Critical Review

New Twist on Neuronal Insulin Receptor Signaling in Health, Disease, and Therapeutics

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Abstract. Long after the pioneering studies documenting the existence of insulin (year 1967) and insulin receptor (year 1978) in brain, the last decade has witnessed extraordinary progress in the understanding of brain region-specific multiple roles of insulin receptor signalings in health and disease. In the hypothalamus, insulin regulates food intake, body weight, peripheral fat deposition, hepatic gluconeogenesis, reproductive endocrine axis, and compensatory secretion of counter-regulatory hormones to hypoglycemia. In the hippocampus, insulin promotes learning and memory, independent of the glucose regulatory effect of insulin. Defective insulin receptor signalings are associated with the dementia in normal aging and patients with age-related neurodegenerative diseases (e.g., Alzheimer’s disease); the cognitive impairment can be reversed with systemic administration of insulin in the euglycemic condition. Intranasal administration of insulin enhances memory and mood and decreases body weight in healthy humans, without causing hypoglycemia. In the hypothalamus, insulin-induced activation of the phosphoinositide 3-kinase pathway followed by opening of ATP-sensitive K⁺ channel has been shown to be related to multiple effects of insulin. However, the precise molecular mechanisms of insulin’s pleiotropic effects still remain obscure. More importantly, much remains unknown about the quality control mechanisms ensuring correct conformational maturation of the insulin receptor, and the cellular mechanisms regulating density of cell surface functional insulin receptors.

Keywords: neuronal insulin receptor, neuroendocrine homeostasis, memory, quality control, up- and down-regulation

Introduction

Physiological roles of insulin, as well as pathological consequences of insulin deficiency and insulin resistance have been extensively studied in adipocyte, hepatocyte, and skeletal myocyte, resulting in the clarification of the pathogenetic mechanisms of diabetes mellitus and diabetic complications. During the last decade, evidence has rapidly accumulated that insulin acts in the brain to regulate food intake, body weight, fat deposition, hepatic gluconeogenesis, energy homeostasis, counter-regulatory hormone secretion to hypoglycemia, and spermatogenesis, as well as ovulation (1 – 3).

Insulin deficiency is known to culminate in diabetic peripheral neuropathy affecting motor, sensory, and autonomic neurons; however, less attention has been paid to the roles of insulin in regulating functions and structures of the peripheral and central nervous systems. Surprising, but compelling evidence has emerged that in developing and adult neurons, insulin and insulin-like growth factor-I (IGF-I) promote myelinization of neuronal axon (4 – 9), formation of synaptic network, synaptic plasticity, cognitive function (e.g., learning /memory) (10, 11), cell survival (12), as well as longevity (13). Although still less recognized by most clinicians, there is now substantial evidence that diabetes mellitus insidiously produces disruptive effects on electrophysiology and structure of the brain, referred to as diabetic encephalopathy (10, 14), a concept proposed in 1950 by DeJong (introduced in Ref. 15).

Levels of insulin and IGF-I in plasma and cerebro-
spinal fluid are abnormal in patients with various neurodegenerative diseases (e.g., Alzheimer’s disease, Parkinson’s disease, and vascular dementia), which are associated with insulin resistance and defective intracellular signalings of insulin receptor and IGF-I receptor; treatment with insulin or IGF-I exerts beneficial effects against various cognitive deficits associated with normal aging and age-related neurodegenerative diseases, independent of the glucoregulatory effect of insulin (14, 16 – 22). In humans, intranasal administration of insulin enhanced memory and mood, while decreasing appetite, with no effect on plasma glucose and insulin levels (23, 24).

