Neuropsychopharmacology (2004) 29, 799–804

www.neuropsychopharmacology.org

Identification of Lithium-Regulated Genes in Cultured Lymphoblasts of Lithium Responsive Subjects with Bipolar Disorder

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Lithium, a common drug for the treatment of bipolar disorder (BD), requires chronic administration to prevent recurrences of the illness. The necessity for long-term treatment suggests that changes in genes expression are involved in the mechanism of its action. We studied effects of lithium on gene expression in lymphoblasts from BD patients, all excellent responders to lithium prophylaxis. Gene expression was analyzed using cDNA arrays that included a total of 2400 cDNAs. We found that chronic lithium treatment at a therapeutically relevant concentration decreased the expression of seven genes in lymphoblasts from lithium responders. Five of these candidate lithium-regulated genes, including alpha1B-adrenoceptor (α1B-AR), acetylcholine receptor protein alpha chain precursor (ACHR), cAMP-dependent 3′,5′-cyclic phosphodiesterase 4D (PDE4D), substance-P receptor (SPR), and ras-related protein RAB7, were verified by Northern blotting analysis in lithium responders. None of these genes were regulated by lithium in healthy control subjects. When we compared the expression of these five genes between bipolar subjects and healthy control subjects at baseline, prior to lithium administration, we found that α1B-AR gene expression was higher in bipolar subjects than in healthy control subjects. Our findings indicate that α1B-AR may play an important role in the mechanism of action of lithium treatment.
Neuropsychopharmacology (2004) **29,** 799–804, advance online publication, 21 January 2004; doi:10.1038/sj.npp.1300383

Keywords: bipolar disorder; lithium; lymphoblast; gene expression; cDNA array; alpha1B-adrenoceptor

INTRODUCTION

Lithium is the most commonly prescribed treatment for bipolar disorder (BD). Prophylactic treatment with lithium prevents recurrences of depression and mania in a substantial proportion of patients with this disorder (Davis *et al*, 1999). Much research has shown that lithium regulates components of signal transduction pathways and their downstream gene transcription factors, an indication that changes in gene expression are likely to underlie the mechanism of action of the treatment (Manji and Zarate, 2002; Bezchlibnyk and Young, 2002). Lithium-induced changes in gene expression have been documented in both cultured cells and animal models (Kalasapudi *et al*, 1990; Williams and Jope, 1994; Manji *et al*, 1995a, b). Several studies have demonstrated that lithium alters the expression of the early response gene c-*fos* through a protein kinase C-mediated mechanism (Divish *et al*, 1991; Manji and Lenox, 1994). For example, incubation of PC-12 cells for 16 h with lithium significantly potentiates c-*fos* expression induced by the muscarinic agonist carbachol. Lithium pretreatment in these cells also potentiates c-*fos* expression in response to phorbol esters, which are known to directly activate protein kinase C (Divish *et al*, 1991). More recently, chronic lithium treatment has been found to induce the gene expression of antiapoptotic factor bcl-2 in the frontal cortex from rat (Chen *et al*, 1999; Chen and Chuang, 1999). Nonetheless, the evidence thus far accumulated on lithium-induced changes in gene expression still provides an incomplete picture of the underlying mechanism of this drug. Therefore, isolation of other genes regulated by lithium is very important, particularly if these genes are found to be involved in the development of BD.

Simultaneous large-scale analysis of gene expression has recently been enabled by the development of DNA array analysis (Marcotte *et al*, 2001; Nguyen *et al*, 2002). Recently, Bosetti *et al* (2002) described gene expression changes in rat brain in animals treated with lithium for 7 and 42 days

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Received 17 August 2003; revised 02 December 2003; accepted 11 December 2003
Published online 17 December 2003 at http://www.acnp.org/citations/Npp12170303372/default.pdf

respectively. The genes showing a change in expression were related to a variety of cell functions and all were downregulated by lithium. In this study we used the cDNA array analysis to investigate the effect of lithium on gene expression in lymphoblasts from patients with BD who have shown response to lithium treatment to further characterize the targets of this drug.

