Drug Development Targeting the Glycogen Synthase Kinase-3β (GSK-3β)-Mediated Signal Transduction Pathway: The Role of GSK-3β in the Maintenance of Steady-State Levels of Insulin Receptor Signaling Molecules and Na,1.7 Sodium Channel in Adrenal Chromaffin Cells

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Abstract. Glycogen synthase kinase-3 (GSK-3) is constitutively active in nonstimulated cells, where the majority of its substrates undergo inactivation/proteolysis by phosphorylation. Extracellular stimuli (e.g., insulin) catalyze inhibitory Ser9-phosphorylation of GSK-3β, turning on signaling and causing other biological consequences otherwise constitutively suppressed by GSK-3β. Regulated and dysregulated activities of GSK-3β are pivotal to health, disease, and therapeutics (e.g., insulin resistance, neurodegeneration, tumorigenesis, inflammation); however, the underlying mechanisms of multifunctional GSK-3β remain elusive. In cultured bovine adrenal chromaffin cells, 1) constitutive and negatively-regulated activities of GSK-3β up- and down-regulated insulin receptor, insulin receptor substrate-1 (IRS-1), IRS-2, and Akt levels via controlling proteasomal degradation and protein synthesis; 2) nicotinic receptor/protein kinase C-α (PKC-α) / extracellular signal-regulated kinase (ERK) pathway up-regulated IRS-1 and IRS-2 levels, enhancing insulin-induced the phosphoinositide 3-kinase (PI3K) / Akt / GSK-3β pathway; 3) inhibition of calcineurin by cyclosporin A or FK506 down-regulated IRS-2 level, attenuating insulin-like growth factor-I (IGF-I)-induced ERK and GSK-3β pathways; and 4) insulin, IGF-I or therapeutics (e.g., lithium) up-regulated the voltage-dependent Na,1.7 sodium channel.

Keywords: glycogen synthase kinase-3β (GSK-3β), LiCl, insulin receptor substrates-1 and -2, Na,1.7 sodium channel, adrenal chromaffin cell

Introduction

In nonstimulated cells, constitutive activity of glycogen synthase kinase-3β (GSK-3β) phosphorylates inactivates a multitude of substrates (e.g., transcription factor β-catenin), translation initiation factor [e.g., eIF2B], and structural proteins [e.g., tau]). Conversely, receptor tyrosine kinases (e.g., insulin receptor), G protein–coupled receptors, Wnt receptors, and hyperglycemia cause Serβ-phosphorylation/inactivation of GSK-3β, relieving the suppression of the aforementioned sub-

strates by GSK-3β (1 – 3). The biological importance of GSK-3β is reinforced by a number of observations. In 1998, Pap and Cooper (4) showed that overexpression of GSK-3β induced apoptosis in rat pheochromocytoma PC12 cells. In 2000, Hoeflich et al. (5) showed that disruption of GSK-3β gene in mice caused embryonic lethality.

Historical milestones about the role of insulin in the central nervous system have been established by the detection of insulin in cerebrospinal fluid by Margolis and Altszuler in 1967 (6) and the discovery of widespread distribution of the insulin receptor in specific brain regions by Havrankova et al. in 1978 (7). However, it is still unknown whether insulin is synthesized in the brain. The larva of the fruit fly Drosophila have insulin-
producing cells in the brain that secrete insulin; ablation of the cells caused developmental delay, growth retardation, and elevated carbohydrate levels in larval hemolymph as seen in diabetes mellitus (8, 9). In adult developed brain, however, it has been generally thought that insulin is transported via a blood–brain barrier after the secretion from pancreatic β-cells; the transport capacity of insulin is decreased by fasting, aging, and dexamethasone administration, while it is abolished in hyperglycemia and during hibernation of marmots (10–12). In normal humans and patients with Alzheimer’s disease, intranasal administration of insulin has been shown to improve memory and mood, while decreasing appetite, with no effect on plasma glucose and insulin levels (12, 13).