Insulin receptor and IGF-I receptor, two members of receptor tyrosine kinase family, are structurally homologous to each other and consist of their cognate two extracellular α- and two transmembrane β-subunits. Insulin receptor and IGF-I receptor can bind both insulin and IGF-I with approximately 100-fold different affinities. Binding of insulin or IGF-I to the α-subunit increases the intrinsic tyrosine kinase activity of the β-subunit and causes autophosphorylation of the β-subunit, triggering tyrosine-phosphorylation of the insulin receptor substrate (IRS) family and Shc. The tyrosine-phosphorylated sites create binding sites for signaling molecules containing the Src homology-2 domain (e.g., phosphoinositide 3-kinase [PI3K] and Grb2), activating cascades of two major signaling pathways, that is, PI3K/Akt (protein kinase B)/glycogen synthase kinase-3 (GSK-3) and Ras/Raf-1/extracellular signal-regulated kinase (ERK) pathways (12). Insulin receptor and IGF-I receptor activate similar intracellular signaling pathways, yet differentially regulate expression of a subset of genes encoding adhesion, transcription, transport, and proliferation molecules (25). In addition, IRS-1 and IRS-2 transduce redundant, yet distinct signaling pathways/biological effects; IRS-1 was indispensable for resisting serum deprivation-induced apoptosis (26), whereas IRS-2 promoted embryonic brain development and inhibited abnormal hyperphosphorylation of tau (27), a pathogenetic hallmark of Alzheimer’s disease brains (14, 17 – 21). Furthermore, IRS-1 and IRS-2 are multifunctional scaffold proteins that integrate signalings from G protein-coupled receptors and cytokine receptors.

In the present review, we briefly summarized historical and recent findings about the novel biological roles of brain insulin in health and disease (Tables 1 and 2). Despite these recent advances, much remains unidentified about the specific downstream effector molecules involved in insulin-induced neuromodulation and the quality control mechanisms regulating cell surface expression of insulin receptors. We then introduced several of our experimental results about these issues in cultured bovine adrenal medullary chromaffin cells (embryologically derived from neural crest) (Fig. 1): 1) stimulation of insulin receptor and IGF-I receptor up-regulated cell surface expression of voltage-dependent Na+ channel via distinct mechanisms, Na+ channel up-

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Fig. 1. Multiple mechanisms regulating cell surface expression of insulin receptor in adrenal chromaffin cells. In the nonstimulated cell, [a] Hsp90 catalyzes homodimerization of monomeric insulin receptor precursor molecule at the endoplasmic reticulum (29). [b] Endoplasmic reticulum Ca<sup>2+</sup>-ATPase activity (31) and [c] peptidyl prolyl cis-trans isomerase activity of immunophilin (30) promote cell surface targeting of insulin receptor. [d] cPKC-α activation up-regulates (33), whereas [e] acetoacetate down-regulates (32), cell surface insulin receptor by altering insulin receptor mRNA levels. CNX, calnexin; cPKC-α, conventional protein kinase C-α; IRS, insulin receptor substrate; pY, tyrosine-phosphorylation.
regulation augmenting opening of voltage-dependent Ca\(^{2+}\) channels and exocytic secretion of catecholamines (28); 2) chaperone functions of 90-kDa heat shock protein (Hsp90) (29) and peptidyl prolyl cis-trans isomerase activity of immunophilin in cytoplasm (30), as well as sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) activity (31), and GSK-3β activity positively maintained the steady-state level of cell surface insulin receptor via distinct mechanisms; 3) ketone body down-regulated cell surface expression of insulin receptor via destabilizing insulin receptor mRNA (32); and 4) activation of conventional protein kinase C-α (cPKC-α) up-regulated cell surface expression of insulin receptor via elevating insulin receptor mRNA level (33).

**Identification of insulin receptor in brain and origin of cerebral insulin**

In 1967, Margolis and Altszuler (34) documented that immunoreactive insulin could be detected in dog cerebrospinal fluids, with its concentration being 27% of plasma insulin; in addition, insulin levels in dog cerebrospinal fluids elevated by up to 10-fold following infusion of bovine insulin into the dog saphenous vein, suggesting that circulating insulin could cross the blood-brain barrier. In 1978, Havrankova et al. (35) provided evidence that the insulin receptor was widely distributed in 16 different rat brain regions, as evidenced by using I-insulin binding assay; I-insulin binding site was enriched, in particular, in the olfactory bulb, cerebral cortex, hippocampus, hypothalamus, and amygdala.

