European Journal of Pharmacology 586 (2008) 82–89

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

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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ARTICLE INFO

Article history:
Received 16 November 2007
Received in revised form 3 February 2008
Accepted 25 February 2008
Available online 4 March 2008

Keywords:
Glycogen synthase kinase-3β
β-Catenin
LiCl
SB216763
Up- and down-regulation of Akt1
Adrenal chromaffin cell

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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doi:10.1016/j.ejphar.2008.02.075

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