It has become increasingly evident that brain region-specific actions of insulin are exquisitely linked to the peripheral actions of circulating insulin, regulating metabolism, appetite, insulin secretion, reproduction, cognition, and neuroprotection (11, 12). At the developing and adult neuronal cell levels, insulin promotes axon myelination, formation of synaptic network, synaptic plasticity, and prevents Alzheimer’s disease-related tau hyperphosphorylation, which are mediated largely via inhibiting GSK-3β (11, 12, 14–18).

**Maintenance of insulin receptor signaling by GSK-3β in adrenal chromaffin cells**

In cultured bovine adrenal chromaffin cells, Nemoto et al. (19) showed that insulin (100 nM) caused a rapid (<5 min) and sustained (up to 24 h) increase of Ser⁹-phosphorylation of GSK-3β up to 104%, which was followed by decreases of insulin receptor substrate-1 (IRS-1) and IRS-2 protein levels up to 72% (Fig. 1). Treatment with LiCl (1–20 mM for ≥12 h) increased Ser⁹-phosphorylated GSK-3β and β-catenin levels up to 59%, while decreasing cell surface ¹²⁵I-insulin binding capacity, cellular levels of insulin receptor, IRS-1, IRS-2, and Akt1 up to 62% in a time- and concentration-dependent manner (19–21). SB216763 (0.1–30 μM for ≥12 h), a selective inhibitor of GSK-3, increased β-catenin level, while decreasing insulin receptor signaling molecule levels, as did LiCl (19–21). LiCl-induced IRS-1 reduction was abolished, while LiCl-induced IRS-2 reduction was partially blocked by β-lactone or

**Fig. 1.** Insulin-induced time-dependent increase of Ser⁹-phosphorylation of GSK-3β and decreases of IRS-1 and IRS-2 protein levels in adrenal chromaffin cells: restoration of these changes by washout of insulin-treated cells. Cells were treated with the vehicle only (−) or with 100 nM insulin (+) for up to 24 h; the cell lysates were subjected to Western blot analysis. p-GSK-3β: Ser⁹-phosphorylated GSK-3β. Wash at 12 h: cells were treated with 100 nM insulin for 12 h, washed, and then incubated without insulin for 24 h. †P<0.01, *P<0.05 vs insulin-nontreated cells; ‡P<0.01, compared between insulin and insulin-washout cells. Modified with permission from Brain Res (Ref. 19).
lactacystin, a proteasome inhibitor; LiCl decreased mRNA levels of insulin receptor, IRS-2, and Akt1 (19 – 21). Changes of these molecules induced by insulin (Fig. 1), LiCl, or SB216763 were all restored to the control levels after washout of either test compound–treated cells at 12 h (19 – 21). Thus, constitutive activity of GSK-3β controls proteasomal degradation of IRS-1 and IRS-2 and mRNA levels of insulin receptor, IRS-2, and Akt, thereby maintaining steady-state levels of these proteins.

**Heterogeneous up- and down-regulations of insulin / insulin-like growth factor-I (IGF-I) / IRS-1 / IRS-2 / GSK-3β pathways in adrenal chromaffin cells**

Up-regulation of IRS-1 and IRS-2 by nicotinic receptor / protein kinase C-α (PKC-α) / extracellular signal-regulated kinase (ERK) pathway: enhancement of insulin receptor / GSK-3β pathway

In cultured bovine adrenal chromaffin cells, Sugano et al. (22) showed that nicotinic receptor stimulation by nicotine (1 – 300 μM for 12 – 48 h) caused time- and concentration-dependent increases of IRS-1 and IRS-2 mRNA and protein levels up to 125%, which was mediated via sequential activation of PKC-α and ERK; in these cells, insulin (100 nM for 10 min)-induced Ser3 phosphorylation of GSK-3β was enhanced.

