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GSK-3 inhibitors and insulin receptor signaling in health, disease, and therapeutics

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1. ABSTRACT

GSK-3 is constitutively active in nonstimulated cells; multiple signalings negatively regulate GSK-3 via GSK-3 phosphorylation, subcellular (i.e. cytoplasmic; nuclear; mitochondrial) localization, and interaction with other proteins. GSK-3α (51 kDa)/-3β (47 kDa) are encoded by different genes. Dysregulated hyperactivity of GSK-3 is associated with various diseases; *in vivo* and *in vitro* studies have increasingly implicated that GSK-3 inhibitors are promising therapeutics in diabetes mellitus, inflammation, tumorigenesis, psychiatric/neurodegenerative diseases, ischemia, and stem cell regeneration. Importantly, GSK-3 is the common target for various classical therapeutic drugs. In adrenal chromaffin cells, GSK-3 inhibition caused up-regulation of voltage-dependent $Na_v1.7$ sodium channel, enhancing voltage-dependent calcium channel gating and catecholamine exocytosis; conversely, chronic treatment with GSK-3 inhibitors caused down-regulation of insulin receptor, IRS-1, IRS-2, and Akt1 levels. In this review, I will focus on these recent topics. Comprehensive review articles about lithium (1), GSK-3 and GSK-3 inhibitors (2-4), and the inhibition of Wnt/GSK-3β/β-catenin signaling pathway by therapeutic drugs (5) are useful. Chemical structures of GSK-3 inhibitors are listed in the review articles (2, 4).

2. INTRODUCTION

GSK-3, a serine/threonine protein kinase originally identified in the late 1970s as an enzyme that regulates glycogen synthesis (6), is now known to control a multitude of physiological events (e.g. cell membrane-to-gene transcription/protein translation; cytoskeletal organization; neuronal polarity; cell survival/apoptosis) (reviewed in 2-5, 7, 8). Consistent with these pleiotropic roles, activity of GSK-3 is tightly regulated via its phosphorylation, subcellular translocation and interactions with GSK-3-binding proteins. GSK-3 is constitutively active in nonstimulated cells and phosphorylates signaling molecules (e.g. glycogen synthase), transcription factors (e.g. β-catenin), translational initiation factor eIF2B and structural proteins (e.g. tau), keeping these GSK-3 substrates in an inactive state or promoting their degradation (reviewed in 2, 3, 5, 7). Receptor tyrosine kinases (e.g. insulin receptor), G protein-coupled receptors, Wnt receptor (reviewed in 2, 3, 5, 7), and hyperglycemia (9) culminate in Ser^{21}/Ser^{9}-phosphorylation of GSK-3α/β, inhibiting catalytic activity of GSK-3α/β to turn on signaling pathways otherwise constitutively repressed by GSK-3α/β in nonstimulated cells. Importantly, GSK-3β knockout in mice caused embryonic lethality due to

Table 1. GSK-3α and GSK-3β: differences and similarities

GSK-3α and GSK-3β		References
1. Tissue distribution		12
2. Different roles	GSK-3β phosphorylation by protein kinase C: c-Jun activation	13
	Phosphatase activation: primed phosphorylation by casein kinase II	14
	Embryonic lethality by GSK-3β knockout: NF-*k*B dysfunction	10, 11, 15
	Prevention of cardiac hypertrophy: inhibition of ERK signaling	16, 17
	GSK-3β inactivation by insulin: muscle glycogen synthase	18
	Cardiomyocyte protection by GSK-3β: mitochondria-mediated death	19
	Transcription by CREB, NF-*k*B, early growth response 1, Smad3/4	10, 11, 20
	Glutamate excitotoxicity: crosstalk between GSK-3α and GSK-3β	21
	Insulin sensitivity in liver	15
	Keratinocyte migration by GSK-3α: inhibition by GSK-3β	22
	Inhibition of sperm motility by GSK-3α	23
3. Similar roles	β-Amyloid production	24, 25
	Axon formation and growth	26, 27
	Wnt/β-catenin pathway	28
	Antiapoptosis	29

hepatocyte apoptosis, resembling dysfunction of nuclear factor-*k*B (NF-*k*B) (10). Embryonic fibroblasts derived from GSK-3β knockout mice were sensitive to apoptosis (11). With respect to the tissue distribution and biological roles (Table 1), differences and similarities between GSK-3α and GSK-3β have been shown in the previous reports (12-29).

More importantly, it has become increasingly evident that dysregulated hyperactivity of GSK-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) (reviewed in 1-4, 7; 30-41). Consistently, lithium and a growing number of synthetic GSK-3 inhibitors have turned out to be effective therapeutics against diabetes mellitus, acute brain injuries, chronic neurodegenerative diseases, inflammation, and cancer (reviewed in 1-4, 7; 30-41; Table 2).