MATERIALS AND METHODS

Subjects

The investigations were carried out on a sample of 12 patients diagnosed with BD and in eight healthy control subjects. The demographic data for both groups are given in Table 1. The patients were diagnosed using Research Diagnostic Criteria, RDC (Spitzer *et al*, 1978), following Schedule for Affective Disorders and Schizophrenia—Lifetime version, SADS-L (Endicott and Spitzer, 1978), interviews. All patients also met DSM-IV criteria for BD type I or II. In addition to diagnosis of BD, they all had to meet criteria of full response to lithium prophylaxis. Patients were considered as excellent lithium responders when the following criteria A, B and C were met:

A. Diagnosis of primary episodic BD based on the SADS-L (lifetime version) interview and Research Diagnostic Criteria (RDC).
B. High recurrence risk (B1 or B2 or B3).
B1: Five or more episodes prior to lithium.
B2: Four episodes prior to lithium; of these two or more during the 2 years preceding lithium.
B3: Three episodes prior to lithium, plus one more within 12 months after lithium discontinuation.
C. Unequivocal lithium response (C1 and C2 and C3).
C1: No recurrence requiring additional biological intervention (ECT, antidepressants, neuroleptics) during the entire observation time on lithium monotherapy.
C2: Minimum period of observation of 3 years.
C3: Average plasma lithium concentration over 0.6 mEq/l.

Table 1 Demographic and clinical data

Variable	Cases	Controls
N	12	8
Males : Females	5 : 7	4 : 4
Age—range (years)	24–72	30–57
Age—mean ± SD (years)	49.8 ± 13.0	46.9 ± 9.0
Bipolar type I *vs* II	8 : 4	—
Age at onset (years)	23.0 ± 7.1	—
Lifetime manic episodes	4.9 ± 2.8	—
Lifetime depressive episodes	4.5 ± 2.0	—
Duration of lithium treatment (years)	10.2 ± 6.8	—

The criteria for determining such response have been described in previous papers (Grof *et al*, 1994; Turecki *et al*, 1998). The patients included in this study had 9.4 ± 3.8 episodes of illness before the treatment and have remained well for 10.2 ± 6.8 years on treatment with lithium alone. The control subjects were recruited from married-in members of probands' families and were interviewed using SADS-L to exclude any persons with psychiatric disorders. For cDNA array hybridization we selected two bipolar subjects, the confirmatory study with Northern blotting analysis based on the entire sample. The project was approved by the Research Ethics Committees at all institutions involved. All subjects gave written informed consent to their participation in the study.

Cell Culture and Drug Treatment

B lymphocytes from patients were transformed with Epstein–Barr virus. Transformed cells were cultured in ISCOV'S medium (GIBCO 12440-053) containing 15% of fetal bovine serum and 2 mM L-glutamine, and maintained at 37°C in the present of 5% CO_2 and 95% air in a humidified incubator. Cells from each subject were divided into two groups, and were treated with vehicle or 1 mM LiCl for 7 days.

RNA Preparation and cDNA Array Analysis

Total RNA from lymphoblasts was isolated using Trizol reagent (Gibco, MD), following the manufacturer's instruction. The concentration of RNA was determined by measuring the OD at 260 nm and the purity was determined by the ratio of 260/280. In all, 3 μg of total RNA were subjected to electrophoresis on a denaturing 1% agarose gel to check the integrity of the RNA (18S and 28S rRNA bands). Gene expression was analyzed using Atlas™ Human 1.2 Array I and II including 2400 cDNAs (Clontech Laboratories). In total, 5 μg of total RNA from vehicle- and LiCl-treated cells were treated with RNase-free DNAse I (Gibco BRL) to digest contaminating genomic DNA. RNA was then reverse transcribed using moloney murine leukemia virus reverse transcriptase in the presence of [α-^{32}P]dATP and a specific CDS primer mix (supplied by Clontech). ^{32}P-labeled cDNA probes were purified from unincorporated nucleotides and small cDNA fragments with a CHROMA SPIN-200 column (Clontech). A set of array membranes were prehybridized at 68°C for 30 min in ExpressHyb solution with salmon sperm DNA (100 μg/ml) and hybridized at 68°C overnight in the same solution with equal amounts of cDNA probes from vehicle and lithium-treated cell, respectively. Membranes were washed three times in 2 × SSC, 1% SDS for 30 min at 68°C and twice in 0.1 SSC, 0.5% SDS for 30 min at 68°C. Membranes were exposed to a Phosphor Screen and scanned using a PhosphorImage (Molecular Dynamics, Inc., Sunnyvale, CA).

