Long Term Lithium Treatment Suppresses p53 and Bax Expression but Increases Bcl-2 Expression

A PROMINENT ROLE IN NEUROPROTECTION AGAINST EXCITOTOXICITY*

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This study was undertaken to investigate the molecular mechanisms underlying the neuroprotective actions of lithium against glutamate excitotoxicity with a focus on the role of proapoptotic and antiapoptotic genes. Long term, but not acute, treatment of cultured cerebellar granule cells with LiCl induces a concentration-dependent decrease in mRNA and protein levels of proapoptotic p53 and Bax; conversely, mRNA and protein levels of cytoprotective Bcl-2 are remarkably increased. The ratios of Bcl-2/Bax protein levels increase by approximately 5-fold after lithium treatment for 5–7 days. Exposure of cerebellar granule cells to glutamate induces a rapid increase in p53 and Bax mRNA and protein levels with no apparent effect on Bcl-2 expression. Pretreatment with LiCl for 7 days prevents glutamate-induced increase in p53 and Bax expression and maintains Bcl-2 in an elevated state. Glutamate exposure also triggers the release of cytochrome c from the mitochondria into the cytosol. Lithium pretreatment blocks glutamate-induced cytochrome c release and cleavage of lamin B1, a nuclear substrate for caspase-3. These results strongly suggest that lithium-induced Bcl-2 up-regulation and p53 and Bax down-regulation play a prominent role in neuroprotection against excitotoxicity. Our results further suggest that lithium, in addition to its use in the treatment of bipolar depressive illness, may have an expanded use in the intervention of neurodegeneration.

For almost half a century, lithium has been the most widely used treatment for bipolar depressive illness, although its therapeutic mechanism of action remains obscure. Among the many biochemical actions of long term lithium treatment in vivo and in vitro are its ability to inhibit phosphoinositide turnover and stimulated levels of calcium, influence signal transduction by modulating G-proteins and protein kinase C, and regulate gene expression involving AP-1 DNA binding activity (1–7). Increasing evidence supports the notion that lithium has neuroprotective effects. We have recently reported that long term treatment of cultured neurons from the central nervous system with therapeutically relevant concentrations of lithium robustly protects against apoptotic cell death associated with glutamate excitotoxicity via N-methyl-D-aspartate (NMDA)1 receptors (8). In cerebellar granule cells (CGCs), the protection afforded by lithium is accompanied by an inhibition of NMDA receptor-mediated calcium influx that is independent of a loss of receptor binding activity or subunit expression (8). In a rat model of ischemia that involves glutamate excitotoxicity, chronic lithium administration was found to markedly reduce brain infarction and neurological deficits induced by occlusion of the middle cerebral artery (9).

In an attempt to elucidate the molecular mechanisms underlying lithium-induced neuroprotection in cultured CGCs, we examined the long term effects of lithium on the expression of key genes involved in proapoptosis and cytoprotection, notably p53, Bax, and Bcl-2. p53, a nuclear protein that binds to specific DNA sequences and functions as a transcriptional activator, promotes the expression of the proapoptotic gene, Bax, but suppresses the expression of the antiapoptotic gene, Bcl-2 (10). Bax has been shown to bind to mitochondrial membranes and thereby to cause the release of cytochrome c, which in turn leads to activation of caspases and degradation of specific protein substrates (11–13). The antiapoptotic action of Bcl-2 is mediated, at least in part, by blocking Bax-induced cytochrome c release and caspase activation (14–16). Thus, it has been proposed that the relative amounts of Bcl-2 and Bax determine the fate of a living cell (17, 18). In this study, we determined the levels of these proapoptotic and antiapoptotic gene products in CGCs pretreated with lithium and/or exposed to glutamate.

EXPERIMENTAL PROCEDURES

Primary Cultures of CGCs and Treatment Conditions—CGCs were prepared from 8-day-old Sprague-Dawley rat pups as described previously (8). Cultures were routinely pretreated with different concentrations (0.5–5 mM) of LiCl for 7 days, starting from the first day in vitro. Glutamate (50–100 μM) was added to the cultures to trigger neuronal apoptosis. Cell viability was determined by measuring the mitochondrial dehydrogenase activity that cleaves 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (8).