3. NEW ASPECTS OF LITHIUM AND GSK-3β IN PSYCHIATRIC DISEASES

Multiple lines of previous *in vitro* studies have demonstrated that direct targets of lithium include GSK-3 (reviewed in 1-3, 7, 42), phosphoinositide 3-kinase (43), protein phosphatase 2A (44), and other enzymes (e.g. inositol monophosphatase). Dopamine is implicated in multiple brain disorders (e.g. Parkinson's disease; schizophrenia; attention deficit hyperactivity syndrome; addiction). In mice suffered from increased dopamine neurotransmission in brain striatum, pharmacological and genetic experiments documented that LiCl-induced behavioral amelioration was attributed to the inhibition of GSK-3β by LiCl (45). O'Brien *et al.* (46) showed that mice fed with chow containing 0.2 or 0.4% LiCl exhibited dose-dependent antidepressant effect of LiCl, as evidenced by the increased mobility in multiple behavioral tests (e.g. forced swim test); LiCl treatment accelerated Wnt/β-catenin-dependent gene transcription *in vivo* in amygdala, hippocampus and hypothalamus. Importantly, these behavioral and biochemical changes seen in LiCl-treated mice were also observed in mice lacking one copy of GSK-3β gene (46). Clinically, Li *et al.* (47) measured Ser^9-phosphorylation levels of GSK-3β in human peripheral blood mononuclear cells obtained from 23 healthy subjects, 9 bipolar patients treated with lithium, and 13 lithium-free bipolar patients. Ser^9-phosphorylation levels of GSK-3β were 8-fold higher in lithium-treated patient group than healthy control group, supporting the association between GSK-3β inhibition and therapeutic responses to lithium treatment.

4. PRECLINICAL EFFICIENCY OF GSK-3 INHIBITORS

4.1. Diabetes mellitus

In biopsy samples of skeletal muscle vastus lateralis from type 2 diabetic patients and nondiabetic individuals, Nikoulina *et al.* (48) showed that GSK-3α/β protein levels and enzyme activities were increased by ~64 and ~286% in diabetic muscles, compared with muscles from lean and weight-matched obese nondiabetic individuals; these values were inversely correlated with glycogen synthase activity and insulin-induced glucose utilization.

Ring *et al.* (49) showed that Chir98014 and Chir99021, two substituted derivatives of aminopyrimidine, inhibited human GSK-3α/β with the IC_{50} values (< 10 nM), the selectivity being at least 500-fold higher for GSK-3α/β, compared to 20 other protein kinases. Chir98014 increased glycogen synthase activity in various rat cells (e.g. hepatocytes; soleus myocytes). Chir98014 or Chir99021 increased glucose transport into soleus myocytes, and lowered blood glucose level without altering plasma insulin level in rodents. Rao *et al.* (50) showed that diabetes model of high-fat fed mice were obese, with impaired glucose tolerance and high plasma insulin level; chronic (~ 20 days) treatment with L803-mts, a peptide inhibitor of GSK-3, decreased plasma insulin level, endogenous glucose production, and insulin resistance, which were accompanied by the increases of glucose uptake, glycogen synthase activity, and net glycogen synthesis.

In terms of atherosclerosis associated with diabetes mellitus, Robertson *et al.* (51) reviewed that chronic hyperglycemia-induced accumulation of intracellular glucosamine may promote atherogenesis via a mechanism involving dysregulated protein folding, activation of endoplasmic reticulum stress, and increased activity of GSK-3; GSK-3 regulates caspases, NF-*k*B, and sterol regulatory element binding proteins that control cellular uptake and synthesis of lipid. In HepG2 hepatoma cells subjected to glucosamine-induced endoplasmic reticulum stress, Kim *et al.* (52) showed that lithium or valproic acid inhibited GSK-3α/β, protecting the cells from the endoplasmic reticulum stress-induced lipid accumulation.

Table 2. Beneficial effects of GSK-3 inhibitors in various diseased states and health

Conditions	Beneficial effects	References
Diabetes mellitus	Increased glycogen synthase activity	49, 50
	Increased glucose transport	49, 50
	Decreased blood glucose level	49, 50
	Prevention of atherosclerosis	51
	Protection of endoplasmic reticulum stress	52
Inflammation	Prevention of lipopolysaccaride shock	30-33
	Increase of anti-inflammatory mediators	30-33
	Decrease of proinflammatory mediators	30-33
	Prevention of hemorrhagic shock	34
	Prevention of peritonitis, arthritis, colitis	35-37
	Prevention of spinal cord inflammation	38
	Bronchial epithelial cell repair	39
	Attenuation of bronchial asthma	40
Cancer	Inhibition of NF-*k*B-induced gene transcription	41
Neurodegeneration	Protection of Alzheimer's disease	54, 56
	Protection of Parkinson's disease	57, 58
	Protection of amyotrophic lateral sclerosis	59, 60
	Protection of Huntington's disease	61
Cardiac ischemia	Reduction of infarct size	62
Neuronal death	Prevention against various stresses	63-68
Stem cells	Stimulation of self-renewal	69, 70
Neural precursors	Neurogenesis, Differentiation	71-73
P-Ser-GSK-3α/β	Increase by 5-HT_1, Decrease by 5-HT_2	74-76
	Antipsychotics	77
	Anesthetics	78

P-Ser-GSK-3α/β, Ser^{21}/Ser^{9}-phosphorylation of GSK-3α/β.