Northern Blot Analysis

Total RNA was run on a 1% agarose gel containing 0.66 M formaldehyde, and applied to a nylon membrane by capillary transfer (Amersham Pharmarcia) and ultraviolet-crosslinked. Membranes were prehybridized at 68°C for

30 min in ExpressHyb hybridization buffer (CLONTECH) and hybridized at 68°C for 1 h in the same buffer with cDNA probes, according to the manufacturer's instruction (Clontech). cDNA probes from their coding region were generated by reverse transcriptase PCR and labeled with [α-^{32}P]dCTP by using a random prime labeling method (Feinberg and Vogelstein, 1983). Membranes were then washed at room temperature for 30 min in 2 × SSC, 0.1% SDS twice and at 50°C for 30 min in 0.1 × SSC, 0.1% SDS twice. Membranes were exposed overnight to a Phosphor Screen and scanned using PhosphorImage (Molecular Dynamics, Inc., Sunnyvale, CA).

Statistical Analysis

AtlasImage Software from Clontech (CA) was used to determine the signal intensities for each DNA spot. The intensity of each spot was adjusted by the global intensity normalization approach. The signal intensity was normalized by adding the values of signal for each spot over the median value of all genes on each array. Data from Northern blotting analysis were obtained by densitometric analysis of autoradiograms using ImageQuant from Molecular Dynamics (CA). Results were expressed as percent of control. Changes in gene expression after drug treatment were expressed as the mean ± SEM from separate experiments. Statistical significance of differences between means was determined by ANOVA with one within (treatment) and one grouping (cases *vs* controls) factors, and by nonparametric methods using the Mann–Whitney test for between-group comparisons, and the Wilcoxon test for within-group comparisons.

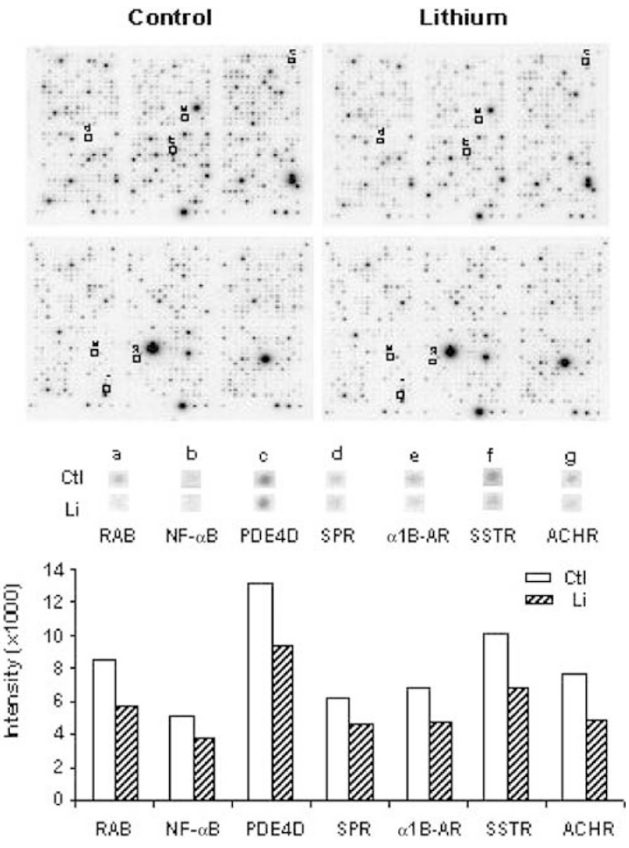

Figure 1 Differential expression of genes between control (Ctl) and lithium-(Li) treated lymphoblasts from lithium responder bipolar disorder subjects. Seven candidate lithium-regulated genes are marked in □. Lithium decreased the expression of (a) ras-related protein RAB7RAB, (b) NF-κB, (c) PDE4D, (d) SPR, (e) α1B-AR, (f) SSTR, and (g) ACHR.

