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Lithium potentiates GSK-3β activity by inhibiting phosphoinositide 3-kinase-mediated Akt phosphorylation

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ABSTRACT

Accumulating evidence has pointed to the direct inhibitory action of lithium, an anti-depressant, on GSK-3β. The present study investigated further insight into lithium signaling pathways. In the cell-free assay Li_2CO_3 significantly inhibited phosphoinositide 3-kinase (PI3K)-mediated phosphorylation of Akt1 at Ser473, but Li_2CO_3 did not affect PI3K-mediated PI(3,4,5)P_3 production and 3-phosphoinositide-dependent protein kinase 1 (PDK1)-mediated phosphorylation of Akt1 at Thr308. This indicates that lithium could enhance GSK-3β activity by suppressing Akt-mediated Ser9 phosphorylation of GSK-3β in association with inhibition of PI3K-mediated Akt activation. There was no direct effect of Li_2CO_3 on Akt1-induced phosphorylation of GSK-3β at Ser9, but otherwise Li_2CO_3 significantly reduced GSK-3β-mediated phosphorylation of β-catenin at Ser33/37 and Thr41. This indicates that lithium directly inhibits GSK-3β in an Akt-independent manner. In rat hippocampal slices Li_2CO_3 significantly inhibited phosphorylation of Akt1/2 at Ser473/474, GSK-3β at Ser9, and β-catenin at Ser33/37 and Thr41. Taken together, these results indicate that lithium exerts its potentiating and inhibiting bidirectional actions on GSK-3β activity.

1. Introduction

Glycogen synthase kinase-3β (GSK-3β) is a multifunctional enzyme to regulate diverse cellular functions including gene expression, neuronal structure, and cell survival [1]. Notably, GSK-3β is a key factor for cognitive and mood disorders [2]. GSK-3β is inactivated by being phosphorylated at Ser9 due to the serine/threonine protein kinase Akt and activated by being phosphorylated at Tyr216. Akt is activated via a pathway along a receptor tyrosine kinase (RTK)/phosphoinositide 3-kinase (PI3K)/3-phosphoinositide-dependent protein kinase (PDK1)/Akt axis. RTK includes a variety of growth factor receptors such as insulin receptor. Amazingly, insulin-like growth factor-I (IGF-I), IGF-II, and insulin are shown to enhance mood [3–6], memory [7,8], neurogenesis [7,8], and angiogenesis [9].

Lithium has been used as an antidepressant since before. Lithium directly inhibits GSK-3β [10–13], to promote β-catenin-dependent transcriptional events [14–16]. The inhibitory effect of lithium on GSK-3β is thought to be due to its direct binding [10,17] or to an increase in the phosphorylation of GSK-3β at Ser9 [18]. Lithium, alternatively, inhibits 5-HT_{2c} receptor [19]. The present study was conducted to gain further insight into lithium signaling. We show here that lithium potentiates GSK-3β activity by inhibiting PI3K-mediated Ser phosphorylation of Akt, while lithium directly suppresses GSK-3β activity.

2. Materials and methods

2.1. Animal care

All procedures have been approved by the Animal Care and Use Committee at Hyogo College of Medicine and were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Western blotting

Rat hippocampal slices (male Wistar, 6 w)(400 μm) were incubated in a standard artificial cerebrospinal fluid (117 mM NaCl, 3.6 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, and 11.5 mM glucose) oxygenated with 95% O_2 and 5% CO_2 in the presence and absence of Li_2CO_3 for 30 min at 34 °C. Then, slices were homogenized by sonication in an ice-cold cell lysis buffer [150 mM NaCl, 20 mM EDTA, 0.5% (v/v) Nonidet P-40 and 50 mM Tris, pH 7.4] containing 1% (v/v) protease inhibitor