4.2. Inflammation

Chronic inflammation is a common pathology of most prevalent diseases, including Alzheimer's disease, multiple sclerosis, mood disorder, diabetes mellitus, and cancer. GSK-3 promotes production of molecules accounting for inflammation and cell migration, while reducing production of anti-inflammatory cytokine interleukin-10 (IL-10). Martin *et al.* (30) documented that GSK-3 is pivotal in inflammation process; in mice subjected to intraperitoneal injection of lethal dose of lipopolysaccharide, intravenous administration of SB216763, an inhibitor of GSK-3, protected over half of the mice from toxic shock and death, even when SB216763 was given 2 hours after the exposure to lipopolysaccaride. In human peripheral blood monocytes or mononuclear cells, they also showed that GSK-3β inhibition was responsible for increasing anti-inflammatory IL-10 production via cyclic AMP-response element-binding protein (CREB), while decreasing proinflammatory interleukin-12 expression via NF-*k*B. In rats, Dugo *et al.* (31) observed that intravenous administration of lipopolysaccharide without or with peptidoglycan caused endotoxemia, renal dysfunction, and hepatocellular, pancreatic and neuromuscular injuries; intravenous injection of GSK-3 inhibitors (TDZD-8; SB216763; SB415286) 30 min before the insults attenuated the organ dysfunction/injury, associated with the reduced levels of mRNAs encoding NF-*k*B-dependent proinflammatory mediators. Remarkably, they found that GSK-3 inhibitors protected multiple organ dysfunction/injury caused by hemorrhagic shock (33, 34). In rats receiving lipopolysaccharide plus peptidoglycan, intravenous administration of insulin attenuated the renal dysfunction and hepatocellular injury presumably via inhibiting GSK-3β, when given before or after the insult (32).

In other inflammatory conditions, GSK-3 inhibitors ameliorated Toll-like receptor 2-induced peritonitis and arthritis in mice (35), type II collagen-induced arthritis in mice (37), and experimental colitis in rats (36). In cultured porcine bronchial epithelial cells, Zhu *et al.* (39) showed that scratching a monolayer of bronchial epithelial cells caused Ser^{9}-phosphorylation of GSK-3β and nuclear translocation of β-catenin, increasing expression of cyclin D1 that promoted cell proliferation for repair of bronchial epithelial cells. In mouse model of bronchial asthma, Bao *et al.* (40) showed that intravenous injection of GSK-3 inhibitor TDZD-8 attenuated ovalbumin-induced inflammatory biochemical and histological impairments, and airway hyperresponsiveness via hampering activation of NF-*k*B. In mouse model of experimental spinal cord trauma, Cuzzocrea *et al.* (38) showed that intraperitoneal injection of GSK-3 inhibitor TDZD-8 inhibited the injury-induced spinal cord inflammation, aberrant pathological expressions of inducible nitric oxide synthase and cyclooxygenase-2, and apoptosis, ameliorating recovery of limb function.

4.3. Neurodegenerative diseases

4.3.1. Alzheimer's disease

GSK-3α/β accounts for formation of neurofibrillary tangles and neuritic plaques, two pathological hallmarks of Alzheimer's disease; GSK-3

inhibitors may be effective in the treatment of Alzheimer's disease, as summarized in previous review articles (1, 53). In mice, overexpression of GSK-3β recapitulated neuropathology of Alzheimer's disease, while transgene shutdown of GSK-3β in symptomatic mice diminished their neuronal death and cognitive deficit (54). In human peripheral blood mononuclear leucocytes prepared from Alzheimer's disease patients and control individuals, Castri *et al.* (55) showed that insulin (100 μg/ml [17.4 μM] for 5 or 10 min)-induced phosphorylation of Akt was significantly reduced in Alzheimer's cells, compared to control cells, when the age and plasma insulin/glucose levels were similar between Alzheimer's group and control group. These results support the hypothesis that impaired control of GSK-3β activity by insulin receptor signaling facilitates hyperphosphorylation of tau, causing neurofibrillary tangle formation.

Interestingly, in transgenic mice overexpressing human tau, Nakashima *et al.* (56) documented that 5-month oral administration of therapeutic concentrations of lithium reduced tau lesions, primarily by promoting tau ubiquitination via an as yet unknown mechanism rather than by inhibiting GSK-3-catalyzed tau phosphorylation.

4.3.2. Parkinson's disease

Pathogenesis of sporadic Parkinson's disease remains unclear; 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) are widely used to model Parkinson's disease. In cultured neuronal cells (rat cerebellar granule cells; PC12; SH-SY5Y), Chen *et al.* (57) showed that 6-hydroxydopamine evoked endoplasmic reticulum stress, with activation of GSK-3β due to decreased Ser^{9}-phosphorylation and increased Tyr^{216}-phosphorylation of GSK-3β; GSK-3 inhibitors (lithium; TDZD-8; L803-mtz) prevented 6-hydroxydopamine-induced events. In Parkinson's disease model mice, Wang *et al.* (58) showed that MPTP caused activation of GSK-3β (decrease in Ser^{9}-phosphorylation of GSK-3β), and increased tau phosphorylation and striatal dopaminergic neuron loss, resulting in behavioral impairment; intraperitoneal injection of GSK-3 inhibitors (indirubin-3'-oxime; AR-A014418) prevented these MPTP-induced pathological events.

