Lithium activates the Wnt and phosphatidylinositol 3-kinase Akt signaling pathways to promote cell survival in the absence of soluble survival factors

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Sinha, Diviya, Zhiyong Wang, Kathleen L. Ruchalski, Jerrold S. Levine, Selvi Krishnan, Wilfred Lieberthal, John H. Schwartz, and Steven C. Borkan. Lithium activates the Wnt and phosphatidylinositol 3-kinase Akt signaling pathways to promote cell survival in the absence of soluble survival factors. Am J Physiol Renal Physiol 288: F703–F713, 2005. First published November 30, 2004; doi:10.1152/ajprenal.00189.2004.—Mouse proximal tubular cells (BUMPT), when cultured in the absence of growth factors, activate a default apoptotic pathway. Although Wnt signaling antagonizes the effect of proapoptotic triggers, its role in regulating the default pathway of apoptosis is less well defined. The present study examines the hypothesis that lithium (Li+) and (2’Z,3’E)-6-bromoindirubin-3’-oxime (BIO), two glycogen synthase kinase-3β (GSK3β) inhibitors, promote survival of growth factor-deprived renal epithelial cells by activating the Wnt pathway. These studies demonstrate that Li+ and BIO activate Wnt signaling as indicated by the following changes: phosphorylation (inhibition) of GSK3β; decreased phosphorylation of β-catenin (a GSK3β substrate); nuclear translocation of β-catenin; specific transcriptional activation of Tcf/catenin-responsive pTopflash constructs; and an increase in the expression of cyclin D1 (indicative of a promitotic cell response). In addition, Li+ or BIO significantly increases the phosphorylation (activation) of Akt, an anti-apoptotic protein, and inhibits apoptosis (decreases both annexin-V staining and caspase-3 activation), during serum deprivation. Inhibition of phosphatidylinositol 3-kinase (responsible for Akt activation) either by wortmannin or LY-294002 prevented Li+- or BIO-induced Akt phosphorylation and reduces cell survival without altering the phosphorylation state of GSK3β. Li+ or BIO also increases the expression of insulin-like growth factor-II (IGF-II), a potent proliferative signaling protein. Li+ or BIO-free conditioned medium harvested from Li+- or BIO-exposed cells also induced Akt phosphorylation, mimicking the protective effect of the two GSK3β inhibitors on serum-starved cells. Furthermore, the effect of conditioned medium on Akt phosphorylation could be inhibited by either LY-294002 or IGF-binding protein. BIO, a specific GSK3β inhibitor, replicated the protective effect of Li+ on cell viability, suggesting that GSK3β activation is important for initiating the apoptotic pathway. Taken together, these data suggest that Li+ or BIO promotes renal epithelial cell survival by inhibiting apoptosis through GSK3β-dependent activation of the Wnt pathway and subsequent release of IGF-II. Extracellular IGF-II serves as an autocrine survival factor that is responsible, in part, for activating the anti-apoptotic phosphatidylinositol-3-kinase-Akt pathway during serum deprivation.

serum deprivation; renal epithelial cells; insulin-like growth factors; β-catenin; glycogen synthase kinase-3β; apoptosis

REGULATION OF CELL SURVIVAL is crucial to the normal physiology of multicellular organisms. Perturbation of cell survival mechanisms (either insufficient or excessive cell death) results in pathological states (3, 7, 54, 58, 73). Apoptosis is an energy-dependent and highly orchestrated process in which cells commit suicide without inducing inflammatory injury to the surrounding tissues (3, 58). Although apoptosis is essential for many physiological functions such as regulation of immune system, tissue homeostasis, and deletion of potentially neoplastic cells, it also contributes to organ dysfunction in response to pathological stimuli (32, 64). Most, if not all, cells undergo apoptosis in the absence of soluble growth factors such as epidermal growth factor (EGF), insulin-like growth factor (IGF), lysophosphatidic acid, vascular endothelial cell growth factor, as well as cytokines including IL-2. Thus absence or deficiency of essential growth (survival) factors leads to apoptosis by triggering a default pathway that is normally under constant inhibition (3, 58, 65).