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cocktail and 1% (v/v) phosphatase inhibitor cocktail and subsequently, homogenates were centrifuged at 800*g* for 5 min at 4 °C. Protein concentrations for the supernatants were determined using a BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride membranes. After blocking with TBS-T [150 mM NaCl, 0.1% (v/v) Tween 20, and 20 mM Tris, pH 7.5] containing 5% (w/v) bovine serum albumin (BSA), blotting membranes were reacted with antibodies against phospho-Thr308/309-Akt (pT308/309-Akt)(Cell Signaling Technology, Inc., Danvers, MA, USA), phospho-Ser473/474-Akt (pS473/474-Akt)(Cell Signaling Technology), Akt1/2 (Cell Signaling Technology), phospho-Ser9-GSK-3β (pS9-GSK-3β)(Cell Signaling Technology), phospho-Tyr216-GSK-3β (pY216-GSK-3β)(BD Biosciences, San Jose, CA, USA), GSK-3β (Cell Signaling Technology), phospho-Ser33/37/Thr41-β-catenin (pS33/37/T41-β-catenin)(Cell Signaling Technology), and β-catenin (Cell Signaling Technology). After washing, membranes were reacted with a horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG antibody. Immunoreactivity was detected with an ECL kit (GE Healthcare, Piscataway, NJ, USA) and visualized using a chemiluminescence detection system (GE Healthcare).

In the cell-free Akt assay, human recombinant Akt1 (Active Motif, Carlsbad, CA, USA) was reacted with PI3K (p110β/p85α)(Sigma, St. Louis, MO, USA) or PDK1 (SignalChem, Richmond, Canada) in the presence and absence of Li_2CO_3 in a medium containing 25 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS)(pH 7.2), 12.5 mM glycerol 2-phosphate, 25 mM $MgCl_2$, 5 mM EGTA, 2 mM EDTA, 0.25 mM dithiothreitol, and 1 mM ATP at 30 °C for 20 min. After termination of the reaction, Western blotting was performed using antibodies against pT308/309-Akt, pS473/474-