4.3.3. Amyotrophic lateral sclerosis

Pathogenesis of amyotrophic lateral sclerosis remains unclear, whereas increased level of GSK-3α/β was detected in spinal cord of patients with sporadic amyotrophic lateral sclerosis (59). In mouse model of amyotrophic lateral sclerosis expressing G93A mutant superoxide dismutase, Koh *et al.* (60) showed that intraperitoneal injection of GSK-3 inhibitor VIII ($C_{12}H_{12}N_4O_4S$) delayed the onset of symptoms, and prolonged the life-span, which were associated with increased levels of survival signals and decreased levels of death/inflammatory signals.

4.3.4. Huntington's disease

Pathology of Huntington's disease is characterized by abnormal expansion of polyglutamine stretch in the Huntingtin gene, producing the toxic misfolded, aggregate-prone proteins. In COS7 and human neuroblastoma SK-N-SH cells transfected with the 74 glutamines, Carmichael *et al.* (61) showed that pretreatment with LiCl or SB216763, or overexpression of dominant-negative GSK-3β mutant reduced polyglutamine-induced nuclear fragmentation and intracellular inclusion formation. Cells expressing polyglutamine had decreased β-catenin level, with retardation in β-catenin/T-cell factor-mediated gene transcription. In COS 7 cells or SK-N-SH cells, LiCl or SB216763 activated β-catenin/T-cell factor-mediated transcription; overexpression of β-catenin inhibited polyglutamine-induced toxicity.

4.4. Cell protection against various stresses

In addition to its well-documented effect on erythropoiesis, administration of erythropoietin has been shown to reduce cardiac necrosis, apoptosis, and ventricular dysfunction after ischemia-reperfusion. Nishihara *et al.* (62) showed that intravenous injection of erythropoietin or cardiac preconditioning with 5-min ischemia/5-min reperfusion reduced infarct size after 20-min ischemia in rat hearts *in situ*, which was correlated with the increased Ser^{9}-phosphorylation of GSK-3β; intravenous injection of SB216763 decreased the infarct size in a dose-dependent manner.

In cultured rat hippocampal and cortical neurons, Kelly *et al.* (63) showed that GSK-3 inhibitor Chir025 reduced cell death caused by glutamate exposure and oxygen-glucose deprivation. In rats subjected to middle cerebral artery occlusion, they also found that intravenous administration of Chir025 decreased brain infarct size, with increased level of brain cytoplasmic Bcl-2 (63). In mice, Roh *et al.* (64) showed that hypoxia selectively decreased Ser^{21}/Ser^{9}-phosphorylation level of GSK-3α/β within 30 sec in brain cortex, hippocampus, and striatum; intraperitoneal injection of imipramine or valproic acid, or oral lithium treatment attenuated hypoxia-induced pathogical dephosphorylation of GSK-3α/β. These results suggest that stabilization of Ser-phosphorylation of GSK-3 by GSK-3 inhibitors contributes to their therapeutic effects.

In cultured rat cerebral cortical neurons, Takadera and Ohyashiki (65) showed that GSK-3 inhibitors (SB216763; alsteropaullone) completely prevented prostaglandin E_2-induced caspase-3 activation and apoptosis. Dysregulated Ca^{2+} overload accounts for neurodegenerative diseases (e.g. ischemia; excitotoxicity; Alzheimer's disease), in which plasma membrane Ca^{2+}-ATPase and sarco(endo)plasmic reticulum Ca^{2+}-ATPase are pivotal in regulating Ca^{2+} homeostasis. In PC12 cells (66) and cultured rat cerebral cortical neurons (67), treatment with thapsigargin, a selective inhibitor of sarco(endo)plasmic reticulum Ca^{2+}-ATPase, caused caspase-3 activation and apoptosis, which were prevented by GSK-3 inhibitors (SB216763; alsteropaullone; azakenpaullone). It is known that rat mature cerebellar granule neurons depend on increased concentration of extracellular K^+ ($[K^+]_o$) for their survival, and they undergo apoptosis when 25 mM $[K^+]_o$ was decreased to 5 mM $[K^+]_o$ (reviewed in 1). In cultured rat cerebellar granule neurons, Chin *et al.* (68) found that 25 mM $[K^+]_o$, cyclic AMP, IGF-

I, lithium, or SB415286 protected 5 mM $[K^+]_o$-induced cell death by inhibiting GSK-3β activity.

4.5. Self-renewal and pluripotency of embryonic stem cells

Human embryonic stem cells can be used for cell replacement therapy against various diseases (e.g. diabetes mellitus; Parkinson's disease; Huntington' s disease). Sato *et al.* (69) documented that GSK-3 inhibitor 6-bromoindirubin-3'-oxime (BIO) induced self-renewal of human and mouse embryonic stem cells, providing a steady supply of embryonic stem cells for regenerative medicine. Activation of Wnt/β-catenin pathway caused by GSK-3 inhibitor was sufficient to maintain the self-renewal, and the undifferentiated and pluripotent state of both embryonic stem cells. Importantly, GSK-3 inhibitor did not lock the cells into undifferentiated state; withdrawal of GSK-3 inhibitor led to normal multidifferentiation programs of both embryonic stem cells.

