these changes did not reach statistical significance. The basal extracellular S100β protein level in the absence of lithium was higher in mixed neuro-astroglia culture (6.56 ± 2.53 µg/mg) when compared to neuronal (0.55 ± 0.11 µg/mg) or astroglial (1.12 ± 0.10 µg/mg) cultures (P<0.05). However, the percent changes from basal extracellular S100β protein levels were not significantly different between three studied cultures following chronic lithium treatment.

Acute lithium treatment had no significant effect on intracellular and extracellular S100β protein levels in neuronal, astroglial and mixed neuro-astroglial cultures in comparison to the respective vehicle-treated cultures (Figures 2 and 3).

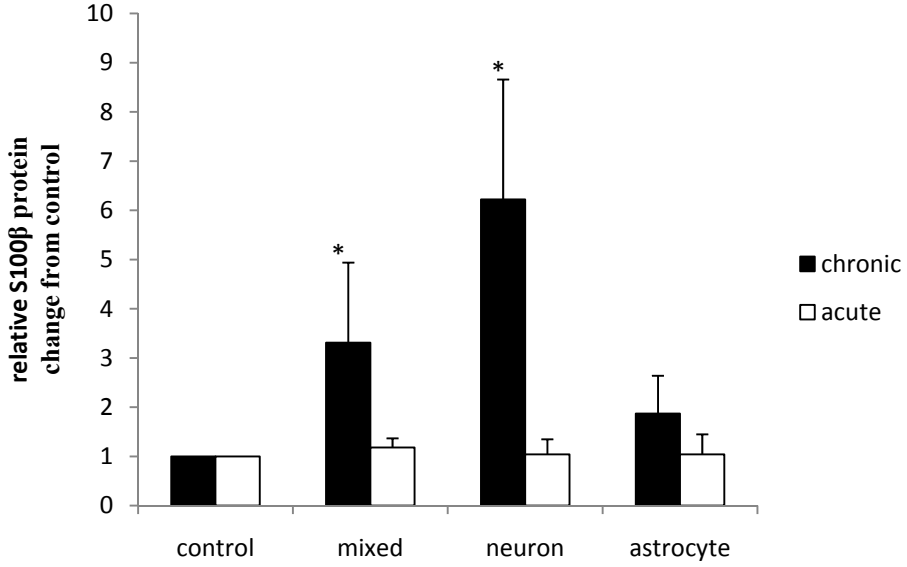

Figure 2. Effects of acute (1 day) and chronic (7days) lithium (1mM) treatment on intracellular S100β protein levels in rat primary mixed neuro-astroglial, neuronal and astroglial cultures in comparison to vehicle-treated cultures (control, 100µl sterile distilled water). Data presented as mean ± SEM. * P< 0.05 was significantly different from control

