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Regulation of Akt mRNA and protein levels by glycogen synthase kinase-3β in adrenal chromaffin cells: Effects of LiCl and SB216763

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ABSTRACT

In cultured bovine adrenal chromaffin cells, where Akt1 is the predominant isoform over Akt2 and Akt3, chronic (≥12 h) treatment with 1–20 mM LiCl, an inhibitor of glycogen synthase kinase-3, decreased Akt1 level by ~52% (EC_{50}=3.7 mM; $t_{1/2}$=12 h); it was associated with LiCl-induced increased levels of Ser^9-phosphorylated glycogen synthase kinase-3β (~37%) and β-catenin (~59%), two hallmarks of glycogen synthase kinase-3β inhibition. The same LiCl treatment did not change phosphoinositide 3-kinase, phosphoinositide-dependent kinase 1, and extracellular signal-regulated kinase-1/2 levels. Treatment with SB216763 [3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione], a selective inhibitor of glycogen synthase kinase-3, lowered Akt1 level by ~67% (EC_{50}=2 μM; $t_{1/2}$=12 h), when SB216763 caused concentration- and time-dependent increase of β-catenin level by ~76%. LiCl- or SB216763-induced Akt1 decrease, as well as increases of Ser^9-phosphorylated glycogen synthase kinase-3β and β-catenin were restored to the control levels of nontreated cells after the washout of LiCl (20 mM for 24 h)- or SB216763 (30 μM for 24 h)-treated cells. LiCl-induced Akt1 reduction was not prevented by β-lactone, lactacystin (two inhibitors of proteasome), calpastatin (an inhibitor of calpain), or leupeptin (an inhibitor of lysosome). LiCl decreased Akt1 mRNA level by 20% at 6 h, with no effect on Akt1 mRNA stability. These results suggest that glycogen synthase kinase-3β inhibition caused down-regulation of Akt1 mRNA and Akt1 protein levels; conversely, constitutive activity of glycogen synthase kinase-3β maintains steady-state level of Akt1 in quiescent adrenal chromaffin cells.

1. Introduction

Glycogen synthase kinase-3 is constitutively active in nonstimulated cells, where the majority of its substrates (e.g., β-catenin) are subjected to inactivation/degradation after phosphorylation (Jope and Johnson, 2004; Meijer et al., 2004; Jope et al., 2007). Receptor tyrosine kinases (e.g., insulin receptor), G protein-coupled receptors, Wnt receptor (Jope and Johnson, 2004; Meijer et al., 2004; Jope et al., 2007), depolarization (Lee et al., 2005), electroconvulsive shock treatment (Roh et al., 2003) and hyperglycemia (Clodfelder-Miller et al., 2005) culminate in Ser^{21}/Ser^9-phosphorylation of glycogen synthase kinase-3α/3β inhibiting catalytic activity of glycogen synthase kinase-3α/3β. Glycogen synthase kinase-3β knockout in mice caused embryonic lethality due to hepatocyte apoptosis, resembling dysfunction of nuclear factor-κB (Hoeflich et al., 2000). Embryonic fibroblasts derived from glycogen synthase kinase-3β knockout mice were sensitive to apoptosis (Takada et al., 2004).

Insulin receptor triggers Tyr-phosphorylation of insulin receptor substrate-1, insulin receptor substrate-2 and Shc, activating two major phosphorylation cascades [i.e., phosphoinositide 3-kinase/phosphoinositide-dependent kinase 1/Akt and Ras/extracellular signal-regulated kinase]. Akt catalyzes inhibitory Ser^{21}/Ser^9-phosphorylation of glycogen synthase kinase-3α/3β (Jope and Johnson, 2004; Meijer et al., 2004; Jope et al., 2007), as well as phosphorylation/inhibition of transcription factor FOXO, proapoptotic Bad, and translation inhibitor tuberin (Manning, 2004). Besides, Akt plays previously unrecognized roles in physiological (e.g., differentiation; polarity; survival; scaffold; pain; reward) and pathological (e.g., tumorigenesis; neurodegeneration) events (Brazil et al., 2004; Song et al., 2005; Stambolic and Woodgett, 2006; Yoeli-Lerner and Toker, 2006; Manning and Cantley, 2007; Russo et al., 2007) by acting in cytoplasm, nucleus (Martelli et al., 2006), endoplasmic reticulum (Hosoi et al., 2007), and mitochondria (Parcellier et al., 2007). Evidence has accumulated that dysregulated hyperactivity of glycogen synthase kinase-3 is associated with insulin resistance, psychiatric (e.g., bipolar mood disorder)/neurodegenerative (e.g., Alzheimer's disease) diseases, tumorigenesis and inflammation (e.g., bronchial asthma, sepsis, shock) (Jope and Johnson, 2004; Meijer et al., 2004; Wada et al., 2005a,b; Dugo et al., 2006; Bao et al., 2007; Jope et al., 2007).

Consistently, lithium and a growing number of synthetic glycogen synthase kinase-3 inhibitors have turned out to be effective against acute brain injuries and chronic neurodegenerative diseases (Chalecka-

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Franaszek and Chuang, 1999; Jope and Johnson, 2004; Meijer et al., 2004; Wada et al., 2005b; Sasaki et al., 2006; Jope et al., 2007).