Transplantation therapy of hematopoietic stem cells has been effectively used to manage hematopoietic malignancies, bone marrow or hematopoietic failure, and immunodeficiency. In recipient mice transplanted with mouse or human hematopoietic stem cells, Trowbridge *et al.* (70) showed that *in vivo* administration of GSK-3 inhibitor CHIR-911 improved neutrophil and megakaryocyte recovery, recipient survival, and enhanced the sustained long-term repopulating capacity of transplantable hematopoietic stem cells, which were mediated via modulating gene targets of Wnt, Hedgehog and Notch pathways.

4.6. Neurogenesis and neuronal differentiation

In adult mammalian (e.g. human) brain, birth of new neurons (i.e. neurogenesis) continuously occurs in subgranular zone of hippocampus and subventricular zone of lateral ventricle; the new neurons are integrated into the functional neuronal network (reviewed in 1). In adult mammalian retina, however, only a limited extent of neurogenesis took place after acute neurotoxic injury *in vivo*; in rodent retinal explant cultures, Osakada *et al.* (71) showed that activation of Wnt/β-catenin signaling caused by GSK-3 inhibitors (SB216763; AR-A014418) promoted proliferation of Müller glia-derived retinal progenitor cells, and accelerated neuronal regeneration after damage or during degeneration.

Neural precursor cells develop into mature neurons; conversely, impaired neuronal development is associated with neurological/psychiatric diseases (reviewed in 1). Ironically, before maturing to functional neurons, significant portions of neural precursor cells are lost due to apoptosis. In cultured neural precursor cells derived from embryonic mouse brain, Emon *et al.* (72) documented that trophic factor withdrawal from culture medium or treatment with camptothecin, a topoisomerase I inhibitor that induces apoptosis via p53-dependent mechanism, caused apoptosis of neural precursor cells, which was associated with Ser^{21}/Ser^{9}-dephosphorylation of GSK-3α/β, as well as activation of apoptosis mediators Bax and caspase-3. Conversely, GSK-3 inhibitors (lithium; kenpaullone; GSK-3 inhibitor II; indirubin-3'-monoxime; SB216763) inhibited trophic factor withdrawal- or camptothecin-induced apoptosis, and activation of Bax and caspase-3. In cultured rat ventral mesencephalon precursor cells, Castelo-Branco *et al.* (73) showed that GSK-3 inhibitors (indirubin-3-monoxime; kenpaullone) promoted differentiation of precursor cells into dopaminergic neurons by ~ 4-fold via increasing β-catenin level; overexpression of β-catenin in ventral mesencephalic precursor cells increased their differentiation into dopaminergic neurons.

5. Ser^{21}/Ser^{9}-PHOSPHORYLATION OF GSK-3α/β BY VARIOUS CLASSICAL THERAPEUTIC DRUGS

It has become increasingly evident that GSK-3 may be a common therapeutic target for different classes of psychiatric drugs (e.g. selective serotonin reuptake inhibitors; antidepressants; monoamine oxidase inhibitors; antipsychotics) (reviewed in 74). In mice, Li *et al.* (75) demonstrated that intraperitoneal injection of d-fenfluramine (to stimulate serotonin secretion and block its reuptake) increased Ser^{9}-phosphorylation of GSK-3β by ~ 500% over control level in prefrontal cortex, hippocampus, and striatum. Treatment with fluoxetine (a selective serotonin reuptake inhibitor) and imipramine (a tricyclic antidepressant) also increased Ser^{9}-phosphorylation of GSK-3β. By using selective agonists and antagonists for serotonin receptor subtypes, it was found that 5-HT_{1A} receptors increased Ser^{9}-phosphorylation of GSK-3β, while 5-HT_{2A} receptors decreased it; endogenous serotonin preferentially increased Ser^{9}-phosphorylation of GSK-3β, when acting simultaneously on both 5-HT_{1A} and 5-HT_{2A} receptors. These results suggest that impaired regulation of GSK-3β activity may be involved in the pathological states, where serotonergic activity is dysregulated (e.g. depression; anxiety; bipolar disorder; autism; schizophrenia). Beaulieu *et al.* (76) generated knockin mice expressing a mutant form of brain serotonin synthesis enzyme, a similar defect being identified in human patients with depression. The mutant mice displayed marked reduction of serotonin production, with behavioral abnormalities and increased activity of GSK-3β/decreased Ser^{9}-phosphorylation of GSK-3β in frontal cortex; inhibition of GSK-3β by intraperitoneal injection of GSK-3 inhibitor (TDZD-8) or genetic approaches alleviated the aberrant behaviors caused by serotonin deficiency.

Atypical antipsychotic drugs have been used in the treatment of mood disorders and schizophrenia. In mice, Li *et al.* (77) showed that intraperitoneal injection of risperidone increased Ser^{21}/Ser^{9}-phosphorylation of GSK-3α/β in the cortex, hippocampus, striatum, and cerebellum in a dose-dependent manner. Similar effects were observed by olanzapine, clozapine, questiapine, and ziprasidone. In addition, treatment of mice with risperidone plus imipramine or risperidone plus fluoxetine elicited a larger increase in Ser^{21}/Ser^{9}-phosphorylation of GSK-3α/β in those various brain regions, compared to each agent alone.