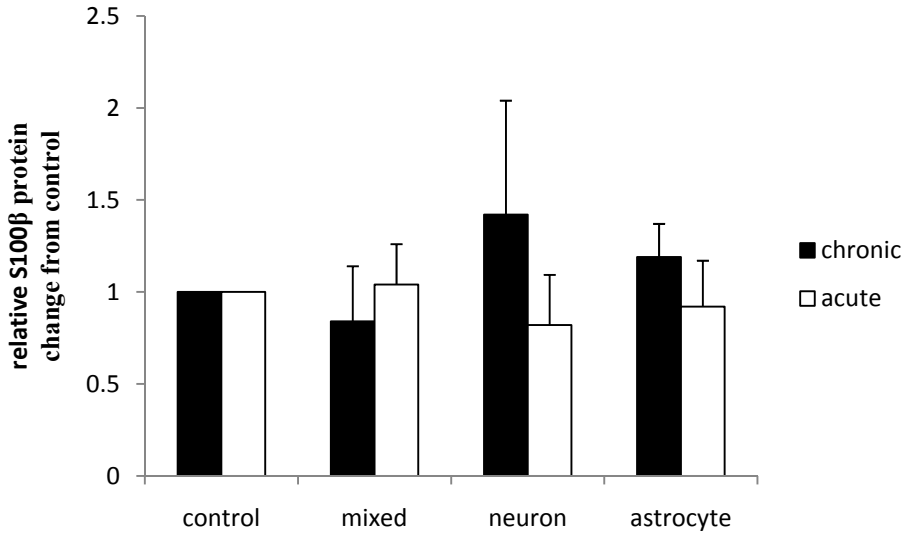

Figure 3. Effects of acute (1 day) and chronic (7days) lithium (1mM) treatment on extracellular protein level of S100β in rat primary mixed neuro-astroglia, neuronal and astroglial cultures in comparison to vehicle-treated cultures (control, 100µl sterile distilled water). Data presented as mean ± SEM

Discussion

The present study showed that chronic lithium treatment at therapeutic concentration increased intracellular S100β protein level in a cell-type specific manner. The findings of this study suggest that lithium may exert its neuroprotective action, at least partly, by increasing neuronal S100ß level, with no effect on astrocytes or interaction between neurons and astrocytes. These findings shed more lights regarding the mechanism of action of lithium as will be discussed below.

To our knowledge, there is only one published study evaluating the effect of lithium on S100β. Thus, in vivo treatment of rats with lithium at blood level of 0.78 mmol/L for 1 month could not significantly change the level of S100β protein in rat prefrontal cortex (10). Conversely, increased S100β level has been observed in CSF of lithium-treated rats by Rocha *et al*., (unpublished observation). Our results are in accordance with Rocha *et al.,* but in disagreement with Dean *et al.,* findings (10). The increased intracellular S100ß protein levels observed in neurons, but not in astrocytes, in the present study suggests that the effect of lithium on S100ß may be cell-type specific. This differential effects of lithium on various cell types may underlie the observation of no change in S100ß levels in tissue homogenates of the prefrontal cortex following lithium treatment (10).

In this study, lithium showed no significant effect on intracellular S100ß in astrocytes because increased it in mixed neuro-astroglia culture. However, the effect of lithium on S100ß in mixed culture was an average of lithium's effect on S100ß in neuron and astrocyte cultures. This suggests that the increased intracellular neuro-astroglia S100ß following chronic lithium treatment may mainly reflect the effect of lithium on neurons.

The differential effects of lithium on S100ß in neurons and astroglial cells may be explained by different characteristics of serotonergic receptors in these cell types (21). Activation of serotonin receptors, especially 5-HT1A receptors, is one of the most important factors for S100ß synthesis (22). Receptor activators were shown to have a higher affinity for neuronal than astroglial serotonin receptors (23). Therefore, the distinct serotonin receptor affinity for lithium, as an activator of 5-HT1A receptor (24), may be proposed to underlie the differential effects of lithium on S100ß levels in neurons and astrocytes. However, further studies are needed to substantiate this notion.

In contrast to increased intracellular S100β protein levels, chronic lithium treatment did not have such an effect on extracellular levels of S100ß in neurons in the current study. In the agreement with this finding, other studies have reported that changes in the intracellular S100ß content were not necessarily associated with changes in S100ß secretion (25, 26). Moreover, neuronal cells have limited secretory activity for S100ß (27) and release S100ß only under stressful conditions (28) as opposed to normal condition used in this study.

Chronic lithium treatment increased S100ßprotein levels with no significant changes in S100ßmRNA levels in neuronal and mixed neuro-astroglia cultures. The exact reason for this inconsistency is elusive. However, it is notable that discord changes in S100β protein and mRNA levels has been observed in prefrontal cortex from bipolar I patients (10) which led to the suggestion that changes in S100β content in CNS are not necessarily related to changes in the rate of gene transcription (10). In addition, this differ effects of lithium on protein and mRNA levels has also been reported for BDNF (29), a neurotrophic factor known to be involved in the mechanism of lithium's action (30).

