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Lithium Inhibits Ceramide- and Etoposide-Induced Protein Phosphatase 2A Methylation, Bcl-2 Dephosphorylation, Caspase-2 Activation, and Apoptosis

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ABSTRACT

Lithium confers cell protection against stress and toxic stimuli. Although lithium inhibits a number of enzymes, the antiapoptotic mechanisms of lithium remain unresolved. Here, we report a novel role of lithium on the blockage of ceramide- and etoposide-induced apoptosis via inhibition of protein phosphatase 2A (PP2A) activity. Overexpression of PP2A resulted in caspase-2 activation, mitochondrial damage, and cell apoptosis that were inhibited by okadaic acid (OA) and lithium. Lithium and OA abrogated ceramide- and etoposide-induced Bcl-2 dephosphorylation at serine 70. Furthermore, ceramide- and etoposide-induced PP2A activation involved methylation of PP2A C subunit, which lithium suppressed. Lithium caused dissociation of PP2A B subunit from the PP2A core enzyme, whereas ceramide caused recruitment of the B subunit. Taken together, lithium exhibited an antiapoptotic effect by inhibiting Bcl-2 dephosphorylation and caspase-2 activation, which involved, at least in part, a mechanism of down-regulating PP2A methylation and PP2A activity.

Lithium has been used for the treatment of bipolar disorders for more than 50 years (Quiroz et al., 2004). Although the mechanisms underlying its actions remain poorly understood, the neuroprotective effects of lithium by promoting cell activation and survival have been proposed. Lithium protected against glutamate-induced neuron damage involving decreased expression of p53 and Bax, enhanced expression of Bcl-2, and activation of Akt (Chalecka-Franaszek and Chuang, 1999; Chen and Chuang, 1999). The neuroprotective actions of lithium have been extended to other apoptotic stimuli, such as ceramide (Mora et al., 2002), staurosporine, and heat shock (Bijur et al., 2000). The antiapoptotic roles of lithium are not fully defined.

Apoptotic cell death induced by ceramide and etoposide has been largely reported (Boesen-de Cock et al., 1999; Tepper et al., 1999; Chmura et al., 2000; Sawada et al., 2000; Caricchio et al., 2002; Kalli et al., 2003; Ogretmen and Hannun, 2004). Ceramide, a product of sphingolipid metabolism, regulates diverse cellular responses in various cell types. Ceramide acts as a second messenger in response to diverse apoptotic stimuli, which activate both protein kinases, such as c-Jun NH_2-terminal kinase, PKCζ, and kinase suppressors of Ras and protein phosphatases, such as protein phosphatase 2A (PP2A) and protein phosphatase 1 (PP1) (Mathias et al., 1998; Hannun and Luberto, 2000; Hreniuk et al., 2001; Caricchio et al., 2002; Ruvolo, 2003). Up-regulation of the endogenous ceramide level induced by etoposide has been demonstrated (Boesen-de Cock et al., 1999; Tepper et al., 1999; Sawada et al., 2000). Ceramide-induced apoptosis has been associated with PP2A activation that in turn causes dephosphorylation of PKCα (Lee et al., 2000), Akt (Schubert et al., 2000), and Bcl-2 (Ruvolo et al., 1999, 2002). Ceramide caused Bcl-2 dysfunction through its dephosphorylation at serine 70 mediated by PP2A (Ruvolo et al., 1999). It is noteworthy that our recent study (Lin et al., 2005) also showed that ceramide- and etoposide-induced mitochondrial damage was initiated

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ABBREVIATIONS: PP2A, protein phosphatase 2A; OA, okadaic acid; DAPI, 4′,6-diamidino-2-phenylindole; PI, propidium iodide; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; OD, optical density; PKC, protein kinase C; pNPP, *p*-nitrophenyl phosphate; $\Delta\Psi_m$, mitochondrial transmembrane potential.

by caspase-2 activation, caspase-2 was regulated by Bcl-2, and Bcl-2 was, at least in part, regulated by PP2A.

PP2A is composed of a dimeric core enzyme, including a 65-kDa scaffolding A subunit and a 36-kDa catalytic C subunit, and variable regulatory B subunits (Zolnierowicz, 2000). The C subunit of PP2A reversibly undergoes methylation on its C terminus (Lee and Stock, 1993; Favre et al., 1994; Xie and Clarke, 1994), which regulates the binding of B regulatory subunits and PP2A phosphatase activity (Bryant et al., 1999). Different B subunits confer different properties of PP2A in dephosphorylating downstream substrates, by which PP2A mediates distinct cellular functions (Kamibayashi et al., 1994; Zhao et al., 1997). Ceramide can activate PP2A; it seemed that ceramide was more effective in activation of heterotrimeric PP2A with the presence of a B subunit compared with that of AC heterodimeric subunits (Dobrowsky et al., 1993). Ceramide induced cerebellar granule cell apoptosis through Akt inactivation and glycogen synthase kinase-3β activation (Stoica et al., 2003). In contrast, lithium blocked Akt and glycogen synthase kinase-3 dephosphorylation induced by ceramide through PP2A (Mora et al., 2002).

In the present study, we demonstrated the blockage of ceramide- and etoposide-induced mitochondrial apoptosis by lithium through inhibiting PP2A methylation and activity, Bcl-2 dephosphorylation, and caspase-2 and -8 activation. The inhibition of ceramide- and etoposide-activated PP2A by lithium may occur by an indirect regulation of PP2A C subunit demethylation. Furthermore, lithium caused dissociation of the B subunit from heterotrimeric PP2A.

Materials and Methods

Materials. Ceramide analog C2-ceramide, etoposide, LiCl, PP2A inhibitor OA, and purified PP2A ($PP2A_1$ composed of the A, B, and C subunits) were purchased from Sigma (St. Louis, MO). Ceramide, etoposide, and OA were dissolved in dimethyl sulfoxide.

Cell Culture. The mouse T hybridoma cell 10I was kindly provided by Dr. M. Z. Lai (Institute of Molecular Biology, Academia Sinica, Taiwan). Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin, and 0.05 mg/ml streptomycin. They were maintained at 37°C in 5% CO_2. Cells were washed with serum-free RPMI 1640 and resuspended in hybridoma serum-free medium (Invitrogen, Carlsbad, CA) before experiments.