In adrenal chromaffin cells (embryologically derived from the neural crest), various agents inhibiting glycogen synthase kinase-3β activity [i.e., insulin (Yamamoto et al., 1996), valproic acid (Yamamoto et al., 1997), insulin-like growth factor-I, lithium, and SB216763 [3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione] (Wada et al., 2005a,b; Yanagita et al., 2007)] up-regulated expression of voltage-dependent sodium channel, augmenting $^{22}Na^{+}$ influx, $^{45}Ca^{2+}$ influx via voltage-dependent calcium channel and exocytosis of catecholamines. Nicotinic acetylcholine receptor/protein kinase C-α extracellular signal-regulated kinase 1/extracellular signal-regulated kinase 2 pathway up-regulated insulin receptor substrate-1/insulin receptor substrate-2 levels, enhancing insulin-induced phosphorylation of phosphoinositide 3-kinase/Akt/glycogen synthase kinase-3β and extracellular signal-regulated kinase 1/extracellular signal-regulated kinase 2 (Sugano et al., 2006). Constitutive and negatively-regulated activities of glycogen synthase kinase-3β, respectively, up- and down-regulated insulin receptor substrate-1/insulin receptor substrate-2 and insulin receptor levels via controlling proteasomal degradation and/or protein synthesis (Nemoto et al., 2006; Yokoo et al., 2007). Here, chronic treatment with LiCl or SB216763 increased β-catenin level or Ser^{9}-phosphorylation of glycogen synthase kinase-3β, while decreasing Akt protein and mRNA levels, without altering phosphoinositide 3-kinase, phosphoinositide-dependent kinase 1 and extracellular signal-regulated kinase 1/extracellular signal-regulated kinase 2 protein levels. These LiCl- or SB216763-induced changes were restored to control levels of nontreated cells after the washout of test compound-treated cells.

2. Materials and methods

2.1. Materials

Eagle's minimum essential medium was from Nissui Seiyaku (Tokyo, Japan). Calf serum, phenylmethylsulfonyl fluoride, leupeptin, aprotinin, sodium orthovanadate, Nonidet P-40, and Tween-20 were from Nacalai Tesque (Kyoto, Japan). LiCl, cytosine arabinoside, *clasto*-lactacystin β-lactone, and actinomycin D were from Sigma (St. Louis, MO). SB216763 [3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione] was from Tocris Cookson (Bristol, UK). Okadaic acid, lactacystin, and calpastatin were from Calbiochem–Novabiochem (San Diego, CA). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody, ECL Plus Western Blotting Detection Reagents, Hybond-N, Hybond-P, Rapid-hyb buffer, and [α-^{32}P]dCTP (>4000 Ci/mmol) were from Amersham Biosciences (Piscataway, NJ). Rabbit polyclonal antibody against p85 subunit of phosphoinositide 3-kinase was from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal antibodies against Ser^{473}-phosphorylated Akt1, Ser^{9}-phosphorylated glycogen synthase kinase-3β or phosphoinositide-dependent kinase 1 were from Cell Signaling Technology (Beverly, MA). Mouse monoclonal glycogen synthase kinase-3β antibody or rabbit polyclonal extracellular signal-regulated kinase antibody was from BD Transduction Laboratories (San Diego, CA). Mouse monoclonal antibodies against Akt1 or β-catenin were from Santa Cruz Biotechnology (Santa Cruz, CA). Bovine adrenal chromaffin cells predominantly express Akt1 isoform, with far lower levels of Akt2 and Akt3 isoforms (Evans et al., 2006); Akt1 antibody is recommended for detection of Akt1 (to a lesser extent, Akt2 and Akt3) by the manufacture's instruction. Can Get signal™ immunoreaction Enhancer Solution-1 and -2 were from TOYOBO (Osaka, Japan). TRIZOL reagent was from Invitrogen (Carlsbad, CA). Oligotex-dT30<Super> was from Nippon Roche (Tokyo, Japan). BcaBEST labeling kit and Noninterfering Protein Assay kit were from Takara (Shiga, Japan). cDNA for human glyceraldehyde 3-phosphate dehydrogenase was from Clontech Laboratories (Palo Alto, CA). Plasmid containing Akt1 cDNA [pBluescript II SK (−)] was generously donated from Dr. Kikkawa, U. (Biosignal Research Center, Kobe University).

2.2. Primary culture of adrenal chromaffin cells: treatment with test compounds

Isolated bovine adrenal chromaffin cells were cultured (4×10^{6} per dish, Falcon; 35 mm diameter) in Eagle's minimum essential medium containing 10% calf serum under 5% CO_2/95% air in a CO_2 incubator (Yamamoto et al., 1996, 1997). Three days (60–62 h) later, the cells were treated in the fresh culture medium without or with LiCl or SB216763 for up to 48 h in the absence or presence of β-lactone, lactacystin, calpastatin, or leupeptin. SB216763, β-lactone, lactacystin, and leupeptin were dissolved in dimethyl sulfoxide; the final concentrations (~0.2%) of dimethyl sulfoxide in the test medium did not affect Akt1 level. The culture medium contained 3 μM cytosine arabinoside to suppress the proliferation of nonchromaffin cells; when chromaffin cells were further purified by differential plating (Yamamoto et al., 1996, 1997), Akt1 level was similar between purified and conventional chromaffin cells. Also, LiCl treatment (20 mM for 12 h) decreased Akt1 level by 23 and 21% in purified and conventional chromaffin cells, compared with nontreated cells within each cell group.

2.3. Western blot analysis of Ser^{473}-phosphorylated Akt1, Akt1, phosphoinositide 3-kinase, phosphoinositide-dependent kinase 1, extracellular signal-regulated kinase and β-catenin

Cells were washed with ice-cold Ca^{2+}-free phosphate-buffered saline and solubilized in 500 μl of 2× sodium dodecyl sulfate electrophoresis sample buffer (125 mM Tris–HCl [pH 6.8], 20% glycerol, 10% 2-mercaptoethanol, and 4% sodium dodecyl sulfate) at 98 °C for 3 min. Total quantities of cellular proteins, as measured by the Noninterfering Protein Assay kit, were not changed between nontreated and test compound-treated cells. The same amounts of proteins (7.0–7.5 μg per lane) were separated by sodium dodecyl sulfate-7.5% or -12% polyacrylamide gel electrophoresis, and transferred onto a nitrocellulose membrane (Hybond-P). The membrane was preincubated with 1% bovine serum albumin in Tween–Tris-buffered saline (10 mM Tris–HCl [pH 7.4], 150 mM NaCl, and 0.1% Tween-20), and reacted overnight at 4 °C in Can Get Signal Solution-1 with mouse or rabbit antibody (1:2000) against Ser^{473}-phosphorylated Akt1, Akt1, phosphoinositide 3-kinase, phosphoinositide-dependent kinase 1, extracellular signal-regulated kinase, or β-catenin (Nemoto et al., 2006; Sugano et al., 2006). After repeated washings, the immunoreactive bands were reacted in Can Get Signal Solution-2 with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody, then visualized by the enhanced chemiluminescent detection system ECL Plus, and quantified by a luminoimage LAS-3000 analyzer (Fuji Film, Tokyo).