Several anesthetics increased Ser^{21}/Ser^{9}-phosphorylation of GSK-3α/β in brain (78). In mice,

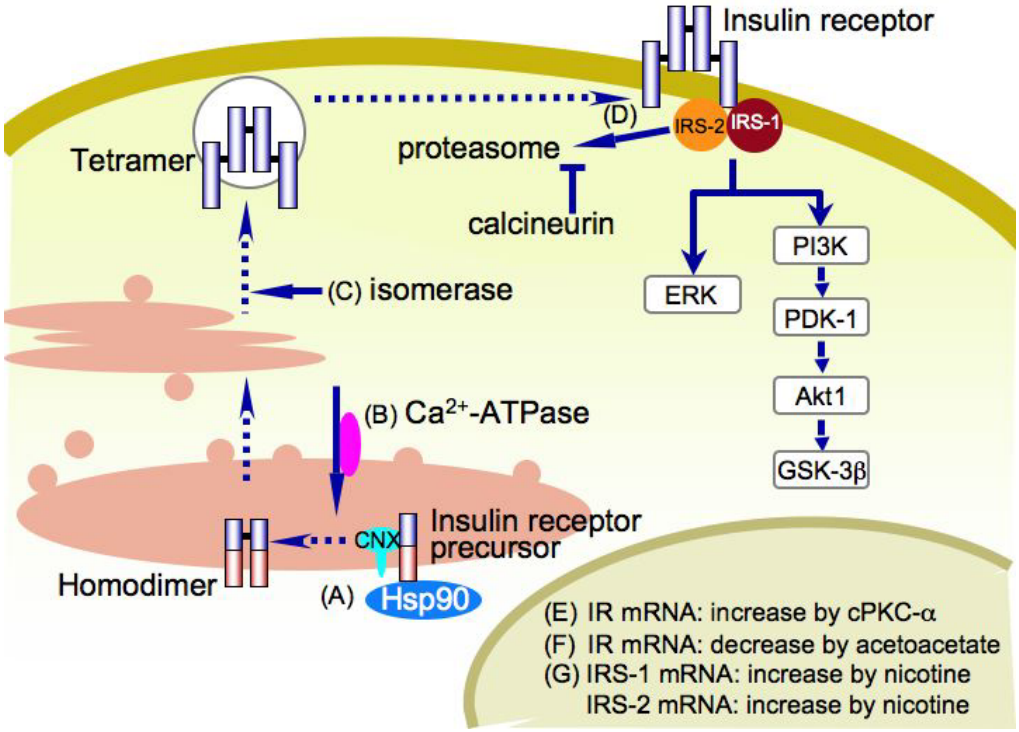

Figure 1. Up- and down-regulation mechanisms of cell surface insulin receptor, IRS-1, IRS-2, and Akt1 in adrenal chromaffin cells. In the nonstimulated cell, cell surface expression of insulin receptor requires (A) Hsp90-catalyzed homodimerization of monomeric insulin receptor precursor at the endoplasmic reticulum (101), (B) endoplasmic reticulum Ca^{2+}-ATPase (102), and (C) peptidyl prolyl *cis-trans* isomerase activity of cytoplasmic immunophilins (103); (D) IRS-2 level was maintained by calcineurin via preventing proteasomal IRS-2 degradation (112). (E) Activation of cPKC-α up-regulates (104), while (F) acetoacetate down-regulates (105) insulin receptor mRNA and protein levels. (G) Nicotinic receptor/cPKC-α/ERK activation up-regulates IRS-1/IRS-2 mRNA and protein levels (109). In addition, Table 3 shows that in the nonstimulated cell, constitutive activity of GSK-3β maintains steady-state levels of insulin receptor (98), IRS-1 (97), IRS-2 (97), and Akt1 (99). Alterations of insulin receptor, IRS-1, and IRS-2 levels regulated strength of insulin/IGF-I-induced PI3K/Akt/GSK-3β and ERK signaling pathways (98, 101, 105, 109, 112). CNX, calnexin; cPKC-α, conventional protein kinase C-α; Hsp90, 90-kDa heat-shock protein.

intraperitoneal injection of pentobarbital or chloral hydrate, or exposure to vapors of halothane rapidly (~ 2 min) increased Ser^{21}/Ser^{9}-phosphorylation of GSK-3α/β in cerebral cortex, hippocampus, striatum, and cerebellum.

6. NEURONAL INSULIN RECEPTOR SIGNALING AND GSK-3

In developing and adult neuronal circuits, insulin plays previously unrecognized pivotal roles (e.g. differentiation of neurites into single axon and multiple dendrites; axon growth cone navigation; formation/maintenance/repair of axon myelination and synapse network; learning/memory; neurogenesis/angiogenesis; cell survival/lifespan; reward) (reviewed in 53). Besides, insulin's actions in brain regulate peripheral functions (e.g. hepatocyte gluconeogenesis; counter-hormone secretion to hypoglycemia; reproductive endocrine axis) (reviewed in 53). Insulin receptor or IGF-I receptor triggers Tyr-phosphorylation of IRS-1, IRS-2 and Shc, leading to activation of phosphoinositide 3-kinase (PI3K)/phosphoinositide-dependent kinase 1 (PDK-1)/Akt pathway and Ras/ERK pathway (Figure 1). IRS-1 and IRS-2 are not functionally interchangeable (reviewed in 79). Intriguingly, IRS-1 and IRS-2 are translocated into nucleus, functioning as transcriptional factors (80, 81). Akt catalyzes inhibitory Ser^{21}/Ser^{9}-phosphorylation of GSK-3α/β, as well as phosphorylation/inhibition of transcription factor FOXO, proapoptotic Bad, and translation inhibitor tuberin (reviewed in 82). Evidence has emerged that Akt plays multiple roles in physiological (e.g. differentiation; polarity; survival; scaffold; pain; reward) and pathological (e.g. tumorigenesis; neurodegeneration) events (reviewed in 83-87; 88) by acting in cytoplasm, nucleus (reviewed in 89), endoplasmic reticulum (90) and mitochondria (reviewed in 91).