Analysis of Cell Apoptosis. Cells were fixed with 70% ethanol in phosphate-buffered saline (PBS) for propidium iodide (PI; Sigma) staining and then were analyzed using flow cytometry (FACScan; BD Biosciences, San Jose, CA). 4′,6-Diamidino-2-phenylindole (DAPI; Sigma) was also used for apoptotic cell staining in 5 μg/ml for 30 min at room temperature and was followed by microscopic detection.

Mitochondrial Functional Assay. The reduction of mitochondrial transmembrane potential ($\Delta\Psi_m$) value was determined using rhodamine 123 (Sigma). Cells were incubated with 5 μM rhodamine 123 in PBS at 37°C for 1 h. After being washed with PBS, cells were analyzed using flow cytometry (FACSCalibur; BD Biosciences). The activity of mitochondrial dehydrogenase was determined using a WST-8 assay kit (Dojindo Laboratories, Kumamoto, Japan).

Detection of Caspase Activation. Cellular caspase activation was determined using the ApoAlert caspase colorimetric assay kits (Clontech, Mountain View, CA) for caspase-3 and -8 and an ApoAlert caspase fluorescent assay kit for caspase-9 all according to the manufacturer's instructions. Caspase-2 activity was detected using a caspase-2 assay kit (Calbiochem, San Diego, CA). Optical density (OD) measurements were made using a microplate reader (Molecular Devices, Sunnyvale, CA), and the substrate activities shown as *p*-nitroanilide (in nanomoles) were calculated for caspase-3 and -9. For caspase-2, -3, and -8, the relative substrate activity was shown by the OD values.

Western Blot Analysis. To detect cytochrome *c* release, cytosolic extract without the mitochondrial fraction was separated using an ApoAlert cell fractionation kit (Clontech) according to the manufacturer's instructions. To detect other proteins, total cell lysate was used. Cells were lysed using a Triton X-100-based lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.5, 5 mM EDTA, 5 mM NaN_3, 10 mM NaF, and 10 mM sodium pyrophosphate) with a protease inhibitor mix and a phosphatase inhibitors cocktail I (Sigma). Cell extract was separated using SDS-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA). After blocking, blots were developed with a series of antibodies as indicated. Rabbit antibodies specific for cytochrome *c*, Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-Bcl-2 serine 70 (Cell Signaling Technology, Beverly, MA), and monoclonal antibodies against PP2A/C, methyl-PP2A, demethylated-PP2A, and the B subunit of PP2A (Upstate Biotechnology, Lake Placid, NY) were used. Mouse antibodies specific for β-actin and α-tubulin (Santa Cruz Biotechnology) were used for internal control. Finally, blots were hybridized with horseradish peroxidase-conjugated goat antirabbit IgG or anti-mouse IgG (Calbiochem) and developed using an AEC substrate kit (Zymed Laboratories Inc., South San Francisco, CA).

Protein Transfection. A Pro-Ject protein transfection reagent kit (Pierce, Rockford, IL) was used to transfect constitutively active PP2A according to the manufacturer's instructions. Pro-Ject reagent was dissolved in 250 μl of methanol and aliquoted in 5 μl and allowed to air dry for 5 h in the laminar flow hood. The Pro-Ject reagent was hydrated with the diluted purified-PP2A (0.02 μg) solution. This mixture was incubated for 5 min at room temperature and then vortexed gently for 5 s at low speed. 10I cells (1.6×10^6/100 μl in serum-free RPMI 1640) were added and incubated for 6 h with or without lithium and OA. Fluorescein isothiocyanate (FITC)-conjugated antibodies were used for the transfection rate, and results showed 97.3% of FITC-positive cells. Cells treated with Pro-Ject reagent were used for mock transfection control.

Immunostaining. For intracellular immunostaining, cells were fixed with 1% formaldehyde in PBS for 10 min at room temperature. After the cells had been washed, a series of antibodies was used as indicated, followed by FITC-conjugated goat anti-mouse and anti-rabbit IgG (Calbiochem) staining. Mouse anti-demethylated PP2A (Upstate Biotechnology) was used for flow cytometric analysis. For confocal microscopy, rabbit anti-phospho-Bcl-2 serine 70 (Cell Signaling Technology), mouse anti-methyl-PP2A, and anti-demethylated PP2A (Upstate Biotechnology) were used. Images were processed and analyzed using MetaMorph software (Universal Imaging Corporation, Downingtown, PA). Mito Tracker Red CMXRos (Invitrogen) was used for mitochondrial staining.

Immunoprecipitation. PP2A and Bcl-2 were immunoprecipitated using protein A-Sepharose with anti-PP2A antibody (Upstate Biotechnology) and anti-Bcl-2 (Santa Cruz Biotechnology), respectively, in phosphatase assay buffer (20 mM imidazole-HCl, 2 mM EDTA, 2 mM EGTA, pH 7.0, and protease inhibitors) for 2 h at 4°C. The beads were washed three times with phosphatase assay buffer and once with pNPP serine/threonine assay buffer (50 mM Tris-HCl, pH 7.0, 100 nM $CaCl_2$; Upstate Biotechnology). PP2A-bound beads were incubated with Bcl-2-bound beads in pNPP serine/threonine assay buffer for 30 min at 30°C. Reactions were terminated with Laemmli sample buffer at 100°C. Proteins were separated using SDS-polyacrylamide gel electrophoresis and Western blot analysis with antibodies specific for phospho-Bcl-2 serine 70 and Bcl-2.

PP2A Activity Assay and Cell-Free in Vitro Phosphatase Assay. A serine/threonine phosphatase assay kit (Upstate Biotechnology) was used to detect PP2A activity according to the manufac-

turer's instructions. In brief, PP2A was immunoprecipitated with monoclonal anti-PP2A antibody and protein A-Sepharose beads. PP2A-bound beads were washed with phosphatase assay buffer and then with pNPP serine/threonine assay buffer. Diluted phosphopeptide in serine/threonine assay buffer (250 μM) was added and then incubated for 5 min at 30°C. After centrifugation, 25 μl of supernatant was transferred to an assay plate, and 100 μl of Malachite Green phosphate detection solution was added for 15 min incubation at 30°C. The relative absorbance was measured at 650 nm.