2.4. Western blot analysis of Ser^{9}-phosphorylated glycogen synthase kinase-3β and glycogen synthase kinase-3β

Cells were washed with ice-cold phosphate-buffered saline, scraped into tube, and centrifuged at 500 ×g for 3 min at 4 °C. After the supernatant was aspirated, the cells were lysed in 100 μl of lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM *O*,*O*'-bis(2-aminoethyl)ethyleneglycol-*N*,*N*,*N*',*N*'-tetraacetic acid (EGTA), 0.2% Nonidet P-40, 1 mM sodium orthovanadate, 100 μM phenylmethylsulfonyl fluoride, 0.2 nM okadaic acid, 10 μg/ml leupeptin, and 10 μg/ml aprotinin), sonicated for 10 s, and centrifuged at 20,000 ×g for 15 min at 4 °C. The supernatant was solubilized in 100 μl of 2× electrophoresis sample buffer at 98 °C for 5 min. The same amount of protein

(20 μg/each lane) was separated by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis, and transferred onto a nitrocellulose membrane. The membrane was preincubated with 1% bovine serum albumin in the Tween–Tris-buffered solution, and reacted overnight at 4 °C in Can Get Signal Solution-1 with Ser^{9}-phosphorylated glycogen synthase kinase-3β antibody (1:2000) (Nemoto et al., 2006; Sugano et al., 2006). The immunoreactive bands were labeled with horseradish peroxidase-conjugated anti-rabbit antibody (1:5000) and analyzed by a luminoimage LAS-3000 analyzer. The membrane was rinsed at 60 °C for 30 min with stripping buffer (100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate, and 62.5 mM Tris–HCl [pH 6.7]) to remove phospho-specific antibody, and used for reprobing with glycogen synthase kinase-3β antibody (1:5000).

2.5. Northern blot analysis of Akt1 mRNA level

Total cellular RNA was isolated from cells by acid guanidine-thiocyanate-phenol-chloroform extraction using TRIzol reagent. Poly(A)$^{+}$ RNA was purified by Oligotex-dT30<Super >, electrophoresed on 1% agarose gel containing 6.3% formaldehyde in buffer (40 mM 3-[*N*-morpholino] propanesulfonic acid [pH 7.2], 0.5 mM ethylenediaminetetraacetic acid (EDTA), and 5 mM sodium citrate), transferred to a nylon membrane (Hybond-N) in 20× saline-sodium citrate (1× saline-sodium citrate=0.15 M NaCl and 0.015 M sodium citrate) overnight, and cross-linked using a UV cross-linker (Funakoshi, Tokyo). cDNA fragment of Akt1 (nucleotides 2–480) was obtained by digestion of pBluescript II SK (−) by BamHI. Akt1 cDNA and glyceraldehyde 3-phosphate dehydrogenase cDNA (1.1 kbp) were labeled with [α-^{32}P]dCTP using the BcaBEST labeling kit (Yamamoto et al., 1996, 1997; Nemoto et al., 2006; Sugano et al., 2006). The membrane was prehybridized with Rapid-hyb buffer at 65 °C, and then hybridized with the Akt1 probe under the same condition for 18 h. It was washed at 65 °C in 2×, 1×, and 0.2× saline-sodium citrate containing 0.1% sodium dodecyl sulfate, each for 30 min twice, and subjected to autoradiography. The same membrane was hybridized with glyceraldehyde 3-phosphate dehydrogenase probe, after it was thoroughly washed in 0.1% sodium dodecyl sulfate at 100 °C to remove Akt1 probe. The autoradiogram was quantified by a bioimage BAS 2000 analyzer (Fuji Film).

2.6. Statistical methods

All experiments were repeated three times (mean±S.E.M.). Significance ($P<0.05$) was determined by one-way or two-way ANOVA with post hoc mean comparison by Newman–Keuls multiple range test. Student's *t* test was used when two means of group were compared.

3. Results

3.1. Selective decrease of Akt1 level by LiCl in adrenal chromaffin cells: no effect of LiCl on phosphoinositide 3-kinase, phosphoinositide-dependent kinase 1 and extracellular signal-regulated kinase 1/extracellular signal-regulated kinase 2 levels

LiCl is a competitive inhibitor of Mg^{2+} between 2 and 20 mM, being a direct reversible inhibitor of Mg^{2+}-ATP-dependent catalytic activity of glycogen synthase kinase-3α/3β (Klein and Melton, 1996; Jope, 2003). In our present study, cells were treated without or with 20 mM LiCl for 24 h; the cell lysates were subjected to Western blot analysis by using antibodies against Akt1, phosphoinositide 3-kinase, phosphoinositide-dependent kinase 1, or extracellular signal-regulated kinase (Fig. 1). LiCl decreased Akt1 level by 54% (left panel, upper blot), without appreciably changing phosphoinositide 3-kinase, phosphoinositide-dependent kinase 1, and extracellular signal-regulated kinase 1/extracellular signal-regulated kinase 2 levels (right panel).