RESULTS

EB virus-transformed B lymphocytes from lithium responders were chronically treated with vehicle (control) or 1 mM LiCl for 1 week. cDNA probes generated from vehicle- or lithium-treated cells were hybridized with two identical Atlas™ Human 1.2 Array I and II cDNA arrays on membranes containing in 2400 genes total, including transcription factors, ion channels, transport proteins, and receptors among others. By comparing signal intensity between vehicle- and drug-treated cells, we identified seven lithium-regulated genes. The experiment was duplicated. To eliminate false positive results, we included only genes with (1) the expression increased or decreased in duplicate samples; (2) signal ratios of lithium- to vehicle-treated cells of $\geqslant$1.3; and (3) signal intensity of $\geqslant$2000 as determined using Atlas Image software (Clontech). As shown in Figure 1, chronic lithium treatment decreased the expression of the following seven genes: alpha1B-adrenoceptor (α1B-AR), acetylcholine receptor protein alpha chain precursor (ACHR), cAMP-dependent 3′,5′-cyclic phosphodiesterase 4D (PDE4D), substance-P receptor (SPR), somatostatin receptor type 2 (SSTR2), nuclear factor kappa-B DNA binding subunit (NF-κB), and ras-related protein RAB7 (Figure 1).

Northern blotting analysis was used to verify lithium-regulated gene candidates in a larger group of lithium responders ($n = 12$). Partial cDNA sequence of α1B-AR, ACHR, PDE4D, SPR, SSTR2, NF-κB and RAB7 was used as a probe to hybridize with Northern blots containing total RNA from vehicle- and lithium-treated cells to confirm their differential expression. Hybridization signal for RAB7 showed two bands on the gel, which have been identified as high weight molecular RAB7H and low weight molecular RAB7L (Vitelli *et al*, 1996), while signal for other genes only showed single bands. We confirmed that chronic lithium treatment significantly decreased the expression of α1B-AR ($P<0.01$), PDE4D ($P<0.01$), ACHR ($P<0.01$), SPR ($P<0.05$), and RAB7H ($P<0.05$) (Figure 2). We did not confirm the effect of lithium on the expression of SSTR2, NF-κB, and RAB7L (data not shown).

To determine whether chronic lithium treatment has similar effect on the expression of α1B-AR, ACHR, PDE4D, SPR, and RAB7 in healthy controls as did in lithium responders, we measured mRNA level of these five genes in vehicle- and lithium-treated lymphoblasts from healthy controls. The results showed that the expression of none of these genes was changed by chronic lithium treatment in healthy controls (Figure 2).

Finally, we compared the expression level of these candidate genes at baseline in lymphoblasts between bipolar subjects and healthy control subjects. We found that while there was no difference of expression for ACHR, PDE4D, SPR, and RAB7, the gene for α1B-AR showed 2.5-fold higher levels of expression in bipolar subjects compared to healthy