It is now generally thought that insulin is not synthesized to any significant amount in adult developed brain (1, 2, 36, 37), although some studies may be consistent with the synthesis of insulin in brain (3). It may be possible that insulin could be synthesized during a specific period of brain development. In the larva of fruit fly Drosophila, insulin-producing cells exist in brain and secrete insulin into the circulatory system; ablation of these cells caused developmental delay, growth retardation, and elevated carbohydrate levels, which are characteristics of human diabetes mellitus (38). In adult developed brain, however, most if not all insulin is transported from the circulation after the secretion from pancreatic β-cells; insulin transport is mediated via a carrier-mediated, saturable process, and occurs presumably at the choroid plexus and capillary endothelial cell, the rates of insulin transport being different among various brain regions (36, 37). The physiological significance of insulin transport machinery is exemplified by the observations that transport of insulin via the blood-brain barrier is decreased in fasting, obesity, aging, and oral dexamethasone administration, while being abolished in hyperglycemia and hibernation of marmots; insulin transport is faster in neonates than in adults, and increased in streptozotocin- or alloxan-induced diabetic rats (36). Alzheimer’s patients have low levels of insulin in cerebrospinal fluid, in spite of the elevated levels of plasma insulin (39); peripheral infusion of insulin, while keeping plasma glucose levels constant, improved learning/memory in Alzheimer’s patients (40). On the contrary, insulin can enhance transport of tyrosine and tryptophan across the blood-brain barrier (36), a condition expected to increase levels of catecholamines and serotonin in brain. Taken together, blood-brain barrier transport of circulating insulin provides a mechanism whereby pancreatic insulin regulates pleiotropic biological events in brain. It still remains, however, elusive whether the insulin transport machinery is related to the signal-transducing insulin receptor consisting of the α/β2 complex (2, 36).

Another layer of complexity exists about the significance of insulin transport via the blood-brain barrier at the arcuate nucleus in the hypothalamus. Insulin decreases food intake by acting at the arcuate nucleus, a brain region located adjacent to the median eminence lacking a blood-brain barrier; however, it is not convincingly determined whether or not arcuate nucleus neurons are protected by the blood-brain barrier (1).

**Novel roles of insulin in brain**

**Feedback catabolic regulation of food intake, fat deposition, and body weight**

Peripheral effects of insulin are anabolic, increasing storage of carbohydrate, lipid and protein, whereas brain insulin has as a catabolic function, decreasing food intake and body weight (1 – 3, 37). In 1979, Woods et al. (41) observed that chronic intracerebroventricular infusion of insulin decreased food intake and body weight of baboons. In contrast, injection of insulin antibody into rat ventromedial hypothalamic caused the hyperphagia characteristic of diabetes mellitus (42). Intravenous infusion of insulin, while controlling plasma glucose level to protect against insulin-induced hypoglycemia, reduced food intake in baboons (43); thus, circulating peripheral insulin can reach the brain, acting as its own counter-regulatory hormone. Subsequent studies confirmed that brain insulin signalings cause an anorexigenic effect, reducing body weight, whereas impairment of brain insulin signalings induces an orexigenic effect, increasing body weight associated with peripheral insulin resistance (44 – 46). Targeted disruption of insulin receptor in the hypothalamic arcuate nucleus in rats, by administration of antisense...
RNA directed against insulin receptor precursor molecule, caused hyperphagia, fat deposition, and body weight gain, as well as attenuating the inhibitory effect of insulin on hepatic gluconeogenesis (45). In the control of energy homeostasis, a gender difference exists; male brains are more sensitive to the catabolic action of low doses of insulin, compared with female brains (24, 47).

For the anorexigenic effect of insulin in the hypothalamus, two laboratories have specified the intracellular signaling pathways of the insulin receptor (1–3, 37). Insulin activated the IRS/Pi3K/Akt signaling pathway in rat hypothalamic arcuate nucleus; prior intracerebroventricular injection of LY294002 or wortmannin, an inhibitor of Pi3K, prevented the reduction of food intake and body weight caused by the subsequent intracerebroventricular administration of insulin (1, 48).

In the hypothalamus of obese rats, insulin-induced autophosphorylation of insulin receptor and subsequent IRS-1/IRS-2 phosphorylation and association with Pi3K as well as Akt phosphorylation were decreased, as compared with those of lean rats (48).

The neurons that regulate food intake in response to insulin were studied. In the hypothalamic arcuate nucleus, anabolic neurons co-express orexigenic peptides neuropeptide Y and agouti-related protein; neuropeptide Y mRNA level was significantly increased in diabetic rats (1, 49). Administration of insulin into the third ventricle decreased neuropeptide Y mRNA level in the arcuate nucleus and lowered neuropeptide Y protein level in the paraventricular nucleus, a target downstream neuron innervated by arcuate nucleus neuron (44, 49). In addition, catabolic neurons in hypothalamic arcuate nucleus express anorexigenic α-melanocyte-stimulating hormone; intracerebroventricular administration of insulin in rats raised the level of pro-opiomelanocortin mRNA encoding for α-melanocyte-stimulating hormone, and melanocortin antagonist prevented reduction of food intake caused by insulin administered into the third ventricle (50). In addition to the paraventricular nucleus, arcuate nucleus neurons project to other important brain regions and are thought to function as primary neurons in various neuronal circuits that regulate food intake, hypothalamus-pituitary function, and sympathetic outflow (1).