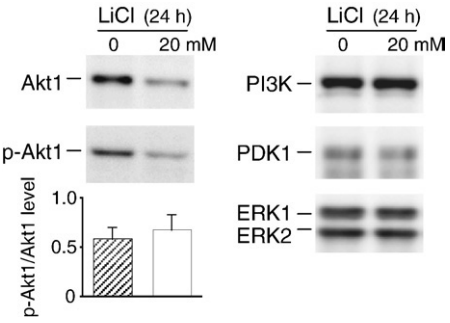

Fig. 1. LiCl-induced selective decrease of Akt1 level in adrenal chromaffin cells. Cells were treated without or with 20 mM LiCl for 24 h; the cell lysates were subjected to Western blot analysis by using antibodies against Aktl, phosphorylated Aktl (p-Akt1), phosphoinositide 3-kinase (PI3K), phosphoinositide-dependent kinase 1 (PDK1), or extracellular signal-regulated kinase (ERK). Blot are typical from 3 independent experiments. Immunoreactive Akt1 and phosphorylated Akt1 (p-Akt1) levels were quantified by a bioimage analyzer, and the relative level of phosphorylated Akt1 (p-Akt1)/Akt1 was calculated. Mean±SEM ($n=3$).

Ser^{21}/Ser^{9}-phosphorylation of glycogen synthase kinase-3α/3β by LiCl is a hallmark of glycogen synthase kinase-3α/3β inactivation, although the exact phosphorylation mechanisms are not yet fully clarified (Jope, 2003). Several investigators showed that LiCl activated phosphoinositide 3-kinase (Chalecka-Franaszek and Chuang, 1999) and inhibited protein phosphatase 2A (Sasaki et al., 2006), which caused activation/phosphorylation of Akt, thus increasing Akt-catalyzed Ser^{21}-/Ser^{9}-phosphorylation of glycogen synthase kinase-3α/3β. Fig. 1 (left pannel, lower blot) shows that LiCl (20 mM for 24 h) decreased Ser^{473}-phosphorylated Akt1 level by 48%; relative level of Ser^{473}-phosphorylated Akt1/Akt1 was not significantly changed between LiCl-treated and nontreated cells (bottom graph).

3.2. LiCl-induced concentration-dependent decreased level of Akt1, as well as increased levels of β-catenin and Ser^{9}-phosphorylated glycogen synthase kinase-3β

It has been shown that cellular accumulation of β-catenin is an index of glycogen synthase kinase-3 inactivation, because of the prevention of glycogen synthase kinase-3-induced proteasomal degradation of β-catenin (Coghlan et al., 2000). Fig. 2A shows that cells were treated without or with 1–20 mM LiCl for 24 h, and subjected to Western blot analysis. LiCl (≥3 mM) decreased Akt1 level in a concentration-dependent manner, developing into 52% reduction at 20 mM LiCl (Fig. 2B). Fig. 2C shows that LiCl (≥3 mM) raised β-catenin level by ~59% in a concentration-dependent manner. As shown in Fig. 2A, LiCl (≥3 mM) increased per se glycogen synthase kinase-3β level (fourth panel), with no discernible effect on phosphoinositide 3-kinase level (bottom panel). We calculated the relative level of Ser^{9}-phosphorylated glycogen synthase kinase-3β/glycogen synthase kinase-3β; LiCl (≥1 mM) increased the relative level by ~37% in a concentration-dependent manner (Fig. 2D), as shown in our previous study (Nemoto et al., 2006).

3.3. LiCl-induced time-dependent decreased level of Akt1 and increased levels of β-catenin and Ser^{9}-phosphorylated glycogen synthase kinase-3β: restoration by washout of LiCl-treated cells

Fig. 3A shows that cells were treated without or with 20 mM LiCl for up to 48 h. LiCl decreased Akt1 level by ~43% between 12 and 48 h (Fig. 3B), while increasing β-catenin and Ser^{9}-phophorylated glycogen

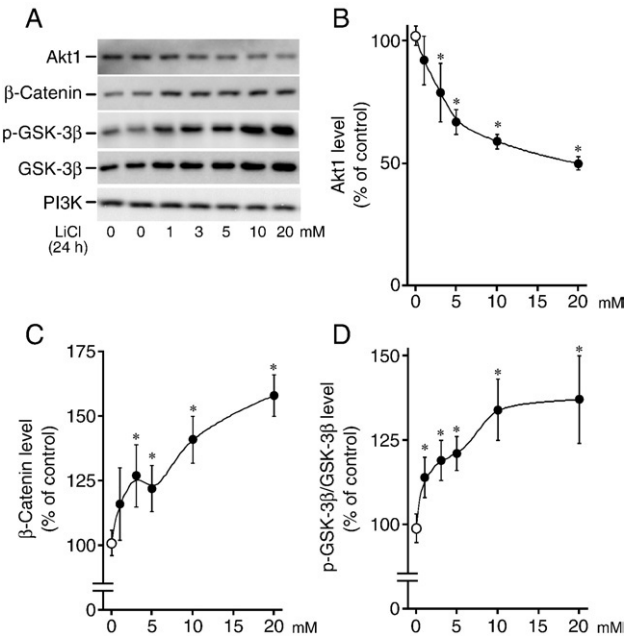

Fig. 2. LiCl-induced concentration-dependent decrease of Akt1 level, as well as increases of β-catenin and Ser[9]-phosphorylated glycogen synthase kinase-3β levels. (A) Cells were treated for 24 h without or with 1–20 mM LiCl, and subjected to Western blot analysis for Akt1, β-catenin, Ser[9]-phosphorylated glycogen synthase kinase-3β (p-GSK-3β), glycogen synthase kinase-3β (GSK-3β and phosphoinositide 3-kinase (PI3K). Blot data are typical from 3 independent experiments with similar results. (B, C and D) Immunoreactivities in panel (A) were quantified by a bioimage analyzer, and relative level of Ser[9]-phosphorylated glycogen synthase kinase-3β/glycogen synthase kinase-3β (p-GSK-3β/GSK-3β at each lane was calculated. (○) None; (●) LiCl. A value of 100% represents the level obtained in the left lane of LiCl-nontreated cells. Mean±SEM (n=3). *P<0.05, compared with LiCl-nontreated cells.

synthase kinase-3β levels by ~49 and ~64% between 6 and 48 h (Fig. 3C and D). In contrast, LiCl (20 mM for ~48 h) did not significantly alter phosphoinositide 3-kinase level (Fig. 3A, bottom panel).