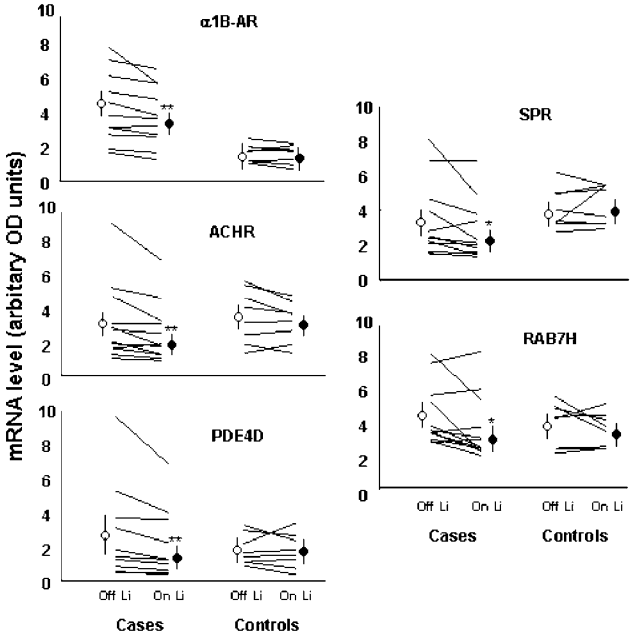

Figure 2 The effect of lithium on the gene expression of α1B-AR, ACHR, PDE4D, SPR, and RAB7H in lithium responders and healthy controls. Total RNA was isolated from lymphoblasts of lithium responders (Cases) or healthy controls (Controls) cultured in vehicle (Off Li) or 1 mM lithium (On Li) for 1 week. RNA (15 g) was analyzed by Northern blot analysis using ^{32}P-dCTP labeled probe of α1B-AR, ACHR, PDE4D, SPR, SSTR2, NF-κB, and ras-related protein RAB7. Autoradiograms were quantitated by densitometry and normalized by 28S ribosome RNA. Results are the mean ± SEM. * indicates $P<0.05$ and ** indicates $P<0.01$ when compared with vehicle-treated cells.

control subjects ($P=0.004$ Figure 2). In exploratory analyses of the α1B-AR gene, both basal- and lithium-regulated expression did not correlate with age, age of onset, lifetime number, and duration of depressive and manic episodes, or duration of lithium treatment (data not shown).

DISCUSSION

In this study, we used DNA array techniques to analyze the expression profile of 2400 genes in lymphoblasts from lithium responder bipolar subjects after chronic lithium treatment *in vitro*. We found that chronic lithium treatment decreased the expression of seven genes including α1B-AR, ACHR, PDE4D, SPR, SSTR2, NF-κB, and RAB7, and that five of these seven genes, α1B-AR, ACHR, PDE4D, SPR, and RAB7, were verified to be differentially expressed between vehicle- and lithium-treated cells by Northern blotting analysis. We also found that lithium decreased the expressions of these same five genes (α1B-AR, ACHR, PDE4D, SPR, and RAB7) in the larger sample of lithium responders, but not in healthy controls. The most interesting finding is that α1B-AR gene expression is higher in lithium responders compared with healthy control subjects. These results suggest that α1B-AR may play a role in the development of BD and the mechanism of action of lithium treatment. The study also demonstrates the utility of this novel gene screening technique for the elucidation of potential candidate genes for lithium treatment.

The α1 receptors include 1A, 1B, 1C, and 1D subtypes and play important roles in many physiological processes, such as myocardial inotropy and chronotropy, smooth muscle contraction, motor activity and motivation (Koshimizu *et al*, 2002). Stimulation of α1 receptors activates Gq/11 coupled phospholipase C, which hydrolyzes phosphatidylinositol 4,5-biphosphate (PIP_2) to yield two second messengers, diacylglycerol, and inositol 1,4,5-triphosphate (IP3), resulting in activation of protein kinase C and release of intracellular calcium (Sawaki *et al*, 1995; Parmentier *et al*, 2002). Many previous studies have indicated an important role for lithium in the regulation of IP3, protein kinase C, and calcium-mediated signaling (Kurita *et al*, 2002; Manji *et al*, 1996). Lithium inhibits myo-inositol monophosphatase, a key enzyme in inositol recycling in various tissues, which is the basis for further inositol depletion (Vadnal and Parthasarathy, 1995). It has been hypothesized that the therapeutic effect of lithium may derive from reduced agonist-induced PI hydrolysis consequent to inositol depletion by inhibition of IMPase and reduction in PI generated second messengers (Berridge, 1989; Berridge and Irvine, 1989; Berridge *et al*, 1992). This hypothesis is supported by findings of inhibition of agonist and GTP-dependent PI hydrolysis and formation of $Ins(1,4,5)P_3$ and/or $Ins(1,3,4,5)P_4$ in rat brain by several groups (Whitworth and Kendall, 1988; Kennedy *et al*, 1989; Godfrey *et al*, 1989; Song and Jope, 1992). Reduction of α1B-AR expression by lithium, which may further result in reduction of PI generated second messengers, suggests that lithium may regulate PI-generated second messengers by not only directly inhibiting IMPase but also by inhibiting α1B-AR expression. Protein kinase C is also a target of lithium treatment. Chronic lithium treatment has been shown to decrease membrane levels of the PKC α isozyme and attenuated protein kinase C-mediated responses, such as phosphorylation of the prominent PKC substrate MARCKS in rat hippocampus (Anderson *et al*, 1988). In addition, Dubovsky *et al* (1991) reported that lithium treatment significantly reduced resting and stimulated intracellular-free calcium in platelets of BD patients. The α1 receptors have also been found to activate other signal transduction systems, such as the mitogen activated protein kinase and cAMP pathways (Michelotti *et al*, 2000; Piascik and Perez, 2001). Much research has demonstrated that lithium treatment affected cAMP pathways (Bezchlibnyk and Young, 2002). Together with our finding that lithium decreased α1B-AR expression, this suggests that the α1B-AR coupled signaling may be a pathway with multiple targets for lithium.