Survival factors have been shown to inhibit the default pathway of apoptosis and promote cell survival via the phosphatidylinositol-3-kinase (PI3K)/Akt signal transduction cascade (34, 57, 65, 71). The PI3K/Akt pathway is activated by growth factors including platelet-derived growth factor (PDGF) (34, 35, 43, 62). Akt (also known as PKB or RAC) is a downstream target of PI3K (2, 13, 14, 69). This enzyme is a multi-isozyme serine/threonine kinase that inhibits apoptosis by many downstream effects. These effects include Jα activating both bcl-xl and bcl2, two proteins that normally heterodimerize with and neutralize the proapoptotic effects of BAD (13) and 2 phosphorlylating and inactivating glycogen synthase kinase-3β (GSK3β), thereby stabilizing β-catenin (11). β-Catenin plays a pivotal role both in cadherin-based cell adhesion (1, 24) as well as in the Wnt signaling pathway (4, 6, 10, 30, 31, 67). Corresponding with its dual functions, β-catenin localizes to two distinct intracellular pools. Most β-catenin is located in the cell membrane, where it binds to the cytoplasmic tail of E-cadherin, a transmembrane protein involved in homotypic cell-cell adhesion (6, 67). Another pool of β-catenin (which is relatively small compared with the E-cadherin-bound fraction) is present within the cytosol. Activation of the Wnt signal leads to translocation of cytosolic β-catenin to the nucleus, where it mediates some of the downstream effects of Wnt signaling (4, 6, 10, 30, 31, 67). In the absence of Wnt signaling, the soluble pool of β-catenin is constitutively degraded by a multigene complex that contains GSK3β, axin, and the tumor suppressor protein adenomatous polyposis coli (APC) (6, 10, 30, 31, 67). This protein complex promotes the phosphorylation of two serine and threonine residues in the NH2-terminal region of

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β-catenin, thereby targeting β-catenin for degradation by the ubiquitin-proteasome pathway (10). Wnt signaling inhibits β-catenin degradation by deactivating (i.e., phosphorylating) GSK3β at Ser3 (21). GSK3β phosphorylation results in dissociation of β-catenin from the APC-containing multiprotein complex. β-Catenin then translocates to the nucleus where it promotes the transcription of Wnt target genes by binding to transcription factors of the TCF-LEF family genes known to stimulate cell proliferation and to inhibit apoptosis (6, 10, 21, 67).

Lithium (Li⁺), an agent used to treat manic depressive illness, inhibits GSK3β and has been shown to mimic the effects of Wnt signaling on gene expression and cell proliferation (28, 73). Li⁺ also exerts robust protective effects against a diverse array of proapoptotic insults including potassium deprivation (47), β-amyloid (7), or anticonvulsant medications (55), and heat shock (33). Furthermore, Li⁺ has recently been reported to protect neuronal cells against glutamate-induced excitotoxicity in vitro and to ameliorate cerebral ischemia in vivo by activating the anti-apoptotic PI3K/Akt pathway (51–53). Whether Li⁺ or (2’Z,3’E)-6-bromoindirubin-3’-oxime (BIO), a specific GSK3β inhibitor (12, 61), prevents apoptosis via the default pathway when cells are deprived of soluble survival factors is presently unknown.

Renal growth factors such as EGF inhibit apoptosis of cultured mouse proximal tubular cells, whereas the withdrawal of growth factors causes apoptosis (65). Apoptosis of tubular cells in response to growth factor deprivation is due, at least in part, to decreased activation of the anti-apoptotic PI3K/Akt pathway (65). The similarity of the signaling pathways involved in protective effects elicited by growth factors and Li⁺ prompted us to hypothesize that Li⁺ or BIO would promote survival in growth factor-deprived, cultured tubular cells by activating the PI3K/Akt pathways. The present study indicates that Li⁺ or BIO alone, in the absence of all other soluble survival factors, maintains the viability of mouse kidney proximal tubular cells and inhibits apoptosis by activating the Wnt and PI3K/Akt pathways.

**MATERIALS AND METHODS**

**Reagents.** All reagents were purchased from Sigma (St. Louis, MO) unless otherwise indicated.

**Antibodies.** Rabbit polyclonal antibodies (Cell Signaling Technology, Beverly, MA) directed against the phospho-serine 173 and phospho-serine 9 were used to detect the active form of Akt and the inactive form of GSK3β, respectively. Total Akt and total GSK3β were determined using specific polyclonal antibodies (Cell Signaling Technology). In addition, phospho-β-catenin (Ser3/Ser77/Thr41, Cell Signaling Technology), total β-catenin (Zymed, Philadelphia, PA), and cyclin D1 (Abcam, Cambridge, MA) and intact caspase-3 (Cell Signaling Technology) were detected using specific mouse monoclonal (total β-catenin) or rabbit polyclonal (phospho-β-catenin and cyclin D1) antibodies. Secondary antibodies conjugated to horseradish peroxidase (Jackson Immunoresearch Laboratories, West Grove, PA) were used in combination with a chemiluminescence detection method (Amersham Pharmacia, Piscataway, NJ). Cy3-conjugated secondary antibody (Jackson Immunoresearch Laboratories) was used to localize β-catenin in intact, fixed cells by immunohistochemistry.