For an in vitro PP2A inhibition assay, phosphopeptide K-R-pT-I-R-R (Upstate Biotechnology) was used. Cell extracts and immunoprecipitated PP2A were prepared in phosphatase assay buffer and incubated on ice with OA, lithium, or protein phosphatase inhibitor cocktail I (Sigma) for 10 min. The mixtures were incubated at 30°C for 30 min. Reactions were terminated and developed with Malachite Green phosphate detection solution (Upstate Biotechnology) according to the manufacturer's instructions.

Results

Lithium Rescues Ceramide- and Etoposide-Induced PP2A-Mediated Mitochondrial Apoptosis. In the present study, the molecular mechanisms of lithium against apoptotic signaling of ceramide and etoposide were investigated. We first sought to determine whether lithium rescues T cells from ceramide- and etoposide-induced apoptosis. In the presence of various doses of LiCl, 10I T cells were treated with C2-ceramide, and results showed that lithium dose-dependently blocked ceramide-induced cell apoptosis (Fig. 1a). Our previous study (Lin et al., 2004) using 10I and Jurkat T cells showed the sequential events of initiator caspase-2 and -8 activation, mitochondria damage followed by downstream caspase-9 and -3 activation, and cell apoptosis after ceramide and etoposide induction. Using lipophilic cationic fluorochrome rhodamine 123 staining, we found that lithium inhibited ceramide-induced mitochondrial transmembrane potential ($\Delta\Psi_m$) reduction in 10I cells (Fig. 1a, left). Likewise, the inhibitory effect of lithium on ceramide-induced reduction of mitochondrial dehydrogenase activity was shown using a WST-8 assay (Fig. 1a, left). Furthermore, lithium dose-dependently blocked both the caspase-9 and -3 activities detected using caspase activity assay kits (Fig. 1a, right), and the processing of procaspase to active form was detected using Western blotting (data not shown). Mora et al. (2002) hypothesized that the neuroprotective action of lithium was due to the inhibition of PP2A activation stimulated by ceramide. We therefore examined whether treatment with PP2A inhibitor OA inhibited ceramide-induced apoptosis.