The increased S100β in neurons, but not astrocyte, following chronic lithium treatment have important implications for understanding the mechanism of action of lithium in treating BD. Activated or damaged astrocytes are the major source of extracellular S100β (31), thus increased serum S100ß has been used as a marker for glial pathology. Remarkably, histopatholigical studies have shown decreased glial density and gray matter volume in bipolar disorder (1). Thus, elevated serum S100ß during depressive and manic episodes of mood disorders (32) may indicate astrocyte alterations and supports the hypothesis of glial pathology in bipolar disorder (33). In this study, however, lithium might indirectly normalize serum S100ß in bipolar patients by reversing glial density and apoptosis. In support of this notion, lithium has increased gray matter volume in BD brain, and increased astroglial bcl2 (14) and GDNF (34) levels in rat primary cortical cultures. Therefore, it can be suggested that lithium by increasing bcl2 and GDNF in astrocytes could prevent glial loss and in turn may normalize extracellular S100ß levels in BD patient. Interestingly, a decline in serum S100ß has been shown following successful antidepressant treatment (33). However, no human studies have yet evaluated serum S100ß during the course of lithium treatment in BD patients. Future studies should address this hypothesis.

The role of extracellular S100β in BD has been investigated by measuring serum S100β from BD patients as indicated above. However, there are limitations in *in vivo* measurements of intracellular S100β in BD patients. Nevertheless, a postmortem study indicated a reduction in regional S100β content in the prefrontal cortex of BD patient (35). In addition, studies in two animal models of depression revealed reductions of S100β protein levels in prefrontal regions (36) and the hippocampus (37) that were reversed by antidepressant treatment with fluoxetine. In accordance with the effects of antidepressants, lithium increased intracellular neuronal S100β in BD brain. The increasing effect of lithium on intracellular neuronal S100β may be favorable in BD in several ways. First, Intracellular S100β is involved in the maintenance of Ca2+ homeostasis (4). Lithium has been shown to modulate disturbed calcium homeostasis (38) in BD. Thus, it can be suggested that lithium by increasing intracellular S100β may reconstitute intracellular calcium homeostasis to restore neuronal abnormality and death. Second, S100β is an important neurotrophic factor which protect neurons against several stressors including excitotoxicity, oxidative stress and ß-amyloid toxicity (31, 39, 40). In parallel, lithium protected neurons against similar stressors (41, 42). Therefore, lithium may exert its neuroprotective effects, at least partly, by increasing neuronal S100β levels. Future studies evaluating the effects of lithium on neuronal S100β levels in the presence of stressors can elucidate this possibility further. Third, S100β is critical for controlling neuroprotection and neuroplasticity in the serotonergic system (43,44). It was suggested that S100β mediates BDNF trophic effects on serotonergic neurons. Accordingly, lithium has been shown to stabilize central serotonergic system (45-47) and increased neuronal BDNF levels (34). Thus, the effect of lithium on S100β may be a linkage between the role of the serotonergic system in the pathophysiology and treatment of BD. Finally, S100β is actively involved in the neuroplasticity and plays important roles in axonal growth and synaptogenesis during development, synaptic remodeling and intracellular signal transduction (31). Consistently, lithium has been shown to have beneficial effects in the treatment of BD by normalizing neuroplasticity (2). Thus, the effect of lithium on neuroplasticity may partly be related to its effect on S100β.

Chronic lithium treatment increased intracellular S100ß protein levels in a cell-type specific manner which may favor its neuroprotective action. Considering the important actions of S100β as a neurotrophic/neuroprotective factor especially in the serotonergic system and previous reports regarding the neuroprotective effect of lithium, it can be suggested that lithium may exert its neuroprotective action, at least partly, by increasing neuronal S100ß level with no effect on astrocyte or neuro-astroglia interaction.

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