Defective insulin receptor signaling is associated with the cognitive dysfunction in normal aging and patients with neurodegenerative diseases (e.g. Alzheimer's disease), which can be ameliorated by intravenous or intranasal administration of insulin or IGF-I in the euglycemic condition (reviewed in 53).

Table 3. Down-regulation mechanisms of insulin receptor, IRS-1, IRS-2, and Akt by GSK-3 inhibitors in adrenal chromaffin cells

Signaling molecules	Protein level	Proteasomal proteolysis	mRNA level	mRNA stability	References
Insulin receptor	Decrease	No change	Decrease	Decrease	98
IRS-1	Decrease	Increase	No change		97
IRS-2	Decrease	Increase	Decrease		97
Akt	Decrease	No change	Decrease	No change	99

7. INSULIN RECEPTOR SIGNALING IN ADRENAL CHROMAFFIN CELLS

7.1. Insulin/IGF-I/GSK-3β pathway: up-regulation of voltage-dependent $Na_v1.7$ sodium channel

In cultured bovine adrenal chromaffin cells, various agents inhibiting GSK-3β activity [i.e. insulin (92); valproic acid (93); IGF-I, lithium, and SB216763 (reviewed in 1, 53; 94)] up-regulated cell surface expression of voltage-dependent $Na_v1.7$ sodium channel via increasing $Na_v1.7$ gene transcription; $Na_v1.7$ up-regulation augmented veratridine-induced $^{22}Na^+$ influx via $Na_v1.7$, $^{45}Ca^{2+}$ influx via voltage-dependent calcium channel and exocytic secretion of catecholamines. New aspects of sodium channel family (e.g. $Na_v1.7$) in neuronal development, pain, and neurodegeneration are summarized in review article (95); multiple roles of $Na_v1.7$ in adrenal chromaffin cells and peripheral nervous system are reviewed in (96).

7.2. Reduction of insulin receptor, IRS-1, IRS-2 and Akt1 levels by GSK-3β inhibitors

In cultured bovine adrenal chromaffin cells, treatment with LiCl, SB216763, or insulin increased Ser^9-phosphorylation of GSK-3β and β-catenin level in a time- and concentration-dependent manner (97, 98). In LiCl-, SB216763-, or insulin-treated cells, cell surface ^{125}I-insulin binding capacity, cellular levels of insulin receptor and insulin receptor precursor molecule were decreased in a time- and concentration-dependent manner; in addition, insulin-induced Tyr-autophosphorylation of insulin receptor was attenuated in SB216763-treated cells (98). LiCl destabilized insulin receptor mRNA, decreasing insulin receptor mRNA level, without altering insulin receptor gene transcription (98). The decreases of ^{125}I-insulin binding capacity and insulin receptor level by LiCl, SB216763, or insulin were restored to the control levels of nontreated cells after the washout of either test compound-treated cells (98). Thus, constitutive activity of GSK-3β maintains steady-state level of insulin receptor via controlling insulin receptor mRNA stability (Table 3; Figure 1).

Treatment with LiCl, SB216763, or insulin decreased IRS-1, IRS-2, and Akt1 levels via controlling proteasomal degradation of IRS-1 and IRS-2, as well as mRNA levels encoding IRS-2 and Akt1; the decreases of IRS-1, IRS-2, and Akt1 levels were restored to the control levels after the washout of either test agent-treated cells (97, 99). Intriguingly, insulin-induced decrease of IRS-2 level occurred rapidly at 5 min (97), as previously reported in the down-regulation of IRS-2 level caused by IGF-I in SH-SY5Y and SH-EP human neuroblastoma cells (100). In contrast, LiCl treatment did not alter cellular levels of PI3K, PDK-1, and ERK1/ERK2 (99).

7.3. Insulin receptor expression by 90-kDa heat-shock protein, endoplasmic reticulum Ca^{2+}-ATPase, peptidyl prolyl *cis-trans* isomerase activity of cytoplasmic immunophilins, protein kinase C-α, and acetoacetate

In nonstimulated adrenal chromaffin cells, steady-state level of cell surface insulin receptor was maintained by chaperone function of 90-kDa heat-shock protein in the endoplasmic reticulum (101) and sarco(endo)plasmic reticulum Ca^{2+}-ATPase activity (102), as well as peptidyl prolyl *cis-trans* isomerase activity of cytoplasmic immunophilins (103). Activation of conventional protein kinase C-α up-regulated (104), while ketone body acetoacetate (but not β-hydroxybutyrate and acetone) down-regulated number of cell surface insulin receptor (105). Down-regulation of cell surface insulin receptor attenuated insulin-induced Tyr-phosphorylation of IRS-1 (101, 105) (Figure 1).