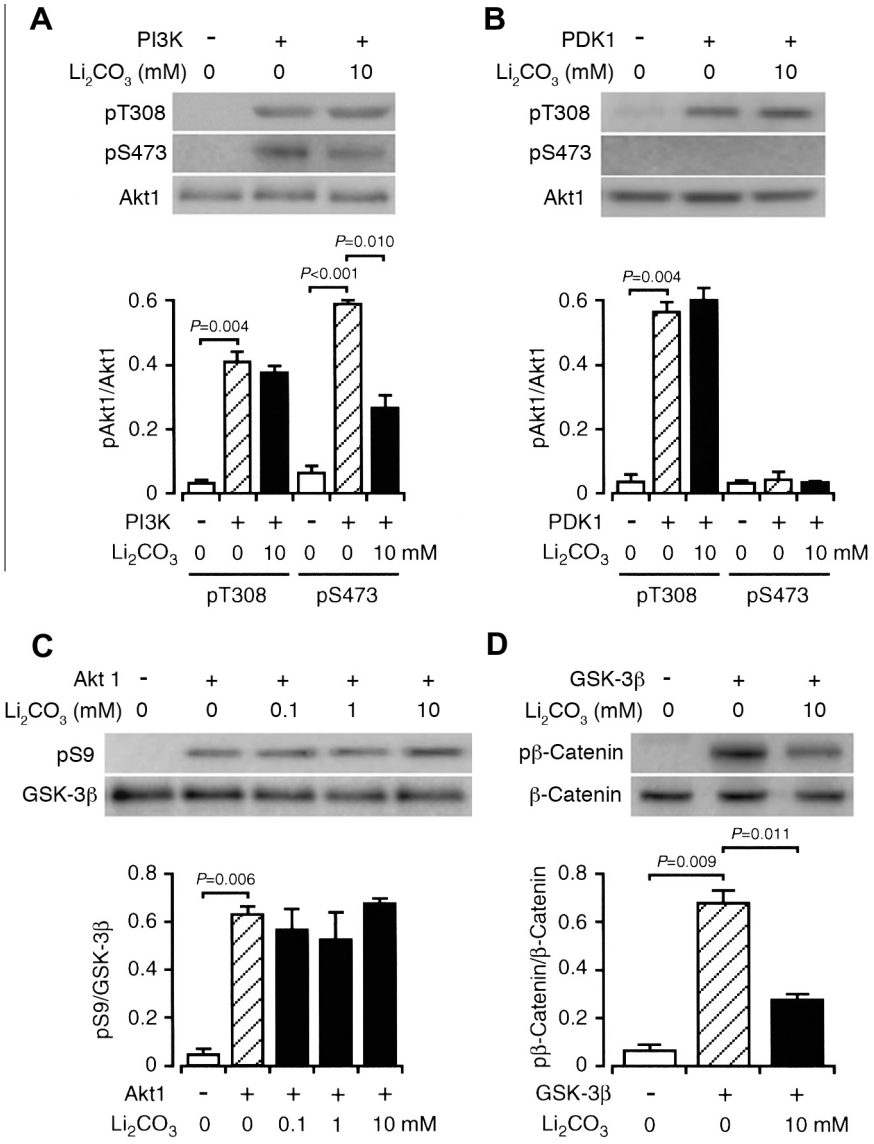

Fig. 1. Effects of lithium on Akt1, GSK-3β, and β-catenin activities under the cell-free conditions. (A) Akt1 was reacted with and without PI3K (1 μg/ml) in the presence and absence of Li_2CO_3 (10 mM), and Western blotting was carried out using antibodies against pT308/309-Akt, pS473/474-Akt, and Akt1/2. (B) Akt1 was reacted with and without PDK1 (1 μg/ml) in the presence and absence of Li_2CO_3 (10 mM), and Western blotting was carried out using antibodies against pT308/309-Akt, pS473/474-Akt, and Akt1/2. (C) GSK-3β was reacted with and without Akt1 (1 μg/ml) in the presence and absence of Li_2CO_3 at concentrations as indicated, and Western blotting was carried out using antibodies against pS9-GSK-3β, and GSK-3β. (D) β-Catenin was reacted with and without GSK-3β (1 μg/ml) in the presence and absence of Li_2CO_3 (10 mM), and Western blotting was carried out using antibodies against pS33/37/T41-β-catenin, and β-catenin. The signal intensities for phosphorylated Akt (pAkt), phosphorylated GSK-3β (pGSK-3β) or phosphorylated β-catenin (pβ-catenin) were normalized by those for Akt1/2, GSK-3β or β-catenin, respectively. In the graphs, each value represents the mean (±SEM) intensity for pAkt, pGSK-3β or pβ-catenin ($n = 4$ independent experiments). *P* values, Dunnett's test.

Akt, and Akt1/2. In the cell-free GSK-3β assay, human recombinant GSK-3β (Sigma) was reacted with human recombinant Akt1 in the presence and absence of Li_2CO_3 in a medium 50 mM Tris–HCl (pH 7.5), 25 mM $MgCl_2$, 0.1 mM EGTA, 10 mM dithiothreitol, and 1 mM ATP at 30 °C for 20 min. After termination of the reaction, Western blotting was performed using antibodies against pS9-GSK-3β, and GSK-3β. In the cell-free β-catenin assay, human recombinant β-catenin (Abcam, Cambridge, UK) was reacted with human recombinant GSK-3β in the presence and absence of Li_2CO_3 in a medium containing 25 mM MOPS (pH 7.2), 12.5 mM glycerol 2-phosphate, 25 mM $MgCl_2$, 5 mM EGTA, 2 mM EDTA, 0.25 mM dithiothreitol, and 1 mM ATP at 30 °C for 20 min. After termination of the reaction, Western blotting was performed using antibodies against pS33/37/T41-β-catenin, and β-catenin.

2.3. Measurement of PI3K activity

PI3K activity was quantified by a PI3K enzyme-linked immunosorbent assay (ELISA) (Echelon Biosciences Inc., Salt Lake City, UT, USA) according to the manufacturer's instructions. Briefly, PI3K (p110β/p85α)(Sigma) was reacted with phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] as a substrate in a reaction buffer without and with Li_2CO_3 at 30 °C for 20 min. After termination of the reaction, a primary phosphatidylinositol (3,4,5)-triphosphate [PI(3,4,5)P_3] detector was added to the reaction mixture followed by 60-min incubation at room temperature. The mixture was transferred to a PI3K ELISA plate, and 60 min later a second detector was added and further incubated at room temperature for 30 min. A tetramethylbenzidine solution was added to the final reaction mixture, and colorimetric signals were measured at 450 nm using a micro-plate reader (SPECTRAmax PLUS384, Molecular Devices, Sunnyvale, CA, USA). PI(3,4,5)P_3 production (pmol/20 min) was regarded as an index of PI3K activity.