Northern Blotting—Total RNA was extracted from CGCs with guanidine thiocyanate and isolated by cesium chloride gradient centrifugation as described previously (19). After electrophoresis on 1% agarose gel containing formaldehyde, RNA was transferred to a Duralose membrane (Stratagene, La Jolla, CA) and hybridized with cDNA probes for p53, Bcl-2, and Bax cDNA probes, separately. The cDNA probes were labeled with [α-32P]dCTP by the random priming method. Hybridizations were performed at 42 °C for 16 h, followed by two washes at room temperature with 2 × SSC/0.1% SDS and then two additional washes at 50 °C in 0.1 × SSC/0.1% SDS. Northern blots were quantified using a Betascope model 603 Blot Analyzer (Betagen Corp., Waltham, MA).

Western Blotting—CGCs were detached by scraping and sonicated for 30 s in lysis buffer containing 10 mM Tris·HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.28 unit/ml aprotinin, 50 μg/ml leupeptin, 1 μM benzamidine, and 7 μg/ml pepstatin A. Proteins were separated by 12% SDS-polyacrylamide gel electrophoresis and then transferred to an Immobilon-P membrane (Millipore, Bedford, MA). Blots were incubated with antibodies against p53 (Phab240), Bcl-2 (N-19), Bax (P-19) (Santa Cruz Biotechnology, Santa Cruz, CA), and lamin B1 (Serotec LTD, Oxford, UK) and then probed with a horseradish peroxidase-conjugated second antibody. Detection was performed by

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1 The abbreviations used are: NMDA, N-methyl-D-aspartate; CGC, cerebellar granule cell.
Lithium Regulates p53, Bax, and Bcl-2 Expression in Neurons

RESULTS

Treatment of CGCs with LiCl induces a concentration-dependent decrease in the levels of p53 mRNA and protein determined by Northern and Western blotting, respectively (Fig. 1, a and b). These effects were detected at therapeutic concentrations of lithium (0.5–1.0 mM), although a more robust decrease was observed at higher concentrations. The lithium (3 mM)-induced decrease in p53 mRNA and protein levels was time-dependent (Fig. 1, c and d). Loss of p53 was observed after treatment for 1 day and reached a maximum after 5–7 days. Similar to the effects on p53, LiCl treatment induced a dose- and time-dependent decrease in Bax mRNA and protein levels (Fig. 2). In contrast, Bcl-2 mRNA and protein levels were markedly increased by LiCl treatment (Fig. 3). The effect was also dose- and time-dependent; 3 days or longer was necessary to observe a significant increase.

In contrast, exposure to 100 μM glutamate for 4 or 8 h markedly increased the mRNA levels of p53 and Bax but had little or no effect on Bcl-2 mRNA (Fig. 4, a, c, and e). Pretreatment with LiCl (3 mM) for 7 days abolished glutamate-induced increase in p53 and Bax mRNA levels, while maintaining elevated levels of Bcl-2 mRNA even in cells exposed to glutamate. Similar effects on protein levels of p53, Bax, and Bcl-2 were observed in CGCs exposed to 50 or 100 μM glutamate for 24 h in the absence or presence of lithium pretreatment (Fig. 4, b, d, and f). Lithium treatment for 5–7 days was found to cause a 5-fold increase in the Bcl-2/Bax protein ratio (Fig. 4g). Conversely, the Bcl-2/Bax ratio was decreased with glutamate exposure for 24 h, and this decrease was prevented by lithium pretreatment for 7 days. Under these experimental conditions, lithium pretreatment effectively protected cells from excitotoxicity (Fig. 4h). It should be mentioned that the effects of glutamate on cell viability and levels of p53, Bax, and Bcl-2 were completely blocked by MK-801 (10 μM), a selective NMDA receptor antagonist (data not shown), confirming our previous report that glutamate-induced apoptosis is entirely mediated...
were pretreated with 3 mM LiCl for 7 days, starting at the first day of treatment. Cells were harvested to determine specific mRNA levels (a–e). Protein levels were determined after 24 h of treatment with 50 or 100 μM glutamate (b, d, and f). Northern or Western blots at the top of each panel are from a typical experiment. Quantified results are the means ± S.E. from three independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001, compared at 12 h after glutamate exposure in a, c, and e and with 50 or 100 μM glutamate in b, d, and f–h.

by NMDA receptors (8, 20).