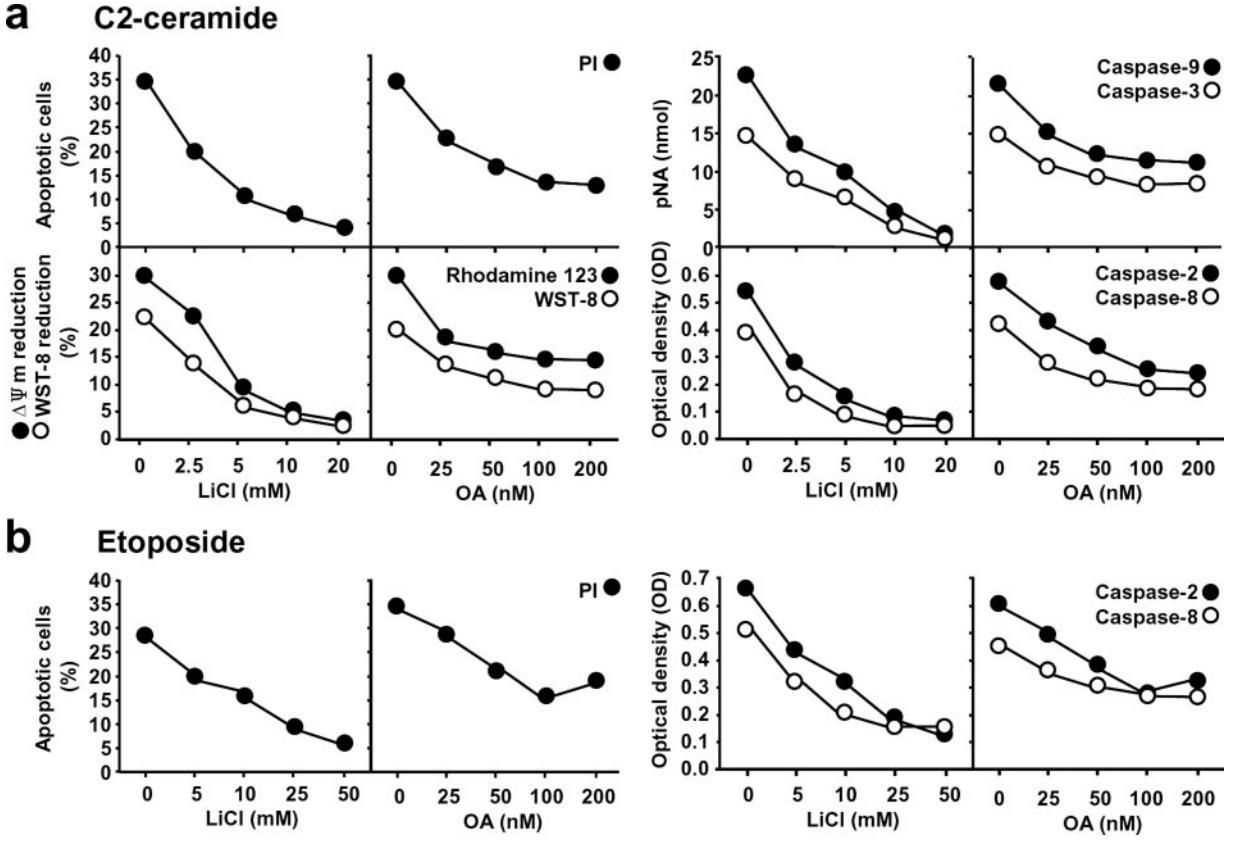

Fig. 1. Lithium and okadaic acid inhibit ceramide- and etoposide-induced caspase activation and mitochondrial apoptosis. 10I cells were treated with C2-ceramide (40 μM) or etoposide (100 μM) plus the indicated doses of LiCl or PP2A inhibitor OA for 6 h. a, the effects of LiCl and OA on ceramide-induced mitochondrial intrinsic pathways of apoptosis. Apoptotic cells were detected using PI staining and then flow cytometric analysis (left top). Mitochondrial disruption was detected in apoptotic cells using transmembrane potential reduction ($\Delta\Psi_m$), and mitochondrial dehydrogenase activity was detected using rhodamine 123 staining and a WST-8 activity assay kit. The percentages of $\Delta\Psi_m$ (●) and WST-8 (○) reduction are shown (bottom, left). The activities of caspase-9 and -3 were determined using caspase activity assay kits with specific substrates. The concentrations of substrate cleaved by active caspase-9 (●) and -3 (○) are shown (top, right). pNA, *p*-nitroanilide. Using specific substrate digestion for the caspase activity assay, the relative activities of caspase-2 (●) and -8 (○) are shown using OD (bottom, right). Data are means of duplicate cultures. b, the effects of LiCl and OA on etoposide-induced cell apoptosis (left) and caspase-2 and -8 activation (right) were examined using PI staining and caspase activity assays, respectively. The percentages of apoptotic cells and the activities of caspase-2 (●) and -8 (○) are shown using OD. Data are means of duplicate cultures.

Results showed that, like lithium, OA inhibited ceramide-induced mitochondrial damage, caspase-9 and -3 activation, and apoptosis (Fig. 1a). OA was not as powerfully inhibitory as lithium, suggesting that inhibiting PP2A activation may represent one of several mechanisms underlying lithium-mediated antiapoptotic effects. Similar results were obtained using etoposide to induce apoptosis (Fig. 1b).

To investigate the signaling events leading to mitochondrial damage, we detected the activation of caspase-2 and -8 in the presence of lithium or OA. Ceramide- and etoposide-induced caspase-2 and -8 activation was reduced after pretreatment with lithium or OA (Fig. 1). This is the first demonstration showing the antiapoptotic role of lithium against mitochondrial apoptosis by inhibiting initiator caspase activation. Furthermore, these data also suggest that activating caspase-2 and -8 requires PP2A activity. To further confirm that lithium and OA both acted upstream of mitochondria, 10I cells were treated with lithium or OA at different time points. We found that treatment with lithium or OA 0, 1, and 2 h, but not 4 h, after ceramide stimulation inhibited cell apoptosis and $\Delta\Psi_m$ reduction (Fig. 2, top). Lithium and OA, when added 2 h after ceramide stimulation, also blocked caspase-2 and -8 activation and the release of cytochrome *c* (Fig. 2, middle and bottom). Taken together, lithium and OA inhibit caspase-2 and -8 activation upstream of mitochondrial damage.

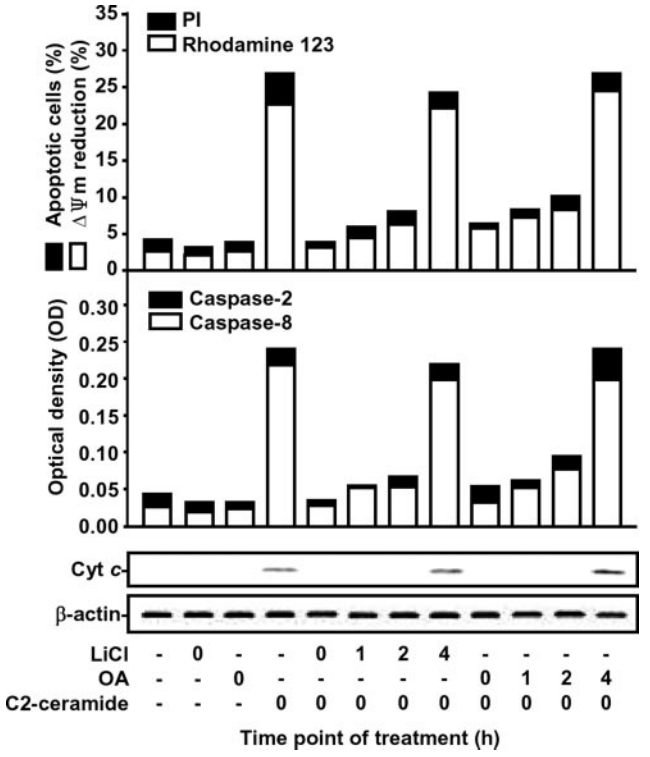

Fig. 2. Time kinetics of lithium- and okadaic acid-mediated inhibition on ceramide-induced initiator caspase activation and mitochondrial apoptosis. 10I cells were treated with C2-ceramide (40 μM); LiCl (20 mM) or OA (100 nM) was added at various time points as indicated. Cell apoptosis and $\Delta\Psi_m$ reduction were determined using PI and rhodamine 123 staining, respectively, and then flow cytometric analysis. The percentages of apoptotic cells (■) and $\Delta\Psi_m$ reduction (□) are shown (top; data are means of duplicate cultures). The activities of caspase-2 (■) and -8 (□) were determined using caspase activity assays with specific substrates (middle; data are means of duplicate cultures). The expression of cytosolic cytochrome *c* released from mitochondria was determined using Western blotting (bottom). The expression of β-actin was an internal control.

Because lithium and OA inhibit ceramide-induced caspase-2 and -8 activation and mitochondrial apoptosis, PP2A might contribute to regulating caspase-2 activation before ceramide-induced mitochondrial damage. The anti-apoptotic effect of lithium against ceramide may target PP2A-regulated caspase-2 activation.

The regulation of caspase-2 by PP2A was further investigated. Cells transfected with PP2A holoenzyme without ceramide stimulation showed apoptotic characteristics with nuclear and DNA fragmentation using DAPI and PI staining, respectively (Fig. 3). The transfection efficiency was 97.3% in this representative experiment. Results showed that PP2A caused cell apoptosis (47.1%), which was partially reduced by lithium (15.7%) and almost completely inhibited by OA (9.5%) compared with the control group (7.9%) (Fig. 3a). A similar pattern was evident in the inhibitory effects of lithium and OA on PP2A-induced $\Delta\Psi_m$ reduction (Fig. 3a, right) and caspase-2 and -3 activation (Fig. 3b). These results indicated that the blockage of PP2A-mediated caspase-2 activa-