**Culture of a continuous mouse proximal tubular cell line.** A conditionally immortalized renal epithelial cell line (BUMPT) was cultured as previously described (65). These cells were maintained for up to 50 passages in DMEM-high glucose medium (GIBCO BRL, Carlsbad, CA) containing 10% FBS and 10% penicillin-streptomycin at 37°C in an incubator containing 5% CO₂.

**Cell viability.** Cell viability was assayed using a modified colorimetric technique that is based on the ability of live cells to convert 3-(4,5 dimethylthiazol)2,5-diphenyl tetrazolium bromide (MTT), a tetrazolium compound, into purple formazan crystals (33, 39–41). MTT (1 mg/ml) was dissolved in Kreb’s-Henseleit buffer (containing in mM) 115 NaCl, 3.6 KCl, 1.3 KH₂PO₄, 25 NaHCO₃, and 1 μM each of CaCl₂ and MgCl₂ and 200 μl of the mixture were added to each well. After 4-h incubation at 37°C, the formazan crystals formed were dissolved in an equal volume of 10% SDS in 0.01 M HCl. After 24-h incubation at 37°C, aliquots from each well were assayed using a Micro ELISA plate reader (test wavelength 570 nm and reference wavelength 650 nm). The number of viable cells exposed to either Li⁺ or BIO (Calbiochem, San Diego, CA) is expressed as a percentage of control maintained in Li⁺ or BIO-free serum containing medium for a comparable time period.

**Serum starvation model.** Monolayers of confluent cells were made quiescent by incubating them for 48 h in FBS-free DMEM medium. Cells were then exposed to Li⁺ (10 mM) or BIO (10–1,000 nM) for 0–24 h. Controls were treated in the same way except for addition of Li⁺ or BIO. In additional experiments, the effect of PI3K on the proliferative action of Li⁺ was examined in the presence of a PI3K inhibitor (either 20 μM LY-294002 or 100 nM wortmannin; Calbiochem) to cells for 16 h before addition of Li⁺ as well as during the period of serum deprivation. At each time point studied, the cells were washed once with 1X PBS before the MTT assay was performed.

**Western blotting.** Cells were harvested at 4°C in a lysis buffer containing (in mM) 20 Tris–HCl, 140 NaCl, 1 sodium orthovanadate, 1 NaF, 1 DTT, 10 PMSF, 1 Na₄P₂O₇ at pH 7.5. In addition, the lysis buffer contained 0.5% Na-deoxycholate, 0.1% SDS, 1% Triton X-100, 10 glycerol, and a cocktail of protease inhibitors (Boehringer Mannheim, Indianapolis, IN) at pH 7.5. The vanadate was activated immediately before use by boiling for 10 min. Cell lysates were incubated for 1 h on ice and then centrifuged at 14,000 g for 15 min at 4°C. The protein concentration of the supernatant was estimated by Bio-Rad assay (Bio-Rad, Hercules, CA) using BSA as a standard. Samples (25 μg) were boiled in 4X SDS sample buffer (Boston Bioproducts, Ashland, MA), resolved using 10% SDS-PAGE under reducing conditions, and then transferred onto a PVDF membrane at 70 V for 80 min at 4°C.

To confirm equivalent loading of the lanes, immunoblots were first probed for the phosphorylated form of each kinase before being chemically stripped and then repeating the immunoblotting procedure using an antibody that assessed total kinase content. The blots were visualized by enhanced chemiluminescence (Pierce, Rockford, IL).

**Immunofluorescence assay.** To assess nuclear translocation of β-catenin, control or Li⁺-treated cells grown on glass coverslips were rinsed twice with 1X PBS and fixed with 3.7% paraformaldehyde for 30 min at 25°C. Cells were then washed three times for 5 min each in TBS containing 50 mM Tris and 150 mM NaCl (pH 7.6) and then in TBS containing 1% BSA for 15 min at 25°C. Cells were incubated with the primary anti-β-catenin antibody (diluted in 1% BSA) for 45 min at 25°C, washed with TBS, and then incubated with a Cy3-conjugated secondary antibody (1:3,000 dilution) for 45 min at 25°C (66). The cells were washed again with TBS, mounted using gelvatol, and then evaluated by immunofluorescence microscopy.

**Flow cytometry to quantify apoptosis and necrosis.** The cells were serum starved from 0–7 days in the presence or absence of Li⁺ or BIO. After 8 h or 7 days of serum deprivation, cells were trypsinized.
and then washed three times with 1× PBS. The cell pellet was then suspended in 200 μl of binding buffer containing annexin-V (10 μl) and propidium iodide (PI; 5 μl; BD Biosciences, San Jose, CA) and then incubated in dark at 25°C for 15 min. The volume of each cell suspension was increased to 500 μl with PBS, pH 7.4, and the percentage of viable, apoptotic and necrotic cells was analyzed with a flow cytometer. Control cells were incubated at 37°C in the complete medium containing 10% FBS. To identify positive staining, confluent BUMPT cells were treated with 3.7% paraformaldehyde for 10 min at 25°C and then stained either with annexin-V or PI.