Subsequent studies examined the downstream molecular mechanisms whereby insulin exerted the anorexigenic effect in the hypothalamus. In isolated hypothalamic neurons supposed to contain neuropeptide Y/agouti-related protein, insulin treatment hyperpolarized (inactivated) neurons via opening of ATP-sensitive K+ channel (KATP channel) (51), a condition inhibiting secretion of these orexigenic peptides. In pancreatic β-cells, insulin caused an opening of KATP channel via activating the PI3K pathway (1, 2). Because the anorexigenic effect of insulin was dependent on insulin-induced activation of PI3K in the hypothalamus (1, 48), these correlative results raise the possibility that insulin-induced activation of the PI3K pathway results in the opening of the KATP channel, thus inhibiting secretion of orexigenic neuropeptide Y/agouti-related protein in the arcuate nucleus and contributing to the insulin-induced anorexigenic effect.

More recent lines of experiments suggest that in addition to its action in the hypothalamus, insulin can act at mesolimbic dopamine signaling pathways implicated in the motivation, reward, and reinforcement of palatable food intake; insulin blunts intake of palatable food, providing new insights into the novel role of insulin in mental disorders (e.g., eating disorders and substance abuse) (37).

Inhibition of gluconeogenesis in hepatocyte

Circulating peripheral insulin directly inhibits glucose output from liver; in addition, insulin acts at brain to suppress gluconeogenesis, which was remarkably blunted in rats deficient in insulin receptor in the hypothalamic arcuate nucleus (45). Infusion of insulin into rat third cerebral ventricle suppressed hepatic glucose production, independent of circulating insulin level; the suppression of glucose production was blunted by third cerebroventricular administration of PI3K inhibitor (i.e., LY290042 or wortmannin) or KATP channel antagonist (glibenclamide or tolbutamine) (52). Pocai et al. (53) documented that insulin-induced opening of KATP channel in mediobasal hypothalamus culminated in the activation of brain stem nuclei (i.e., dorsal motor vagal nuclei and nucleus of solitary tract), which generated efferent vagal impulses to hepatocytes and inhibited hepatic glucose output into the bloodstream, decreasing blood glucose level; the inhibitory effect of central insulin on hepatic gluconeogenesis was negated by infusion of glibenclamide within the hypothalamus or surgical resection of the hepatic branch of the vagus nerve. In addition, the inhibitory effect of systemic insulin on hepatic gluconeogenesis was halved by the surgical resection of hepatic vagus branch (53), suggesting the involvement of the hypothalamus in circulating insulin-induced restraint of hepatic gluconeogenesis. Consistent with these results, mice lacking SUR1, a subunit of the octameric KATP channel, are resistant to the inhibitory action of insulin on gluconeogenesis (53). These correlative results suggest that insulin inhibits hepatic glucose production by both a classical direct mechanism and a newly identified indirect pathway, involving PI3K-dependent opening of hypothalamic KATP channels and activation of the
vagus nerve.

Maintenance of counter-regulatory hormone secretion to hypoglycemia

A common finding in insulin-deficient type 1 diabetic patients is a defective compensatory response to hypoglycemia, exacerbating frequency and severity of insulin-induced hypoglycemic episodes (54). In rats subjected to bilateral lesions of ventromedial hypothalamus, hypoglycemia-induced compensatory secretion of epinephrine, norepinephrine, and glucagon was attenuated by up to 80% (55). In knockout mice lacking brain/neuron-specific insulin receptor (NIRKO mice), hypoglycemia-induced secretion of norepinephrine (but not glucagon and corticosterone) was almost totally absent (56). Hypoglycemia-induced rapid secretion of catecholamines and glucagon was prevented, when rat ventromedial hypothalamus was perfused with D-glucose (but not nonmetabolizable L-glucose) (57). The hypothalamus contains specific glucose-excited neurons and glucose-inhibited neurons (58). Glucose-excited neurons increase their own electrical activity at high extracellular glucose level by closing $K_{\text{ATP}}$ channels; in contrast, glucose-inhibited neurons increase their activity at low extracellular glucose level via an as yet unidentified mechanism (58). In rat arcuate and ventromedial hypothalamus, Spanswick et al. (51) demonstrated that insulin hyperpolarized (inactivated) a subpopulation of these glucose-responsive neurons by opening $K_{\text{ATP}}$ channels. In ventromedial hypothalamus of awake rats, Evans et al. (59) documented that closure of $K_{\text{ATP}}$ channel by glibenclamide or tolbutamide suppressed compensatory secretion of epinephrine and glucagon in response to brain glycopenia or systemic hypoglycemia. Although the exact relationship between $K_{\text{ATP}}$ channel opening-induced suppression of neuronal activity and counter-regulatory hormone secretion remains elusive, these correlative results may raise the possibility that insulin-induced opening of $K_{\text{ATP}}$ channels in hypothalamus is involved in hypoglycemia-induced compensatory secretion of counter-regulatory hormones.