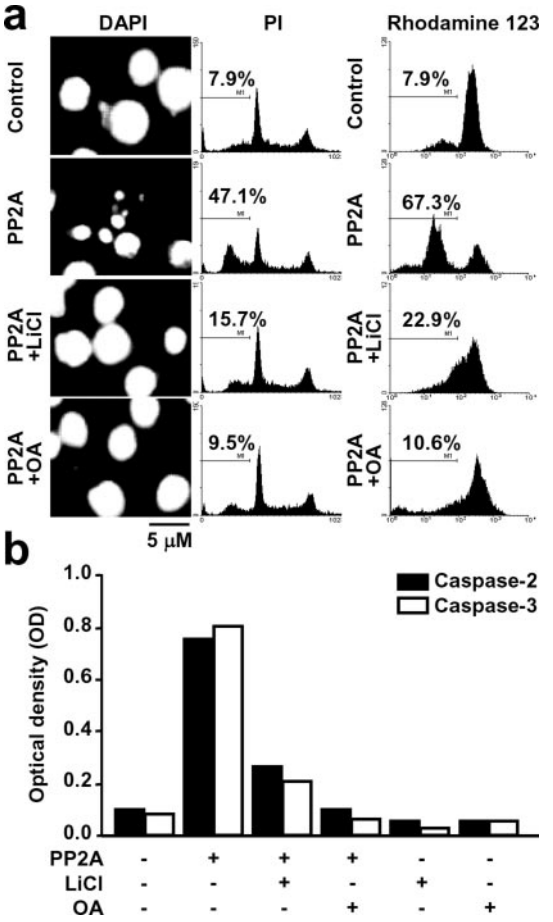

Fig. 3. Lithium reduces PP2A-induced mitochondrial apoptosis and caspase activation. 10I cells were transfected with purified PP2A (0.02 μg) for 6 h, with or without LiCl (20 mM) or OA (100 nM). a, cell apoptosis characterized by DNA fragmentation was determined using DAPI staining (left) and PI staining followed by flow cytometric analysis (middle). The $\Delta\Psi_m$ reduction was detected using rhodamine 123, and then flow cytometric analysis (right). The histogram and the percentages of apoptotic cells and $\Delta\Psi_m$ reduction are shown as marked. Cells treated with transfection reagent were used as the control. b, the caspase-2 and -3 activities were determined using caspase activity assay kits. The substrate activities for caspase-2 (■) and -3 (□) are shown using OD. Data are means of duplicate cultures.

tion by lithium was, at least in part, through inhibiting PP2A-induced downstream apoptotic events.

Lithium Rescues Ceramide- and Etoposide-Induced Bcl-2 Dephosphorylation. To address the effects of lithium on PP2A, the PP2A-regulated downstream substrate Bcl-2 was investigated. Ceramide activated a mitochondrial PP2A-mediated Bcl-2 dephosphorylation at serine 70 in association with cell apoptosis (Ruvolo et al., 1999). Our recent study (Lin et al., 2005) showed that the loss of Bcl-2 phosphorylation by ceramide-activated PP2A caused caspase-2 activation before mitochondrial damage. In the presence of lithium or OA, ceramide- and etoposide-induced PP2A-mediated Bcl-2 dephosphorylation was rescued (Fig. 4a). Confocal microscopic analysis revealed that ceramide- and etoposide-induced Bcl-2 dephosphorylation in both cytosolic (green in Merge) and mitochondrial (yellow in Merge) fractions were reversed by lithium and OA pretreatment (Fig. 4b). In addition, immunoprecipitated PP2A from ceramide-treated cells directly dephosphorylated Bcl-2 at serine 70, whereas PP2A from cells pretreated with lithium or OA failed to dephosphorylate Bcl-2 (Fig. 4c).

Lithium Inhibits PP2A Indirectly. Because lithium and OA both showed inhibitory effects on ceramide-mediated apoptosis and Bcl-2 dephosphorylation, we next examined whether lithium, like OA, caused PP2A inactivation. First, immunoprecipitation experiments showed that ceramide-activated PP2A activity was reduced when cells were pretreated with lithium or OA (Fig. 5a). In other words, lithium inhibited ceramide-induced PP2A activation in vivo. To further determine whether lithium-inhibited PP2A was through a direct effect, an action similar to that of OA, the activity of PP2A immunoprecipitated from ceramide-treated cells was measured in the presence of lithium. This in vitro study showed that lithium did not cause an inhibition on ceramide-activated PP2A (Fig. 5b). These results indicated that, unlike OA, lithium may indirectly inhibit PP2A activity.

Lithium Suppresses PP2A through PP2A C Subunit Demethylation and B Subunit Dissociation. Because lithium and OA inhibit ceramide-activated PP2A activity in different manners, we next investigated the mechanism through which lithium suppresses PP2A activity. PP2A holoenzyme assembly requires methylation of PP2A catalytic C subunits in a conserved C-terminal leucine residue, which increases binding of regulatory B subunits with the AC core enzyme and PP2A activity (Kowluru et al., 1996; Tolstykh et al., 2000; Yu et al., 2001). We then examined the effects of lithium on PP2A C subunit methylation and complex formation after ceramide and etoposide treatment. Using monoclonal antibody that recognized only demethylated PP2A C subunit, the steady-state unmethylated level of PP2A C subunit after ceramide (Fig. 6a, top) or etoposide (data not shown) treatment was detected using flow cytometry. The percentages of cells expressing higher levels (as marked in Fig. 6a, top, using 50% of untreated cells for gating) of unmethylated-