The α1B-AR gene showed a significantly higher level of expression in patients compared to controls as well as more prominent effect of lithium. This makes the gene an interesting potential target for further study in patients with BD. The receptor and its gene, however, have not been studied in depression or BD before, and relatively little is known about their role in mood regulation. The receptor is present in a variety of tissues including high levels of expression in the cerebral cortex. Its density can be influenced by drugs with a known effect on mood, such as reserpine or desipramine, producing up- and downregulation, respectively (Civantos Calzada and De Artinano, 2001). α1B-AR is recognized to be involved in adrenoceptor-

mediated active behavior, which has been used as an indicator in studies of movement and mood disorders. Stone *et al* found that inhibition of α1B-AR blocked cage changing-induced gross and small movement in mice. α1B-AR knockout mice also showed decreased exploratory activity and fear-motivated behavior (Knauber and Muller, 2000; Stone *et al*, 2001). The gene for alpha 1B receptor maps to 5q33, a region investigated with suggestive results in several linkage studies of BD (Shink *et al*, 2002). It is interesting that α1B-AR expression is higher in bipolar patients than controls, but decreased by lithium in our study. These findings suggest that α1B-AR may not only be involved in pathological development but also may be a potential target for lithium treatment.

The second interesting finding in this study is the inhibitory effect of lithium on the expression of the phosphodiesterase (PDE 4D) gene. The enzyme is important for cAMP signaling, which may be particularly relevant for the mechanism of action of lithium. Lithium has multiple effects on cAMP signaling including inhibition of adenylyl cyclase activity—an effect that we were able to confirm in the same cell lines as those used in the present study (Sun *et al*, 2002). Inhibition of PDE activity has been shown to produce an antidepressant effect (Wachtel, 1983; Fujimaki *et al*, 2000; Zhang *et al*, 2002). Furthermore, an administration of a PDE4 inhibitor rolipram has been found to potentiate antidepressant-induced BDNF expression and neurogenesis in rat hippocampus (Fujimaki *et al*, 2000).

Studies in cultured lymphoblasts from BD patients have certain advantages: lymphocytes are easily obtained from the subjects; studies can be performed with lymphocyte from live patients from whom response to lithium treatment can be isolated; we can perform comparison between before and after drug treatment with cultured lymphocytes in the same subject. A number of mood stabilizer and mood disorder studies have been published on lymphocytes and platelets, but the study with lymphocytes must be interpreted with caution. The biological response may be different in EBV transformed cells when compared with untransformed cells, and peripheral lymphocyte may not fully represent changes that occur in regionalized brain tissue. However, a number of studies have been published on transformed lymphoblasts from BD patients, and similar biological responses as in these cells are also found in CNS (Manji *et al*, 1995a, b; Schreiber *et al*, 1991; Young *et al*, 1991, 1993, 1994).

In conclusion, using DNA array analysis we identified a number of lithium responsive genes including α1B-AR in lymphocytes from BD subjects. Since previous studies have shown that lithium regulates IP3 and protein kinase C signal transduction pathways, the regulation of α1B-AR expression by lithium suggests that this drug may have multiple targets for α1-AR and coupled downstream signaling pathways.

α1B-AR is highly expressed in BD subjects, but its expression is decreased by lithium treatment, indicating that α1B-AR may play an important role in the action of lithium treatment.

ACKNOWLEDGEMENTS

This work is supported by a grant from Canadian Institutes of Health Research (MA and LTY), and by Independent Investigator Award from the National Alliance for Research in Schizophrenia and Depression (MA).

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