β-Catenin/Tcf luciferase assay. The pTopflash (wild type) and the pFopflash (mutant) constructs were a generous gift of Carl Vogelstein (National Institutes of Health, Bethesda, MD). These constructs contain a luciferase reporter under the control of two repeats each (National Institutes of Health, Bethesda, MD). These constructs contain a luciferase reporter under the control of a constitutively active promoter, was cotransfected with either pTopflash or pFopflash using the Lipofectamine plus reagent (Invitrogen, Cambridge, UK). A β-galactosidase reporter plasmid, under the control of a constitutively active promoter, was cotransfected in each well to control for potential variations in well-to-well cell number and viability. Cell lysates were harvested 24 h after transfection and the levels of luciferase and β-galactosidase were determined. Luciferase activity in each well was normalized to the β-galactosidase activity. Luciferase activity specifically due to the presence of Tcf binding in response to Li⁺ exposure in pTopflash-positive cells was reported after subtracting background (pFopflash) activity. Samples from each experiment were assayed in triplicates and the results of three separate experiments are presented.

Autocrine growth factor release in response to Li⁺ or BIO exposure. Confluent donor cells were made quiescent by incubation in FBS-free DMEM for 48 h followed by an 8-h period of exposure to 10 mM Li⁺ or BIO (100 nM). The Li⁺- or BIO-exposed cells were washed twice with 1× PBS and incubated with fresh medium for an additional 24 h at 37°C. This medium was collected and designated as “conditioned medium.” In addition, the Li⁺- or BIO-containing medium removed before the PBS wash was extensively dialyzed (using a membrane with a 3.5-KDa cut off) against 1× PBS to remove residual Li⁺ and was designated as “dialyzed, conditioned medium.”

Recipient cells that were FBS starved for 48 h in the presence or absence of Li⁺ were incubated for 20 min at 37°C either with the conditioned medium or dialyzed, conditioned medium. Donor and conditioned medium-exposed cells were washed with 1× PBS and harvested in lysis buffer. The cell lysates were subjected to immunoblot analysis using anti-phospho-Ser473-specific Akt antibodies.

Analysis of IGF-I and IGF-II gene expression. Total RNA was isolated from control or Li⁺- or BIO-treated cells using TRIzol reagent and reverse transcribed using “Ready to Go” RT-PCR beads (Amersham Biosciences). PCR amplification was performed for IGF-I (forward primer, 5'-aaatggccacgctggatcc-3'; reverse primer, 5'-ctgaaggaaggcagttgctc-3'), IGF-II (forward primer, 5'-ctcgccgaggctgatccacg-3'; reverse primer, 5'-aggtggctgcgctgttgct-3'), and GAPDH (forward primer, 5'-ctggtctgcgctttgctg-3'; reverse primer 5'-cgtcctcctcctcctcct-3'). RT-PCR contained an initial reverse transcription step performed at 42°C for 30 min followed by 5-min incubation of the reaction mixture at 95°C to inactivate reverse transcriptase and denature the template. This was followed by 36 amplification cycles (95°C for 1 min, 55°C for 1 min, 72°C for 2 min) and a final elongation step (72°C for 15 min). PCR products were resolved on a 1% agarose gel and visualized by ethidium bromide during UV excitation.

RESULTS

Li⁺ or BIO increases survival and inhibits apoptosis after serum deprivation. Compared with control (grown in the presence of serum), serum deprivation decreased cell survival by 32% (Fig. 1A), whereas Li⁺ (2, 5, and 10 mM) significantly increased the number of viable cells by 28, 34, and 16%, respectively. Cell number after serum deprivation was also significantly increased in the presence of BIO (12, 61), a specific GSK3β inhibitor (Fig. 1B). In the presence of either 50 or 100 nM BIO, MTT absorbance exceeded 150% of control. In contrast, forskolin and sodium valproate [mood stabilizers that also inactivate GSK3β but have different chemical structures than Li⁺ (73)] failed to improve renal cell viability after serum deprivation (data not shown). Serum starvation activated caspase-3 (i.e., decreased the content of inactive procaspase-3), an enzyme responsible for the execution phase of apoptosis (Fig. 1C, lane 1). Compared with untreated cells, Li⁺ or BIO inhibited caspase-3 activation (Fig. 1C, lanes 2 and 3). Increased survival of Li⁺ or BIO-exposed serum-starved cells was confirmed by flow cytometric analysis of cells stained with annexin-V and propidium iodide (Fig. 1, D and E). In serum-replete cells at baseline, only 2.8 ± 0.6% of cells were annexin-V positive. In contrast, annexin-positive cells comprised 14.1 ± 2.4 and 38.2 ± 5.1% of the total cell population after 8 h or 7 days of serum starvation, respectively. Treatment with Li⁺ or BIO significantly reduced the number of apoptotic cells to 2.4 ± 0.6 and 4.0 ± 1.8% after 8 h of serum deprivation and to 10.7 ± 2.8 and 12.3 ± 2.1% following 7 days without serum (P < 0.05; n = 3). Less than 1% of cells were necrotic (propidium iodide positive, annexin-V negative) 8 h after serum removal. After 7 days, 15.6 ± 7.9% of cells were necrotic. Neither Li⁺ nor BIO significantly reduced the number of necrotic cells at either time point. These observations demonstrate that Li⁺ or BIO inhibits apoptosis.