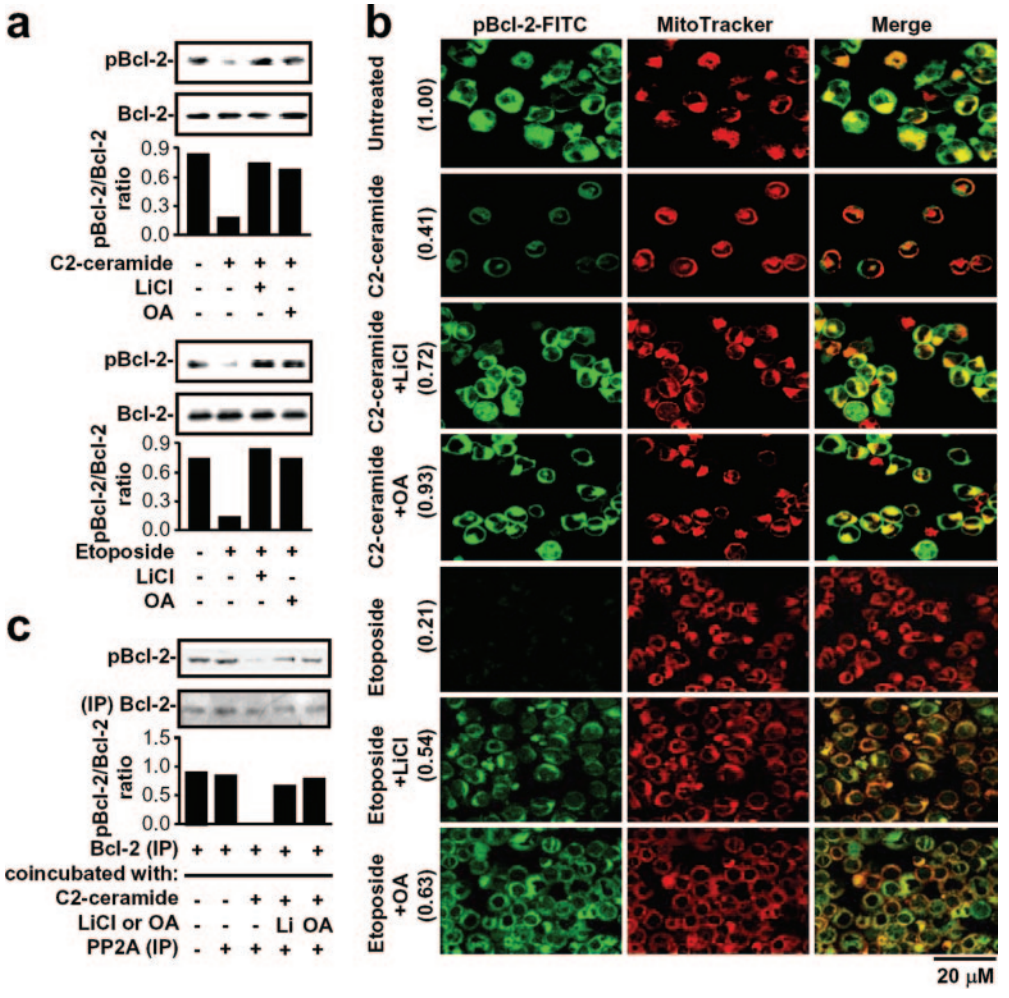

Fig. 4. Lithium and okadaic acid abolish ceramide- and etoposide-induced Bcl-2 dephosphorylation. 10I cells were treated with C2-ceramide (40 μM) and etoposide (100 μM) with or without LiCl (20 mM) or OA (100 nM) for 6 h. The phosphorylation of Bcl-2 at serine 70 was determined using Western blot analysis (a) and immunostaining plus confocal microscopic observation (b). The protein expression of Bcl-2 was an internal control. For confocal microscopic observation, Mito Tracker dye (red) was added to cells, which were then incubated with anti-phospho-Bcl-2 and then FITC-conjugated secondary antibody (pBcl-2-FITC; green). The relative fluorescence intensity compared with untreated control was quantified by imaging analysis using MetaMorph software. c, the relative ratio of phosphorylated Bcl-2 and Bcl-2 protein. To verify PP2A directly affected Bcl-2 dephosphorylation, PP2A was immunoprecipitated and then coincubated with precipitated Bcl-2 from untreated cells. The phosphorylation of Bcl-2 (pBcl-2) at serine 70 was determined using Western blot analysis. The expression of precipitated Bcl-2 was an internal control.

PP2A (Fig. 6a, middle) and mean fluorescence intensity (Fig. 6a, bottom) were quantified. Ceramide and etoposide treatment reduced the percentages of cells expressing demethylated PP2A. In other words, ceramide and etoposide stimulated the methylation of PP2A. It is noteworthy that lithium and OA reversed this effect. Similar findings were obtained using Western blot analysis with antidemethylated PP2A (Fig. 6b) and confocal microscopy with FITC-conjugated antidemethylated and antimethylated PP2A antibodies, respectively (Fig. 6, c and d).

We next examined the involvement of PP2A B subunits. Coimmunoprecipitated PP2A C subunits from untreated or ceramide-treated cells with or without lithium indicated that lithium caused dissociation of PP2A B55 subunit, whereas ceramide caused recruitment of B55 subunit (Fig. 6e). Because ceramide promoted translocation of PP2A B56α subunit from cytosol to mitochondria and Bcl-2 dephosphorylation (Ruvolo et al., 2002), we also examined the lithium effect on PP2A C and B56 association. Results showed an increase in the association of B56 subunit with PP2A C after ceramide stimulation, and lithium caused an inhibitory effect on B56 subunit recruitment (data not shown). Nevertheless, with or without ceramide and/or lithium, PP2A A subunit was consistently associated with PP2A C (data not shown). These results demonstrated a mechanism of lithium-mediated PP2A inhibitory effects on PP2A methylation and B subunit recruitment.

Discussion

Caspase-2 has been shown to act upstream of mitochondria in stress-induced apoptosis. Our studies (Lin et al., 2004, 2005) showed that ceramide and etoposide induced mitochondrial apoptosis by initiating the activation of caspase-2. Bcl-2 was dephosphorylated at serine 70 after ceramide and etoposide treatment, and PP2A inhibitor OA rescued Bcl-2 dephosphorylation and blocked caspase-2 activation. In the present study, we further showed that, like OA, lithium blocked the apoptotic signaling of ceramide and etoposide. Furthermore, the inhibitory effect of lithium on ceramide- and etoposide-induced apoptosis was due, at least in part, to an inhibition of PP2A activity. By in vitro assay, lithium may suppress PP2A in an indirect manner. Based on our results, we hypothesize that the antiapoptotic role of lithium is mediated via PP2A inactivation, Bcl-2 phosphorylation, and caspase-2 inhibition. Most importantly, this study is the first to demonstrate the inhibitory effects of lithium on PP2A methylation and the heterotrimer holoenzyme assembly (Fig. 7).