As shown in Fig. 3A (upper right labeled by Wash at 24 h), cells were treated without or with 20 mM LiCl for the first 24 h, washed at 24 h with culture medium, and incubated in the continuous absence of LiCl for up to 48 h. LiCl-induced decreased level of Akt1 (Fig. 3B), as well as increased levels of β-catenin (Fig. 3C) and Ser[9]-phophorylated glycogen synthase kinase-3β (Fig. 3D) were restored to the control levels of nontreated cells at 48 h.

3.4. SB216763-induced concentration- and time-dependent decrease of Akt1 level and increase of β-catenin level: restoration by washout of SB216763-treated cells

SB216763 [3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione] is a selective inhibitor for glycogen synthase kinase-3; previous *in vitro* assay showed that among 25 different protein kinases, SB216763 inhibited only glycogen synthase kinase-3β by 96% at 10 μM (Coghlan et al., 2000). In adrenal chromaffin cells, our previous Western blot analysis showed that 12 h-treatment with SB216763 increased β-catenin level by ~47% (EC_{50}=1 μM) (Nemoto et al., 2006). In Figs. 4A (upper blot) and 4B, 12 h-treatment with SB216763 decreased Akt1 level by ~67% (EC_{50}=2 μM). Fig. 4A (lower two blots) shows that SB216763 caused time-dependent decrease of Akt1 level ($t_{1/2}$=12 h) (Fig. 4C) and increase of β-catenin level ($t_{1/2}$=8.2 h) (Fig. 4D).

As shown in Fig. 4A (lower two blots; upper right labeled by Wash at 24 h), cells were treated without or with 30 μM SB216763 for the first 24 h, washed, and incubated in the continuous absence of SB216763 for up to 48 h; SB216763-induced reduction of Akt1 level (Fig. 4C) and elevation of β-catenin level (Fig. 4D) were restored toward the control levels of nontreated cells at 48 h.

3.5. No prevention of LiCl-induced decrease of Akt1 level by proteolysis inhibitors

In adrenal chromaffin cells, our previous study showed that LiCl (20 mM for 12 h) decreased protein levels of insulin receptor substrate-1 and insulin receptor substrate-2, which were prevented by the concurrent treatment of β-lactone (20 μM) or lactacystin (1 μM) (two inhibitors of proteasome) (Nemoto et al., 2006). By using cell surface [^{3}H]saxitoxin binding assay, we also observed that simultaneous 24 h-treatment with 1 μM calpastatin (calpain inhibitor) blocked reduction of cell surface sodium channel number caused by an increased concentration of cytoplasmic Ca^{2+} (Shiraishi et al., 2001). In cultured microglial cells, Takai et al. (1998) showed that 100 μM leupeptin (lysosome inhibitor) prevented apoptosis caused by 6-hydroxydopamine (100 μM for 24 h). In our present study, Fig. 5 shows

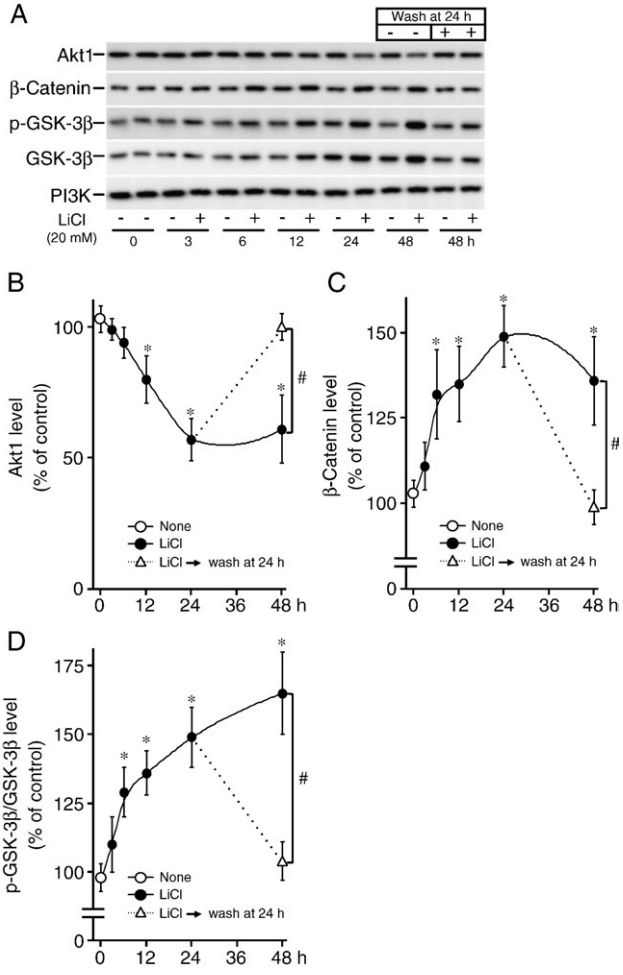

Fig. 3. LiCl-induced time-dependent decrease of Akt1 level, as well as increases of β-catenin and Ser[9]-phosphorylated glycogen synthase kinase-3β levels: restoration by washout of LiCl-treated cells. (A) Cells were treated without (−) or with (+) 20 mM LiCl for up to 48 h. In parallel experiment, cells were treated without (−) or with (+) 20 mM LiCl for the first 24 h, washed at 24 h (upper right labeled by Wash at 24 h), and incubated in the continuous absence of LiCl for up to 48 h. Blot data are typical from 3 independent experiments with similar results. (B, C and D) Immunoreactivities in panel (A) were quantified by a bioimage analyzer, and relative level of Ser[9]-phosphorylated glycogen synthase kinase-3β/glycogen synthase kinase-3β (p-GSK-3β/GSK-3β at each lane was calculated. (○) None; (●) LiCl; (△) LiCl → wash at 24 h. A value of 100% represents the level obtained in the left lane of LiCl-nontreated cells at each incubation time. Mean±SEM (n=3). *P<0.05, compared with LiCl-nontreated cells; #P<0.05, compared between LiCl and LiCl wash out cells.