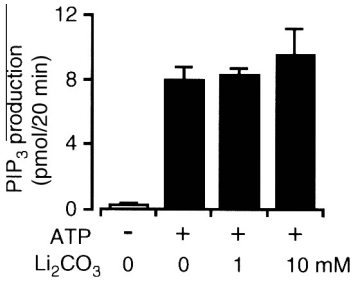

Fig. 2. Effect of lithium on PI3K activity. PI3K was reacted with PI(4,5)P_2 in the presence and absence of Li_2CO_3 for 20 min and PI(3,4,5)P_3 production was measured with a competitive ELISA. In the graph, each value represents the mean (±SEM) PI(3,4,5)P_3 production (pmol/20 min) (n = 4 independent experiments).

2.4. Statistical analysis

Statistical analysis was carried out using Dunnett's test.

3. Results and discussion

3.1. Lithium enhances GSK-3β activity by inhibiting PI3K-mediated Akt1 activation

Akt1/2 is activated by being phosphorylated at Thr308/309 and Ser473/474 via a pathway along a RTK/PI3K/PDK1/Akt axis. We initially examined the effect of lithium on Akt activity. In the cell-free assay, PI3K increased phosphorylation of Akt1 both at Thr308 and Ser473 (Fig. 1A). PDK1, on the other hand, increased phosphorylation of Akt1 only at Thr308 without affecting Ser473 phosphorylation (Fig. 1B). Li_2CO_3 (10 mM) significantly suppressed PI3K-mediated phosphorylation of Akt1 at Ser473, although Thr308 phosphorylation was not affected (Fig. 1A). No effect on PDK1-mediated Thr308 phosphorylation of Akt1 was obtained with Li_2CO_3 (10 mM) (Fig. 1B), indicating that lithium has no effect on PDK1. Collectively, these results indicate that lithium reduces Akt activity by inhibiting PI3K-mediated Ser473 phosphorylation of Akt.

In the cell-free ELISA analysis, PI3K apparently phosphorylated PI(4,5)P_2 to produce PI(3,4,5)P_3, and the effect was abrogated in the absence of ATP (Fig. 2). Li_2CO_3 (1 and 10 mM) had no significant effect on PI3K-mediated PI(3,4,5)P_3 production (Fig. 2). Taken together, these results suggest that the inhibitory effect of lithium on PI3K-mediated Ser473 phosphorylation of Akt is not due to the direct inhibitory action on PI3K.

Akt phosphorylates GSK-3β at Ser9, to inactivate GSK-3β. Then, we postulated that lithium could enhance GSK-3β activity in association with lithium-induced inhibition of Akt activity. To address