PP2A has a highly conserved catalytic subunit containing six residues, TPDYFL, at its carboxyl terminus, which plays an important role in phosphatase regulation (Ogris et al., 1997). Phosphorylation of Tyr307 in this sequence by receptor or nonreceptor tyrosine kinases resulted in a 90% decrease in PP2A phosphatase activity (Chen et al., 1992). In addition, the reversible methyl esterification of the carboxyl-terminal Leu309 in the C subunit of PP2A affects the binding affinity of the AC heterodimers with B subunits (Tolstykh et al., 2000; Yu et al., 2001). Moreover, two specific enzymes, AdoMet-dependent PP2A methyltransferase (Lee and Stock, 1993) and PP2A methylesterase (Lee et al., 1996), control the carboxyl methylation mechanism. Methylation of the highly conserved catalytic domain of PP2A exhibits up to a 2-fold increase in specific activity (Favre et al., 1994; Kowluru et al., 1996). PP2A is activated by ceramide stimulation (Ruvolo, 2003), but the mechanisms are not clear. Recent studies (Ruvolo et al., 1999, 2002) have indicated that ceramide promotes translocation of the B56α subunit of PP2A to the mitochondrial membrane and colocalization with its downstream substrate Bcl-2, resulting in direct dephosphorylation of Bcl-2 at serine 70. Thus, the role of PP2A in ceramide-induced mitochondrial apoptosis was suggested.

In this study, we demonstrated that PP2A methylation increased after ceramide and etoposide stimulation in 10I cells. One report (Kowluru and Metz, 1997), however, showed that ceramide did not affect the carboxyl methylation of PP2A C subunit in vitro in an insulin-secreting INS-1 cell cytosolic fraction. This and our data suggest that, unlike OA, ceramide itself may not directly act on the PP2A catalytic subunit to modulate methylation activity.

During ceramide-induced PP2A activation, the B56α(B′) subunit of PP2A translocated from cytosol to mitochondria and dephosphorylated the downstream substrate, Bcl-2 (Ruvolo et al., 2002). The different B subunits of the PP2A holoenzyme may provide PP2A to dephosphorylate distinct substrates. Because the reversible methylated modification regulates PP2A B subunits associated with AC heterodimers, the regulation of the recruitment of B subunits by ceramide and lithium is intriguing. We found that ceramide also regulated B55 (B) subunit

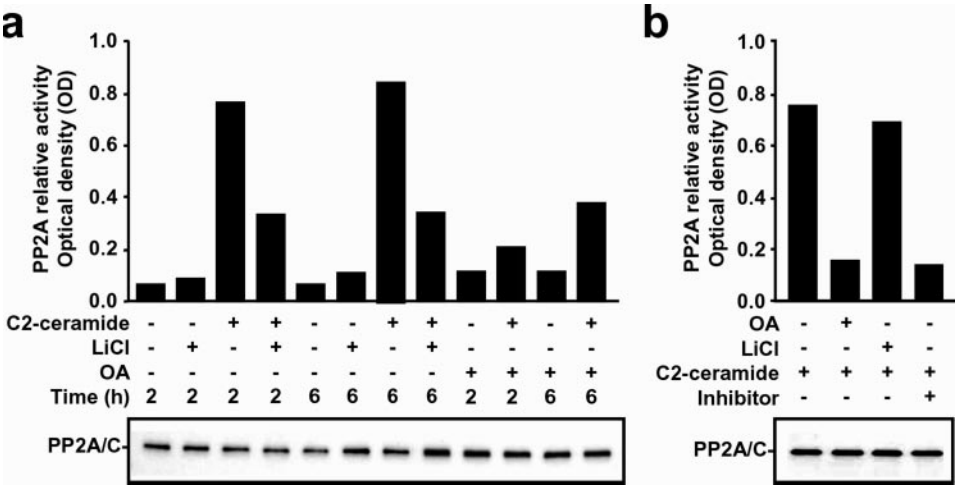

Fig. 5. Lithium indirectly abolishes ceramide-induced PP2A activation. a, 10I cells were treated with C2-ceramide (40 μM) with or without LiCl (20 mM) or OA (100 nM) for 2 and 6 h and then analyzed using a PP2A activity assay. b, immunoprecipitated PP2A from ceramide-treated cells were coincubated with or without LiCl (20 mM), OA (100 nM), or protein phosphatase inhibitor cocktail I (5 μM). The activities of PP2A are shown using OD (top). Data are means of duplicate cultures. Meanwhile, immunoprecipitated PP2A protein amounts are shown using PP2A/C immunoblot analysis (bottom).

engagement. Ceramide-increased PP2A methylation was associated with the recruitment of the regulatory B55 subunit. Previous studies (Lee et al., 2000; Ruvolo et al., 2001) indicated that PP2A dephosphorylated PKCα during ceramide stimulation, which resulted in Bcl-2 dysfunction. In addition, the PP2A induced in this Bcl-2 dysfunction was identified as a membrane-associated heterotrimer that included subunits A, B55, and C (Millward et al., 1999).

Based on these previous studies, together with our findings, we speculate that in intact cells, two distinct forms of PP2A heterotrimer, ABC and AB′C, are involved in response to ceramide stimulation. In the presence of lithium or OA, Bcl-2 dephosphorylation and caspase-2 activation were blocked. Studies have reported that PP2A methyltransferase (Lee and Stock, 1993) and PP2A methylesterase (Lee et al., 1996) regulated carboxyl methylation in the PP2A C subunit.