Because Bax and Bcl-2 modulate the release of cytochrome c from mitochondria (11, 14, 15), we studied the effects of glutamate and lithium on cytochrome c protein levels in the cytosolic and mitochondrial fractions. Glutamate exposure for 6 or 12 h caused a time-dependent increase in the levels of cytochrome c in the cytosol, with a concomitant decrease of cytochrome c protein levels in the mitochondrial fraction (Fig. 5a and b). Lithium pretreatment completely antagonized glutamate-induced changes in cytochrome c levels in both subcellular fractions. Additionally, lithium pretreatment suppressed the degradation of lamin B1 (Fig. 5c), a nuclear membrane protein and a substrate of caspase-3. This effect is consistent with the finding that NMDA receptor-mediated excitotoxicity in CGCs involves the activation of caspase-3 (21).

**DISCUSSION**

Bcl-2 family plays a prominent antiapoptotic role by acting upstream of caspase activation. Specifically, Bcl-2 may inhibit the Bax-mediated release of cytochrome c or AIF (apoptosis-inducing factor) from mitochondria, thereby preventing caspase activation and cell death (14, 22). In addition, Bcl-2 may physically interact with Apaf-1 (apoptotic protease-activating factor-1) to prevent Apaf-1-mediated caspase activation (23, 24). Lithium-induced neuroprotection against glutamate excitotoxicity is likely mediated in part by Bcl-2 up-regulation, whereas glutamate-induced apoptosis involves the overexpression of p53 and Bax. In support of the role of p53 in mediating glutamate excitotoxicity, we found that cultured CGCs derived from p53-deficient mice are resistant to the glutamate insult. We also found that glutamate triggers the release of cytochrome c from mitochondria and the cleavage of lamin B1, a nuclear membrane and substrate of caspase-3. Moreover, these glutamate-induced effects are almost completely blocked by long term lithium pretreatment.

Other actions of Bcl-2 may also contribute to lithium-induced neuroprotection against excitotoxicity. For example, Bcl-2 down-regulates the activity of transcription factor NF-κB induced during apoptosis (25) and inhibits abnormal release of calcium from the endoplasmic reticulum, thus reducing calcium loading to mitochondria and subsequent oxidative stress (26, 27). Bcl-2 overexpression could also be related to the lithium protection against apoptosis induced by anticonvulsants (carbamazepine and phenytoin) in CGCs (28) and by brain damage resulting from focal ischemia in rats (9).

This study shows for the first time that lithium influences the expression of p53 and some Bcl-2 family members. Moreover, we show that these changes in gene expression are associated with neuronal survival. In this context, Bcl-2 levels appear to be increased in the frontal cortex of rats chronically treated with lithium and valproate, another drug used to treat manic depressive illness. It is well established that p53 positively regulates Bax but negatively regulates Bcl-2 expression (19, 20). Thus, lithium-elicited changes in the mRNA and protein levels of Bax and Bcl-2 are likely the results of the inhibition of p53 expression. Consistent with this possibility, we found that only 1 day of lithium treatment is needed to observe a decrease in p53 levels but 3 days are needed to observe a change in Bax and Bcl-2 levels. However, it is also possible that lithium has a direct regulatory role in the expression of Bcl-2 and/or Bax.

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Lithium has been shown to increase basal transcription factor binding to AP-1 and CRE sites (6, 7) but decreases stimulated binding to the AP-1 site in cultured cells and the brain (5). These lithium-induced changes in transcription factor binding may be related to the ability of lithium to inhibit the activity of glycogen synthase kinase (29), which phosphorylates transcription factors such as c-Jun, causing a loss of its DNA binding activity (30). Lithium modulation of transcription factor DNA binding activity may underlie the effects of this drug on the expression of genes involved in apoptosis. Regardless of the mechanisms involved in lithium-induced gene expression, our results suggest that lithium, in addition to treating bipolar disorder, may also be useful in the treatment of neurodegenerative diseases, particularly those involving glutamate excitotoxicity.

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