Proteasomal degradation of IRS-2 by calcineurin inhibition: attenuation of IGF-I receptor / GSK-3β pathway

In cultured bovine adrenal chromaffin cells, Satoh et al. (23) showed that calcineurin inhibition by cyclosporin A (0.001 – 30 μM for 3 – 48 h) or FK506 (0.001 – 30 μM for 3 – 48 h) caused a time- and concentration-dependent decrease of IRS-2 up to 50% via proteasomal degradation, thereby attenuating IGF-I (10 nM for 10 min)-induced Ser3-phosphorylation of GSK-3β.

Up-regulation of voltage-dependent Na1.7 sodium channel in adrenal chromaffin cells by inhibition of GSK-3β

In addition to generating and propagating action potentials in established neuronal network, sodium channel activity is essential to sculpting neuronal architecture from early in embryonic development through adulthood (e.g., differentiation of multiple neurites into a single axon and multiple dendrites, elaborating neuronal polarity; myelination; growth cone navigation; cell survival; experience-driven cognition) (24, 25). In adrenal chromaffin cells, the sodium channel α-subunit is Na1.7 isoform encoded by SCN9A gene, which is widely distributed among peripheral neuronal cells (24, 25).

Gain-of-excess activity/loss-of-normal activity mutations of Na1.7 in human patients proved that Na1.7 is causally involved in intolerable pain (24, 25). More importantly, Na1.7 and the sympathetic neuron–catecholamine system are responsible for various pain syndromes in humans (e.g., inflammation, diabetic neuropathy, nerve injury, fibromyalgia) (24, 25).

In dorsal root ganglion neurons, PC12 cells, and NG108-15 cells, Na1.7 was localized predominantly in the axon growth cone, a neuronal compartment being pivotal to correct synapse elaboration and repair following neuronal injury (24 – 26). Besides, the cell surface number of Na1.7 was up-regulated during neuronal differentiation of PC12 (27) and NG108-15 cells (26) by growth factors (e.g., nerve growth factor [NGF]). NGF- or IGF-I–induced Ser3-phosphorylation of GSK-3β or GSK-3 inhibitors (e.g., lithium, SB216763, and valproic acid) promoted axon–dendrite neuronal polarity, increasing axon growth cone size (28 – 33). However, much remains unknown about whether GSK-3β can regulate cell surface expression of Na1.7 in any given tissue.

In cultured bovine adrenal chromaffin cells, insulin (100 nM for ≥12 h) (34) or IGF-I (12, 35) increased the cell surface number of Na1.7 up to 49%, as evidenced by cell surface [3H]saxitoxin binding assay. By using phosphoinositide 3-kinase (PI3K) inhibitors, LiCl, SB216763, or valproic acid, we showed that up-regulation of Na1.7 was mediated by PI3K-induced GSK-3β inhibition and involved increased levels of Na1.7 mRNA and auxiliary β1-subunit mRNA (12, 35). Up-regulation of Na1.7 augmented veratridine-induced 22Na+ influx via Na1.7, 45Ca2+ influx via voltage-dependent calcium channel, and exocytic secretion of catecholamine (12, 35).

In cultured adrenal chromaffin cells, Shepherd and Holzwarth (2001) showed that gating of sodium channel by veratridine increased elaboration of growth cone and its outgrowth, culminating in synapse-like contacts with neighboring cells (36); consistently, our recent study showed that veratridine-induced Na+ influx via Na1.7 decreased Ser396-phosphorylated tau level via GSK-3β inhibition (the authors’ unpublished observation), a condition promoting axon elongation (37, 38).

**Conclusion**

Evidence has accumulated that multiple signaling pathways (e.g., insulin) converge on GSK-3β, thus GSK-3β being pivotal to health, disease, and therapeutics. Therefore, both (1) regulation of insulin receptor signaling molecules by GSK-3β and (2) modification of
GSK-3β signaling strength by various stimuli are the current issues of great interest.

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