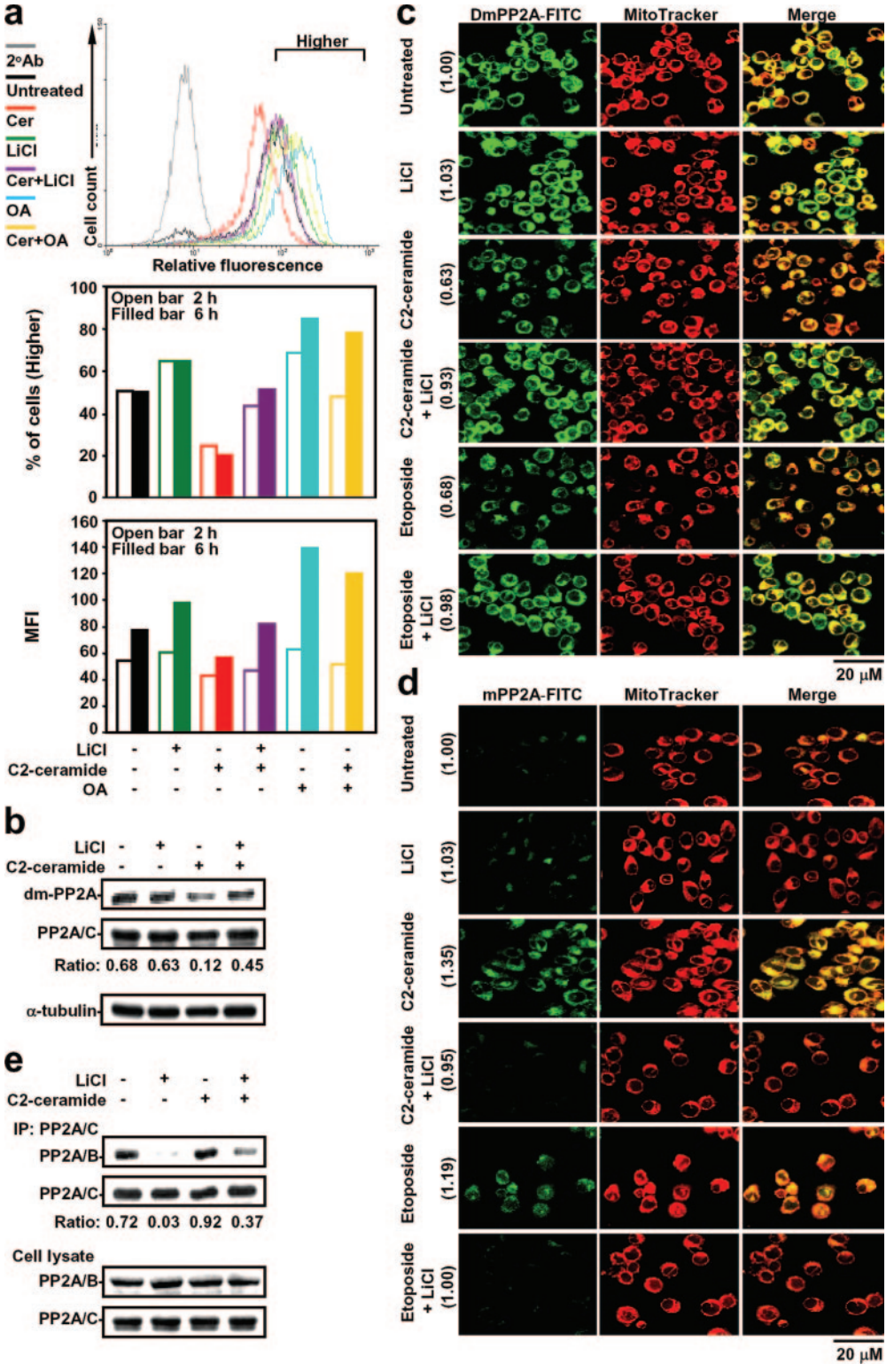

Fig. 6. Lithium blocks ceramide- and etoposide-induced PP2A activation by inhibiting PP2A C subunit methylation and PP2A B subunit recruitment. a, with or without LiCl (20 mM) or OA (100 nM), 10I cells were treated with C2-ceramide (40 μM) for 2 and 6 h. The demethylation of PP2A was determined using a demethylated PP2A-specific antibody and then flow cytometric analysis. The histogram shows the results of 6 h of stimulation (top). The percentages of cells expressing higher levels of unmethylated-PP2A (as marked in the top) and mean fluorescence intensity are shown. Data are means of duplicate cultures. b, the demethylation of PP2A at 6 h was analyzed using Western blot. The levels of PP2A C subunit (PP2A/C) and α-tubulin were used as controls. c and d, for confocal microscopic observation, Mito Tracker dye (red) was added to cells. After fixation, cells were incubated with demethylated (c) or methylated (d) PP2A-specific antibody and then FITC-labeled secondary antibody (green). The relative fluorescence intensity compared with untreated control was quantified by imaging analysis using MetaMorph software. e, PP2A was immunoprecipitated from cells using anti-PP2A/C antibody. The presence of PP2A B55 subunit (PP2A/B) and PP2A/C in the pull-down PP2A complexes were determined using Western blot analysis with specific antibodies. The levels of PP2A/B and PP2A/C in cell lysate were used as control.

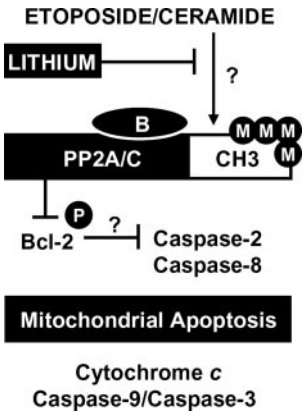

Fig. 7. Lithium inhibits ceramide- and etoposide-induced PP2A-mediated mitochondrial apoptosis. Ceramide and etoposide induce caspase-2 and -8 activation, mitochondrial damage, cytochrome *c* release, and then caspase-9 and -3 activation. During apoptosis, PP2A causes Bcl-2 dephosphorylation that promotes caspase-2 activation via an unknown mechanism. Although the mechanisms of ceramide- and etoposide-induced PP2A activation remain unclear, it is clear that they involve the methylation of the PP2A C subunit and the recruitment of the B subunit. Our findings suggest that lithium inhibits ceramide- and etoposide-induced caspase-2-mediated mitochondrial apoptosis by blocking PP2A activity. Lithium indirectly inhibits PP2A. The mechanisms of PP2A demethylation and inactivation by lithium require further investigation.

In the presence of ebelactone B, which inhibited PP2A methylesterase, the blockage of lithium on ceramide-induced mitochondrial apoptosis was partially inhibited (C.-L. Chen, C.-F. Lin, C.-W. Chiang, and Y.-S. Lin, unpublished data). This result implied the upstream regulatory role of lithium on PP2A methylation via PP2A methylesterase. Whether lithium also acts on PP2A methyltransferase needs further investigation.

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