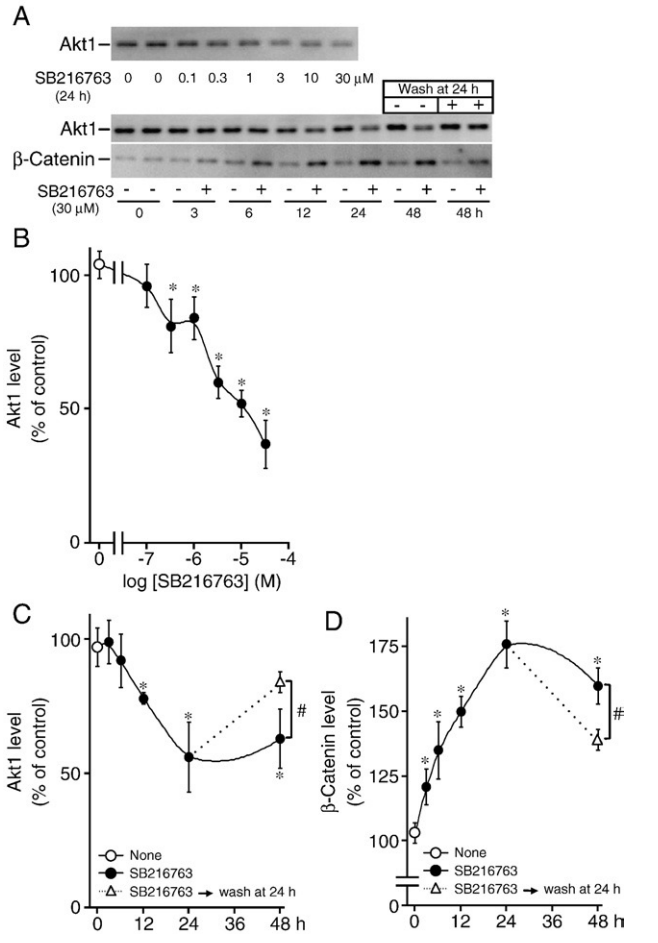

Fig. 4. SB216763 [3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione]-induced decrease of Akt1 level and increase of β-catenin level: concentration- and time-dependent effects, and restoration by washout of SB216763-treated cells. (A) Cells were treated without or with (upper blot) 0.1–30 μM SB216763 for 24 h, or (lower two blots) 30 μM SB216763 for up to 48 h. In the lower two blots, cells were treated without or with 30 μM SB216763 for the first 24 h, washed at 24 h (upper right labeled by Wash at 24 h), and incubated in the continuous absence of SB216763 for up to 48 h. Blot data are typical from 3 independent experiments with similar results. (B, C and D) (○) None; (●) SB216763; (△) SB216763 → wash at 24 h. A value of 100% represents the level obtained in the left lane of SB216763-nontreated cells (B) at 24 h or (C and D) at each incubation time. Mean±SEM (n=3). *P<0.05, compared with SB216763-non-treated cells; #P<0.05, compared between SB216763 and SB216763 wash out cells.

that LiCl (20 mM for 24 h) decreased Akt1 level by ~37% in the absence of proteolysis inhibitor (lanes 1 and 2). β-lactone, lactacystin, calpastatin, or leupeptin alone elevated Akt1 level by 32, 28, 11, or 2% (lane 3, 5, 7, or 9), compared to dimethyl sulfoxide (lane 1), but these proteolysis inhibitors failed to prevent LiCl-induced reduction of Akt1 level.

3.6. LiCl-induced reduction of Akt1 mRNA level: no effect of LiCl on Akt1 mRNA stability

We examined whether LiCl could decrease Akt1 mRNA level. Northern blot analysis (Fig. 6A) shows that 20 mM LiCl decreased relative level of Akt1 mRNA/glyceraldehyde 3-phosphate dehydrogenase mRNA by ~27% between 6 and 12 h.

Steady-state level of mRNA is dependent on gene transcription, processing of heterogeneous nuclear RNA to mRNA and mRNA degradation. We then measured the degradation rate of Akt1 mRNA by using actinomycin D, an inhibitor of RNA synthesis. Fig. 6B shows that cells were treated for the first 3 h without or with 20 mM LiCl, then exposed to actinomycin D for 6 h in the continuous absence or

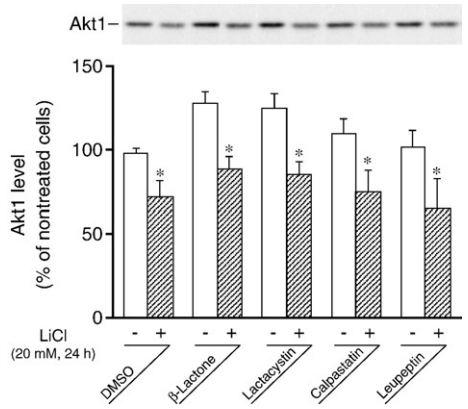

Fig. 5. LiCl-induced decrease of Akt1 level: no prevention by proteolysis inhibitors. In the absence dimethyl sulfoxide (DMSO) or presence of 20 μM β-lactone, 1 μM lactacystin, 1 μM calpastatin, or 10 μM leupeptin, cells were treated without (−) or with (+) 20 mM LiCl for 24 h. Blot data are typical from 3 independent experiments with similar results. A value of 100% represents Akt1 level in cells subjected to 24 h-incubation without dimethyl sulfoxide (DMSO) (blot not shown here). Mean±SEM (n=3). *P<0.05, compared with LiCl-nontreated cells within each cell group.

presence of LiCl, and subjected to Northern blot analysis at the indicated times. Akt1 mRNA level declined by ~29% at 6 h in LiCl-treated cells, as in nontreated cells.