Li⁺ and BIO activate the Wnt signaling pathway. Prior studies suggest that Li⁺ ameliorates or prevents apoptosis (28, 25, 51–53) by inhibiting GSK3β, a component of the Wnt signaling pathway (27, 36, 73). To evaluate the potential protective role of Wnt signaling during serum deprivation, the effect of Li⁺ and BIO on this pathway was investigated.

Li⁺ and BIO phosphorylate and inactivate GSK3β. After 48-h serum deprivation, cells were incubated with Li⁺ (10 mM) for 0–24 h. Inhibition of GSK3β (i.e., GSK3β phosphorylation) was assessed by immunoblot analysis using a phospho-serine-specific antibody. The level of inactive phosphorylated GSK3β was relatively low before the addition of Li⁺ (Fig. 2A: t = 0; lane 1) or BIO (Fig. 2B: t = 0; lane 1). One hour or more of Li⁺ exposure significantly increased the content of inactive, phosphorylated GSK3β (Fig. 2A: lanes 2–8). The level of phosphorylated (i.e., inactive) GSK3β could also be enhanced by BIO in a dose-dependant manner (Fig. 2B, lanes 3–8). In contrast, neither Li⁺ nor BIO exposure altered total GSK3β content (Fig. 2, A and B, bottom). Exposure to forskolin or valproate did not affect GSK3β phosphorylation (data not shown).

To provide additional evidence that Li⁺ inactivated GSK3β in these cells, the phosphorylation state of β-catenin (a substrate of GSK3β) was examined. A reduction in β-catenin...
LITHIUM AND CELL SURVIVAL

A

MTT absorbance (% control)

[Li⁺] (mM)

0 2 5 10

110 100 90 80 70 60 50 40

* * *

B

MTT absorbance (% control vs. untreated cells)

Li⁺ BIO

10 50 100

200 150 100 50 0

C

pro-caspase 3

Ctrl Li⁺ BIO

D

8 hr

Control Lithium (5mM) BIO (100nM)

7 days

Control Lithium (5mM) BIO (100nM)

E

Percentage cells

8 hr 7 days

Ctrl Li⁺ BIO Ctrl Li⁺ BIO

† † † † ** **
Li⁺ deprivation for 7 days reduced cell viability by 32%

Phosphorylation of GSK3β (ser9) increased with the duration of exposure to either Li⁺ or BIO in a concentration-dependent manner (top) in the absence of changes in total GSK3β. Results are representative of 5 individual experiments.

Li⁺ or BIO activates the PI3K/Akt survival pathway. Activation of Akt mediates the survival activity of virtually all extracellular survival factors (16, 17, 65). Conversely, dephosphorylation and inactivation of Akt lead to apoptosis (16, 17, 65). In renal epithelial cells, the level of phosphorylated (i.e., active) Akt was relatively low in cells deprived of serum for 48 h (Fig. 7A, lane 1) but progressively increased after 0–24 h of Li⁺ treatment (lanes 2–8). Compared with control (lane 1), the level of phosphorylated (i.e., activated) Akt also increased in a dose-dependent manner following BIO treatment (Fig. 7B, lanes 3–8).

Li⁺ activates Akt through PI3K. To evaluate the involvement of PI3K in Li⁺-stimulated Akt phosphorylation, LY-294002 and wortmannin, two specific PI3K inhibitors, were tested. These inhibitors are structurally unrelated and exert their effects on PI3K activity by distinct mechanisms (29). Compared with untreated control (lane 1), inhibition of PI3K with either 8-h (lanes 5 and 6) or 24-h (lanes 8 and 9) exposure to LY-294002 or wortmannin prevented Akt phosphorylation (Fig. 8A) and decreased cell survival (Fig. 8C) associated with Li⁺ exposure. These observations suggest that PI3K activates Akt and that Akt activation is responsible for the increased cell survival afforded by Li⁺ in serum-deprived cells.