7.4. Up-regulation of IRS-1 and IRS-2 by nicotinic receptor/protein kinase C-α/ ERK pathway

Activation of neuronal nicotinic receptor rapidly evokes excitatory postsynaptic potentials and Ca^{2+}-dependent exocytosis of neurotransmitters, while generating longer-lasting multiple effects (e.g. synaptic plasticity; learning/memory; cell survival) via transcription- and translation-dependent mechanisms (reviewed in 106). Conversely, aberrant down-regulation of neuronal nicotinic receptor accounts for cognitive deficits in normal aging and age-related neurodegenerative diseases (e.g. Alzheimer's disease; Parkinson's disease; Lewy body dementia) (reviewed in 107), with impairment of acetylcholine synthesis in Alzheimer's disease brain (108). Stimulation of neuronal nicotinic receptor is the mainstay for the treatment of these cognitive deficits, while the therapeutic mechanisms remain elusive at the cellular level (reviewed in 107) (Figure 1).

In cultured bovine adrenal chromaffin cells, stimulation of neuronal nicotinic receptor caused time (> 12 h)- and concentration (EC_{50} = 3.6 and 13 μM)-dependent increases in IRS-1 and IRS-2 levels by ~ 125%, without changing cell surface number of insulin receptor (109). The IRS-1 and IRS-2 increases by nicotinic receptor stimulation was prevented by a cell membrane-permeable Ca^{2+} chelator, cycloheximide or actinomycin D. Nicotine caused sequential phosphorylation/activation of conventional protein kinase C-α and ERK1/ERK2, thereby increasing IRS-1 and IRS-2 mRNA levels by ~ 57%. In nicotine (10 μM for 24 h)-treated cells, insulin (100 nM for 10 min)-induced Tyr-phosphorylation of IRS-1/IRS-2 and recruitment of PI3K to IRS-1/IRS-2 were augmented by ~ 63%; in addition, insulin-induced phosphorylation of Akt, GSK-3β and ERK1/ERK2 was enhanced by ~ 62%.

Selective activation of conventional protein kinase C-α by thymeleatoxin mimicked these effects of nicotine.

7.5. Proteasomal degradation of IRS-2 by calcineurin inhibition

Calcineurin is an important regulator of numerous physiological events (e.g. cytoskeletal structure/function; exocytosis/endocytosis; Ca^{2+} homeostasis; gene expression), but conversely, aberrant calcineurin activity is associated with impaired behavior/learning/memory in normal aging and neurodegenerative diseases (e.g. Alzheimer's disease) (reviewed in 110). Clinically, inhibition of calcineurin activity by cyclosporin A or FK506 is indispensable for immunosuppressive therapy, but frequently associated with toxicities via unknown mechanisms (e.g. new-onset of diabetes mellitus; seizure) (111) (Figure 1).

Chronic (≥ 3 h) treatment of cultured bovine adrenal chromaffin cells with cyclosporin A or FK506 inhibited calcineurin activity (IC_{50} = 500 or 40 nM), and decreased IRS-2 protein level by ~50% (IC_{50} = 200 or 10 nM), without changing IRS-2 mRNA level, and insulin receptor, IGF-I receptor, IRS-1, PI3K/PDK-1/GSK-3β and ERK1/ERK2 protein levels (112). Rapamycin, an FK506-binding protein ligand unable to inhibit calcineurin, failed to decrease IRS-2 level, but reversed FK506-induced decreases of calcineurin activity and IRS-2 level. Pulse-label followed by polyacrylamide gel electrophoresis revealed that cyclosporin A or FK506 accelerated IRS-2 degradation rate ($t_{1/2}$) from > 24 h to ~4.2 h, without altering IRS-2 synthesis. IRS-2 reduction by cyclosporin A or FK506 was prevented by proteasome inhibitor lactacystin, but not by calpain inhibitor calpeptin or lysosome inhibitor leupeptin; cyclosporin A or FK506 increased Ser-phosphorylation of IRS-2 and ubiquitination of IRS-2. In cyclosporin A- or FK506-treated cells, IGF-I-induced phosphorylations of GSK-3β and ERK1/ERK2 were attenuated due to the reduction of IRS-2 level by cyclosporin A or FK506; these reductions of IGF-I-induced phosphorylation events were protected by lactacystin or rapamycin.

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Abbreviations: CNX, calnexin, cPKC-α, conventional protein kinase C-α, ERK, extracellular signal-regulated kinase, GSK-3, glycogen synthase kinase-3, Hsp90, 90-kDa heat-shock protein, IGF-I, insulin-like growth factor-I, IRS, insulin receptor substrate, PI3K, phosphoinositide 3-kinase, MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, NF-*k*B, nuclear factor-*k*B

Key Words: GSK-3 inhibitor, GSK-3 phosphorylation, Diabetes mellitus, Inflammation, Neurodegenerative diseases, Cell protection, Neurogenesis, Insulin receptor, IRS-1, IRS-2, Akt, Protein kinase C, Calcineurin, Ca^{2+}, Chaperone, Review

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