4. Discussion

4.1. Akt1 down-regulation and glycogen synthase kinase-3β inhibition by LiCl and SB216763

In adrenal chromaffin cells, LiCl or SB216763 [3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione] decreased Akt1 level, while increasing β-catenin or Ser^9-phosphorylated

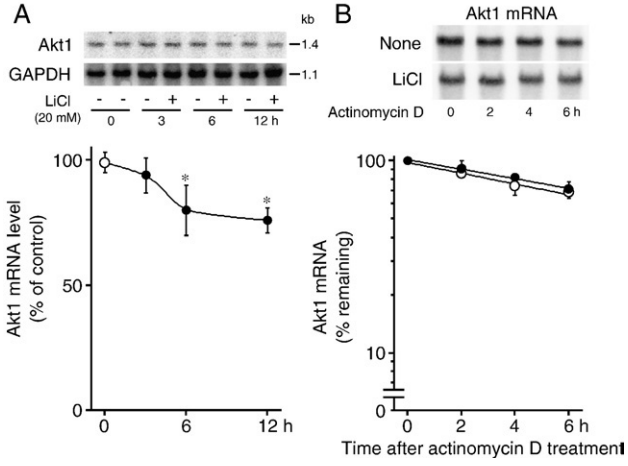

Fig. 6. LiCl-induced reduction of Akt1 mRNA level: no effect of LiCl on Akt1 mRNA degradation. (A) Cells were treated without (−,○) or with (+,●) 20 mM LiCl for up to 12 h; poly(A)+RNA was subjected to Northern blot analysis. Blot data are typical from 3 independent experiments with similar results. Levels of Akt1 mRNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were quantified and the relative level of Akt1 mRNA/glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA is shown in lower graph. The relative level obtained in the left lane at each incubation time is assigned a value of 100%. (B) Cells were pretreated without (○) or with (●) 20 mM LiCl for 3 h. To measure degradation of Akt1 mRNA, the cells were incubated with 10 μg/ml actinomycin D in the continuous absence or presence of LiCl for up to 6 h; at the indicated times, poly(A)$^+$RNA was subjected to Northern blot analysis. Blot are typical from 3 independent experiments with similar results. A value of 100% represents Akt1 mRNA level in each cell group before addition of actinomycin D (see Fig. 6A). Mean± SEM (n=3). *P<0.05, compared with LiCl-nontreated cells.

glycogen synthase kinase-3β level in a concentration- and time-dependent manner. The concentration-response curve of LiCl or SB216763 for Akt1 decrease was inversely related to that for increased level(s) of β-catenin and/or Ser^9-phosphorylated glycogen synthase kinase-3β, as in our previous study (Nemoto et al., 2006). These inverse relationships implicate that the inhibition extent of glycogen synthase kinase-3β is tightly linked to the down-regulation extent of Akt1 in a quantitative manner.

LiCl-induced down-regulation of Akt1 was not prevented by β-lactone, lactacystin, calpastatin, or leupeptin. LiCl decreased Akt1 mRNA level between 6 and 12 h, which was followed by LiCl-induced down-regulation of Akt1 protein level between 12 and 48 h. The degradation rate of Akt1 mRNA was comparable between nontreated and LiCl (20 mM for 9 h)-treated cells. These correlative results suggest that inhibition of glycogen synthase kinase-3β activity retards Akt1 mRNA synthesis, decreasing Akt1 protein level.

Transcriptional and posttranscriptional regulation of Akt1 mRNA synthesis remains elusive in any given tissue. Only a limited number of studies have documented that *Akt*1 gene transcription was stimulated by Src/signal transducer and activator of transcription-3 (STAT3) in various normal cells (e.g., mouse fibroblast NIH3T3 cells) (Park et al., 2005), and by Wnt/β-catenin signaling in colorectal cancer cells (Dihlmann et al., 2005). In mouse macrophages, *Akt*1 gene transcription was promoted by the interaction between cyclic AMP response element-binding protein and cyclic AMP response element in the promoter of *Akt*1 gene (Misra and Pizzo, 2007). Glycogen synthase kinase-3β caused phosphorylation of cyclic AMP response element-binding protein, its DNA binding activity being stimulated in PCl2 cells (Fiol et al., 1994) and inhibited in SH-SY5Y neuroblastoma cells (Grimes and Jope, 2001).

4.2. Constitutive activity of glycogen synthase kinase-3β in nonstimulated cells: positive regulation for steady-state level of Akt1

Adrenal chromaffin cells were treated without or with 20 mM LiCl or 30 μM SB216763 for the first 24 h, then washed, and incubated in the absence of LiCl or SB216763 for up to 48 h. LiCl- or SB216763-induced decreased level of Akt1 and increased level(s) of β-catenin and/or Ser^9-phosphorylated glycogen synthase kinase-3β were all returned to their control levels of nontreated cells at 48 h. These results favor the view that constitutive activity of glycogen synthase kinase-3β is crucial to maintaining steady-state levels of Aktl mRNA and Akt1 protein.

It has been shown that Ser^9-phosphorylation of glycogen synthase kinase-3β is catalyzed by Akt, protein kinase C, cyclic AMP-dependent protein kinase, p70 ribosomal S6 kinase, p90 ribosomal S6 kinase, and integrin-linked kinase, in response to receptor tyrosine kinases, G protein-coupled receptors, Wnt receptor (Jope and Johnson, 2004; Meijer et al., 2004), depolarization (Lee et al., 2005), mood-stabilizing electroconvulsive shock treatment (Roh et al., 2003), and hyperglycemia (Clodfelder-Miller et al., 2005). In SH-SY5Y neuroblastoma cells, rat embryonic hippocampal cells, and PC12 cells, Lee et al. (2005) showed that KCl-induced membrane depolarization caused multiple cycles of undulating Ser^9-phosphorylation/Ser^9-dephosphorylation of glycogen synthase kinase-3β in parallel with β-catenin level fluctuation. These previous and our present results may raise the question of whether Akt level could be fluctuated, depending on the glycogen synthase kinase-3β activity regulated by various extra- and intra-cellular signals.