Phosphorylation of GSK3β by Li⁺ occurs by a mechanism independent of the PI3K/Akt pathway. GSK3β phosphorylation is inhibited by Ser9 phosphorylation (6, 21). In serum-deprived cells, Li⁺ treatment was associated with a significant increase in GSK3β phosphorylation (Fig. 2B). GSK3β is a downstream target of Akt that inactivates the enzyme by Ser9 phosphorylation (22, 63, 75). Hosing that Li⁺ activates Akt and inhibits GSK3β, we next examined whether the effect of Li⁺

(determined by immunoblot analysis) progressively increased over 48 h in response to Li⁺ (Fig. 6). This observation is consistent with transcriptional activation of β-catenin-responsive TCF-LEF genes in response to Li⁺ (7).

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(determined by immunoblot analysis) progressively increased over 48 h in response to Li⁺ (Fig. 6). This observation is consistent with transcriptional activation of β-catenin-responsive TCF-LEF genes in response to Li⁺ (7).
on GSK3β is mediated by PI3K/Akt activation. Cells were exposed to Li⁺ in the presence of the PI3K inhibitor LY-294002. The increase in GSK3β phosphorylation caused by Li⁺ was minimally inhibited by pretreating the cells with LY-294002, a PI3K inhibitor (Fig. 8B). These data suggest that Li⁺ phosphorylates GSK3β-independent of the PI3K/Akt pathway.

Li⁺- or BIO-induced IGF-II expression serves as an autocrine factor that stimulates the PI3K/Akt pathway. Recent investigations suggest that the Wnt proteins inhibit apoptosis by inducing the expression and secretion of growth factors (e.g., IGFs) that act by an autocrine or paracrine mechanism to activate the anti-apoptotic PI3K/Akt pathway (28, 43–45). Therefore, the effect of Li⁺ and BIO on expression and secretion of growth factors was examined. In recipient cells, medium conditioned with Li⁺ (Fig. 9A) and BIO (Fig. 9B) strongly stimulated the phosphorylation of Akt (lane 1 vs. lane 2). This effect was observed even after conditioned medium was dialyzed (Fig. 10, lane 4). The effect of conditioned medium on p-Akt was inhibited by LY-294002, a PI3K inhibitor (Fig. 10, lanes 3 and 5 vs. lanes 2 and 4). However, no change in the content of p-GSK3β was observed in recipient cells following treatment with the conditioned medium harvested from the Li⁺-treated donor cells (Fig. 10, bottom). The lack of GSK3β phosphorylation reflects the absence of Li⁺ in the dialyzed, conditioned medium as well as in the dialysate.

Binding of autocrine growth factor(s) including IGFs to their receptors triggers a signaling cascade that includes the activation of PI3K, phosphoinositide-dependent protein kinase-1 (PDK-1), and ultimately, Akt phosphorylation (5, 23, 50). Active Akt then phosphorylates several target proteins whose activation (e.g., IkB kinase) or inactivation (e.g., bad, a BCL2 family member, forkhead and caspase-9) promotes cell survival (5). Given the potent growth factor activity exhibited by conditioned media harvested from Li⁺- or BIO-treated cells, a PI3K-dependent event was suspected. In fact, LY-294002 prevented Akt activation (i.e., phosphorylation) induced by Li⁺ exposure in both donor cells (lane 3 vs. 2, Fig. 9, A and B) and in recipient cells incubated with conditioned media obtained from Li⁺- or BIO-treated cells decreased Akt phosphorylation (lane 3 vs. 2, Fig. 9, A and B). This observation suggests that the autocrine or paracrine growth factor(s) present in the conditioned medium activate Akt in a PI3K-dependent manner.

To evaluate the potential involvement of IGFs in the activation of the PI3K/Akt pathway in recipient cells, IGF binding protein-4 (IGFBP), a known inhibitor of IGFs (43), was tested. IGFBP-4 significantly decreased PI3K/Akt phosphorylation attributable to conditioned media in both donor (Fig. 9, A, top, lane 4 vs. lane 2) and recipient cells (lane 4 vs. lane 2, Fig. 9, A and B). This indicates that the IGFs mediate the effect of Li⁺-activated Wnt signaling pathway by altering PI3K-mediated Akt phosphorylation.

Li⁺ or BIO stimulates IGF-II expression. To test the hypothesis that Li⁺ and BIO stimulate Tcf-Lef-mediated transcription of anti-apoptotic genes including IGF-I and IGF-II, RT-PCR analyses were performed on RNA extracted from
control and Li\textsuperscript{+}-treated cells. Relative to GAPDH, Li\textsuperscript{+} (Fig. 11A) or BIO (Fig. 11B) exposure increased the expression of IGF-II compared with control. In contrast, IGF-I expression could not be detected in either control or Li\textsuperscript{+}-exposed cells (Fig. 11A).