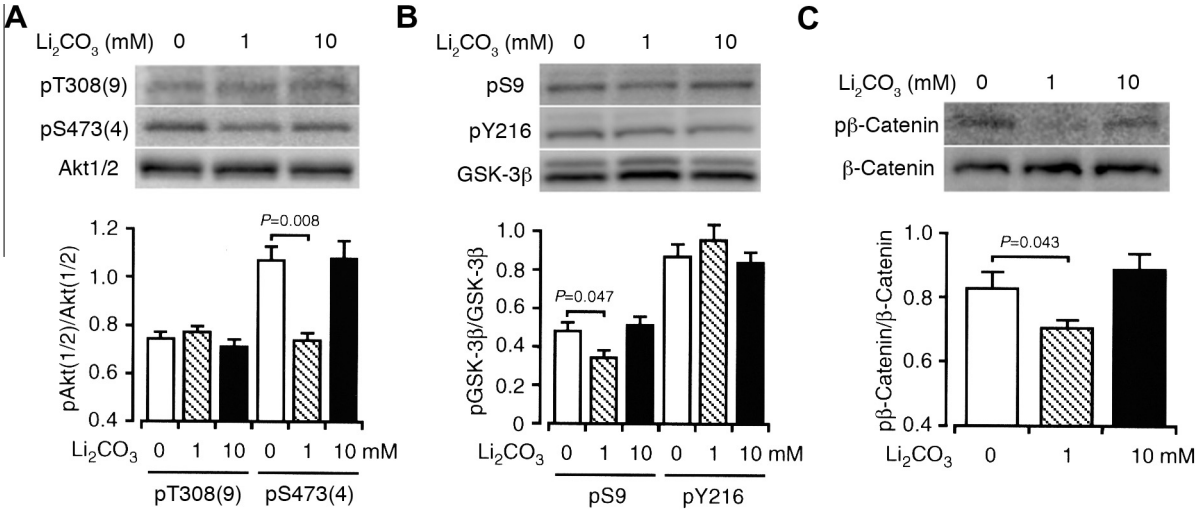

Fig. 3. Effects of lithium on Akt1, GSK-3β, and β-catenin activities in rat hippocampal slices. Slices were untreated and treated with Li_2CO_3 at concentrations as indicated, and Western blotting was carried out using antibodies against pT308/309-Akt, pS473/474-Akt, Akt1/2 (A), pS9-GSK-3β, pY216-GSK-3β, GSK-3β (B), pS33/37/T41-β-catenin, and β-catenin (C). The signal intensities for pAkt, pGSK-3β or pβ-catenin were normalized by those for Akt1/2, GSK-3β or β-catenin, respectively. In the graphs, each value represents the mean (±SEM) intensity for pAkt, pGSK-3β or pβ-catenin (n = 4 independent experiments). P value, Dunnett's test.

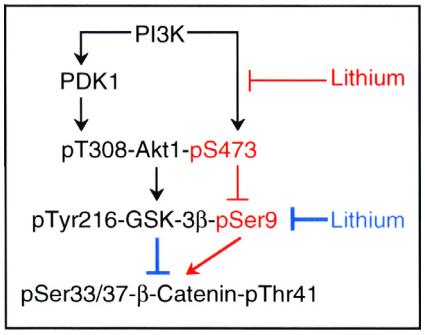

Fig. 4. A schematic paradigm for the sites of action of lithium.

this issue, we next examined the effect of lithium on GSK-3β. In the cell-free assay, Akt1 significantly increased phosphorylation of GSK-3β at Ser9, but the effect was not affected by Li_2CO_3 at concentrations ranging from 0.1 to 10 mM (Fig. 1C). In rat hippocampal slices Li_2CO_3 (1 mM) significantly inhibited phosphorylation of Akt1/2 at Ser473/474 under the basal conditions without stimulation, but not at Thr308/309 (Fig. 3A). Moreover, Li_2CO_3 (1 mM) significantly inhibited phosphorylation of GSK-3β at Ser9, although Tyr216 phosphorylation, to activate GSK-3β, was not affected (Fig. 3B). Overall, these results lead to a conclusion that lithium could enhance GSK-3β activity by inhibiting PI3K-mediated Akt activation (Fig. 4). To our knowledge, this is the first to provide evidence for the enhancing action of lithium on GSK-3β activity.

3.2. Lithium directly inhibits GSK-3β

β-Catenin is a substrate of GSK-3β. In the cell-free assay, GSK-3β significantly increased phosphorylation of β-catenin at Ser33/37 and Thr41, and the effect was clearly inhibited by Li_2CO_3 (10 mM)(Fig. 1D). This, in the light of the finding that Li_2CO_3 did not affect Akt1-mediated Ser9 phosphorylation of GSK-3β (Fig. 1C), indicates that lithium directly inhibits GSK-3β, to reduce GSK-3β-mediated phosphorylation of β-catenin (Fig. 4). In rat hippocampal slices, Li_2CO_3 (1 mM) significantly prevented phosphorylation of β-catenin at Ser33/37 and Thr41 under the basal conditions (Fig. 3C). This further supports the note for the direct inhibitory effect of lithium on GSK-3β (Fig. 4).

Amazingly, a higher concentration (10 mM) of Li_2CO_3 exhibited no inhibitory effect on Ser473/474 phosphorylation of Akt1/2, Ser9 phosphorylation of GSK-3β, or Ser33/37 and Thr41 phosphorylation of β-catenin in rat hippocampal slices (Fig. 3A-C). This suggests the concentration-dependent different actions of lithium on Akt and GSK-3β activities.

In conclusion, the results of the present study show that lithium exerts its enhancing and inhibiting bidirectional actions on GSK-3β activity, possibly due to suppression of PI3K-mediated Akt activation for the former action and to direct GSK-3β inhibition for the latter action (Fig. 4).

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