4.3. Physiological significance in maintaining Akt level

In neurons, differentiation of neurites into single axon and multiple dendrites (i.e., neuronal polarity) is a critical initial step in neuronal maturation; Akt-induced phosphorylation/inactivation of glycogen synthase kinase-3β promotes axon differentiation, whereas finely regulated-proteasomal degradation of Akt within specific cell compartments favors glycogen synthase kinase-3β-induced dendrite development (Wada et al., 2005a,b; Yan et al., 2006). In embryonic dorsal root ganglion neurons, nerve growth factor/Akt increased axon caliber and branching (Markus et al., 2002). In cultured Schwann cells, insulin-like growth factor-I/Akt promoted Schwann cell differentiation and axon myelination (Ogata et al., 2004). In adult dorsal root ganglion neurons, noxious stimulation-induced depolarization caused Akt phosphorylation, linking to pain behavior (Pezet et al., 2005; Sun et al., 2007). In midbrain, Russo et al. (2007) found that Akt maintained mesolimbic dopaminergic neurons involved in the motivation, drug reward (e.g., morphine), and reinforcement of palatable foods.

In adrenal chromaffin cells, Evans et al. (2006) documented that nicotinic receptor-induced Ca^{2+} influx via voltage-dependent calcium channel caused Akt phosphorylation, increasing catecholamine exocytosis. In pancreatic β-cells, glucose-induced insulin exocytosis required Akt (Bernal-Mizrachi et al., 2004).

Akt-catalyzed phosphorylation of type A γ-aminobutyric acid receptor increased cell surface type A γ-aminobutyric acid receptor, enhancing the inhibitory synaptic transmission (Wang et al., 2003). In ciliary ganglion neurons, growth factors (e.g., transforming growth factor-β1) caused Akt phosphorylation, promoting cell surface trafficking of K_{Ca} channel (Chae et al., 2005).

Much remains unknown whether Akt isoforms play redundant or distinct biological effects (Song et al., 2005; Stambolic and Woodgett, 2006). In mice, target disruption of Akt1 or Akt3 reduced the brain size to the comparable degree; however, Akt1 deficiency decreased cell number, whereas Akt3 deficiency decreased cell size of individual neurons (Easton et al., 2005). In hippocampal neurons, Akt1 acted as scaffold protein for c-Jun N-terminal kinase-interacting protein 1, protecting excitotoxicity by sequestering c-Jun N-terminal kinase-interacting protein 1 from the complex formation (Kim et al., 2002).

4.4. Decreased level and function of Akt in neurodegenerative diseases: neuroprotection by Akt activation

In Huntington's disease model, insulin-like growth factor-I prevented neuronal death caused by mutant huntingtin, which was mediated by Akt-catalyzed phosphorylation of huntingtin (Humbert et al., 2002). In human Huntington's patients, phosphorylation/activation of Akt was defective; full-length active Akt (60 kDa) was cleaved into an inactive Akt (49 kDa) by caspase 3 (Humbert et al., 2002; Colin et al., 2005), the latter functioning as a dominant negative to promote cell death (Xu et al., 2002). Mutant huntingtin impaired proteasome function; it was protected by Akt-catalyzed phosphorylation of arfaptin 2, a protein interacting with ADP-ribosylation factor (Rangone et al., 2005).

In amyotrophic lateral sclerosis, Akt level and activity were decreased in mouse spinal cord prior to the neuronal loss (Warita et al., 2001), and in human patient skeletal muscles (Léger et al., 2006); intrathecal or intramuscular administration of insulin-like growth factor-I in mice increased Akt phosphorylation in spinal motor neurons, improving motor performance and survival (Kaspar et al., 2003; Nagano et al., 2005).

In Alzheimer's disease models of cultured rat hippocampal neurons and PC12 cells, transfection with mutant presenillin-1 caused neuronal apoptosis and Akt activity reduction; the apoptosis was rescued by expression of Akt (Weihl et al., 1999). In rat cerebral cortical neurons, acetylcholinesterase inhibitors used for the treatment of Alzheimer's disease patients increased Akt phosphorylation (Takada-Takatori et al., 2006).

In human postmortem brains from schizophrenia patients, Akt level was decreased; conversely, intraperitoneal injection of haloperidol, a therapeutic drug, increased Akt phosphorylation in mouse brains (Emamian et al., 2004).

4.5. Akt down-regulation by glycogen synthase kinase-3β inhibition: implications for glycogen synthase kinase-3 inhibitor treatment in various diseases

It has been noted that lithium up-regulates cell survival molecules (e.g., Bcl-2), while down-regulating proapoptotic molecules (e.g., Bax), promoting neurogenesis in acute brain injuries (e.g., excitotoxicity) and chronic neurodegenerative diseases (e.g., Huntington's disease) (Wada et al., 2005b). Multiple lines of *in vitro* studies have demonstrated that direct targets of LiCl include glycogen synthase kinase-3 (Klein and Melton, 1996; Jope, 2003; Jope and Johnson, 2004; Meijer et al., 2004; Wada et al., 2005b; Jope et al., 2007), phosphoinositide 3-kinase (Chalecka-Franaszek and Chuang, 1999), protein phosphatase 2A (Sasaki et al., 2006), and other enzymes (e.g., inositol monophosphatase) (Gurvich and Klein, 2002). *In vivo* experiments in normal and dopamine-associated diseased mice documented that LiCl-induced behavioral changes and increase of hypothalamic β-catenin level were related to glycogen synthase kinase-3β inactivation by LiCl (Beaulieu et al., 2004; O'Brien et al., 2004). In addition to LiCl, synthetic glycogen synthase kinase-3 inhibitors have been expected as therapeutics against various diseases (Jope and Johnson, 2004; Meijer et al., 2004; Wada et al., 2005a,b; Jope et al., 2007). Our present and previous studies showed that chronic treatment of adrenal chromaffin cells with LiCl or SB216763 decreased Akt1, insulin receptor substrate-1/insulin receptor substrate-2 (Nemoto et al., 2006), and cell surface insulin receptor levels (Yokoo et al., 2007). Taken together, our studies may provide a new avenue for judicious therapeutic uses for glycogen synthase kinase-3 inhibitors against various diseased states.

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