**DISCUSSION**

All cells possess a constitutively expressed “default pathway” capable of initiating apoptotic cell death unless it is constantly and specifically inhibited by growth or other survival factors (17, 18, 57, 65, 68). We previously showed that primary cultures of mouse proximal tubular cells undergo apoptosis when deprived of soluble survival factors (65) and that the presence of growth factors such as EGF and IGF prevents activation of this default pathway (41). In the absence of growth factors, this prior study demonstrated that apoptosis is associated with a progressive decrease in the activity of Akt, a kinase critical to cell survival (41). The present study examines the regulation of the default pathway in a conditionally immortalized renal epithelial cell line that possesses the structural and functional features of a homogenous population of differentiated proximal tubular cells (66). Immortalized cells, like primary cultures of tubular cells (65), undergo apoptosis over a 7- to 10-day time period when deprived of growth factors (Fig. 1). Li\textsuperscript{+} and BIO have been reported to improve survival in neuronal cells by activating the PI3K/Akt anti-apoptotic pathway as well as by activating Wnt signaling through inhibition of GSK3\textbeta (8, 9, 19, 21, 25, 36). Given the similarity between the mechanism of action of growth factors and Li\textsuperscript{+}, the survival effect of Li\textsuperscript{+} or BIO on renal epithelial cells was assessed.

The current study demonstrates several key findings. First, the addition of Li\textsuperscript{+} or BIO to the medium significantly increased cell number in the absence of soluble growth or survival factors and other anti-apoptotic stimuli as shown by the MTT assay, analysis of annexin-V staining and caspase-3 activation (Fig. 1). Second, Li\textsuperscript{+} or BIO increased the number of viable cells by inhibiting GSK3\textbeta (Fig. 2) and then sequentially activating Wnt signaling followed by the activation of the anti-apoptotic PI3K/Akt pathway (Fig. 7, A and B). A central role for GSK3\textbeta in the cell survival pathway is supported by the observation that BIO, a GSK3\textbeta inhibitor, also increased the...
number of viable cells (Fig. 1B) and activated Wnt signaling (Fig. 2B and Ref. 61). Third, pharmacological inhibition of the PI3K/Akt pathway (Fig. 8A) had no effect on Li⁺-induced GSK3β inhibition (Fig. 8B) but completely abrogated the prosurvival effect of Li⁺ (Fig. 8C). This suggests that the beneficial effect of PI3K/Akt activation is a downstream effect of Wnt signaling as outlined in Fig. 12. Fourth, exposure to Li⁺ and BIO increased the expression of Tcf-Lef genes including IGF-II (Fig. 11), a known activator of the PI3K/Akt pathway (28, 43–45) as well as cyclin D1 (Fig. 6), a protein that promotes cell cycle entry (31, 37, 49, 70). Finally, conditioned medium harvested from Li⁺- or BIO-treated (B) donor cells activated the PI3K/Akt pathway (Figs. 9 and 10), an effect that was inhibited by LY-294002. Both p-Akt and total Akt content were examined by immunoblot analysis in each cell group. Each lane contains 25 μg of total protein. Individual blots were stripped and then probed with total Akt antibody to confirm equivalent sample loading (n = 5).

On the basis of these findings, we hypothesize that in response to Li⁺, Wnt-dependent modulation of Akt activity requires the assembly of several signaling molecules including GSK3β, β-catenin, β-catenin-responsive Tcf-Lef elements, cyclin D1, IGF-II, PI3K, and Akt. A summary of these putative signaling events is schematically shown (Fig. 12). GSK3β is a highly conserved protein kinase thought to be constitutively active in differentiated cells (15, 59). It is an important component of Wnt signaling and its inhibition plays a crucial role in cell proliferation during embryogenesis (26, 59). Constitutive GSK3β activity can be suppressed by a variety of stimuli that cause ser9 phosphorylation, including Wnt ligands, EGF, and FGF (11, 26). Conversely, GSK3β is activated in a variety of cell types by noxious stimuli including hypoxia (42), serum starvation (59), hypertonic stress (59), and potassium deprivation (12). Altered GSK3β activity has also been implicated in several human conditions including diabetes mellitus, cancer, and Alzheimer’s disease (46).

Fig. 9. A and B: effect of insulin-like growth factor-binding protein (IGFBP) and LY-294002 on Akt phosphorylation in Li⁺- or BIO-treated donor and recipient cells exposed to conditioned medium. Donor (top) or recipient (bottom) cells were incubated with the conditioned media obtained from Li⁺ (A) or BIO-treated (B) donor cells in the presence or absence of either IGFBP-4 (2.5 nM) or LY-294002. Both p-Akt and total Akt content were examined by immunoblot analysis in each cell group. Each lane contains 25 μg of total protein. Individual blots were stripped and then probed with total Akt antibody to confirm equivalent sample loading (n = 5).

Fig. 10. Effect of medium harvested from Li⁺-treated cells on p-Akt and p-GSK3β in the presence and absence of LY-294002. Recipient cells were subjected to 48-h serum deprivation and were then incubated either with conditioned medium or dialyzed, conditioned medium for 30 min in the presence or absence of LY-294002, a PI3K inhibitor. The content of phospho-ser473 Akt (top) and phospho-ser9 GSK3β (bottom) was examined by immunoblot analysis. Each lane contains 25 μg of total protein. Both types of media increased p-Akt in a PI3K-dependent manner in the absence of changes in GSK3β phosphorylation. These results are representative of 3 separate experiments.

Fig. 11. A and B: effect of Li⁺ or BIO on IGF-I and IGF-II expression. IGF-I, IGF-II, and GAPDH expression was assessed in samples of total RNA isolated from serum-starved control (Control) or Li⁺ (10 mM; A) or BIO-treated cells (B) by RT-PCR as described in MATERIALS AND METHODS. Products were separated on a 1.5% agarose gel in 1× Tris acetate EDTA and were visualized by UV excitation after ethidium bromide staining. In contrast to IGF-I (a product of similar size as IGF-II), IGF-II mRNA was increased by Li⁺ treatment. These results are representative of 5 separate experiments.
The ability of Wnt signaling to inhibit apoptosis has been described in nonrenal cells. Inhibitors of GSK3β [e.g., Li+ (74), SB-216763, SB-415286 (7, 12), and BIO (61)] activate Wnt signaling and promote cell survival. The observation that BIO exposure markedly increased MTT absorbance in serum-deprived cells compared with serum-replete control (Fig. 1) suggests that GSK3β inhibition promotes proliferation. Although the proliferation rate has not been formally assessed in this study, this hypothesis is consistent with the proliferative role of the Wnt pathway (6, 67) as well as the observed stimulation of cyclin D1 (Fig. 6). Furthermore, BIO has been recently shown to maintain an undifferentiated phenotype in human and mouse embryonic stem cells (HESCs and MESCs) manifested as the sustained expression of pleuripotent state-specific expression of transcription factors (61). In addition, Wnt signaling is endogenously activated in undifferentiated MESCs and is downregulated during differentiation (61). Importantly, BIO-mediated Wnt activation is reversible, as withdrawal of this compound leads to normal multidifferentiation programs in both HESCs and MESCs (61). Collectively, these data define a ubiquitous mechanism by which Wnt-mediated gene expression regulates both apoptosis and proliferation in a variety of cell types. Therefore, the enhanced expression of β-catenin-responsive TCF-Lef genes (e.g., cyclin D1) in response to Li+ in a nonpleuripotent renal epithelial cell line suggests that Li+ exposure drives these cells toward pleuripotency. This hypothesis is supported by recent reports of renal epithelial cell dedifferentiation into mesenchymal cells following injury (60, 76).

Both IGF-I and IGF-II have been reported to act through a PI3K-dependent mechanism to protect 3T3-L1 cells from apoptosis during serum deprivation (5, 23, 43, 50). IGF-I and IGF-II activate anti-apoptotic pathways in a number of cell types and tissues (43). The anti-apoptotic kinase Akt is phosphorylated and activated in response to IGF-I in a PI3K-dependent manner, suggesting that Akt may be central to IGF-mediated survival in 3T3-L1 cells (23). Interestingly, both IGF-I and IGF-II have been shown to influence β-catenin stability by inhibiting its phosphorylation and can therefore influence β-catenin-mediated gene expression (23, 48, 56). These events indicate that Wnt signaling and IGF expression may have both primary and secondary effects on gene expression (48, 56).

IGF-II (also known as multiplication stimulating activity or MSA) is a potent mitogenic growth factor. In contrast to IGF-I (somatomedin A and somatomedin C) that has important postnatal functions, the growth-promoting function of IGF-II is limited to embryonic development (20). Therefore, increased expression of IGF-II caused by Li+--catenin-mediated gene expression (23, 48, 56). These events indicate that Wnt signaling and IGF expression may have both primary and secondary effects on gene expression (48, 56).

In summary, exposure of renal epithelial cells to Li+ or BIO in a medium deprived of growth and survival factors is associated with inhibition of GSK3β and activation of Wnt signaling. Wnt activation is evidenced by the decreased phosphorylation and nuclear translocation of β-catenin with subsequent transcription of genes encoding for IGF-II, an autocrine survival factor. Newly synthesized IGF-II activates the PI3K/Akt pathway and inhibits apoptosis. Manipulation of this pathway could contribute new strategies for improving renal epithelial cell survival after stress.

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