Mechanisms of $K_{\text{ATP}}$ channel opening by PI3K pathway

In hypothalamic arcuate nucleus (60) and CRI-G1 insulinoma cells (61), two laboratories have examined the underlying mechanisms for PI3K-induced opening of $K_{\text{ATP}}$ channel. By using agents that destabilize or stabilize cytoskeletal actin filament, they proposed that PI3K-catalyzed formation of phosphoinositide 3,4,5-trisphosphate from phosphoinositide 4,5-bisphosphate resulted in actin filament depolymerization, and the subsequent cytoskeletal remodeling was involved in opening of $K_{\text{ATP}}$ channels.

Treatment of diabetic patients with oral hypoglycemic sulfonylurea: potential adverse effects due to closing of $K_{\text{ATP}}$ channels by sulfonylurea

As stated above, insulin-induced opening of $K_{\text{ATP}}$ channels in hypothalamus has been shown to be essential to insulin-induced reduction of food intake and suppression of hepatic gluconeogenesis (51 – 53); in addition, opening of $K_{\text{ATP}}$ channels was necessary for triggering secretion of counter-regulatory hormones to brain glycopenia or systemic hypoglycemia (59). In pancreatic $\beta$-cells, however, closing of $K_{\text{ATP}}$ channels is the cellular mechanism whereby glucose or sulfonylurea increases secretion of insulin. Sulfonylurea has been used routinely to lower blood glucose level in type 2 diabetic patients; if oral administration of sulfonylurea can gain access to and inhibit hypothalamic $K_{\text{ATP}}$ channels, it might potentially exert multiple unwanted effects. Clearly, a wide variety of critical studies are required to address these clinically important issues.

Maintenance of reproductive endocrine axis

Mice with targeted disruption of brain insulin receptor exhibited impaired spermatogenesis and defective ovarian follicle maturation because of the hypothalamic dysregulation of luteinizing hormone (46). Diabetic female rats have decreased ovulation, sexual behavior, and luteinizing hormone secretion; increasing peripheral insulin level restored the phenotype to normal (62). In vivo intracerebroventricular administration of insulin in diabetic female rats (62) or increasing circulating insulin level in male mice (63) elevated luteinizing hormone level in plasma; in cultured hypothalamic cells, insulin treatment stimulated secretion and expression of gonadotropin-releasing hormone (63).

Promotion of learning/memory and neuroprotection in health and neurodegenerative diseases

In 1976, De Castro and Balagura (64) found that in rats subjected to hippocampal lesions, pretreatment with insulin significantly protected against memory deficits caused by the lesions, as evaluated in an active avoidance learning test. In 1989, Voll et al. (65) documented that following 10-min controlled systemic hypotension coupled with bilateral carotid artery clamping in rats, subsequent subcutaneous injection of insulin for 1 week prevented ischemia-induced learning deficits when those rats were subjected to water maze place navigation training for 1 – 2 months after the ischemic insult; the insulin treatment also significantly reduced CA1 hippocampal necrosis caused by the ischemia. In 1999, Zhao et al. (11, 66) showed that water maze
training in rats was associated with up-regulation of insulin receptor gene expression, IRS-1, and She protein levels, as well as enhancement of ERK1/ERK2 phosphorylation in hippocampal CA1 pyramidal neurons. In 2001, Kern et al. (67) showed that systemic 6-h infusion of insulin under euglycemic condition in healthy human volunteers enhanced their abilities of verbal memory and selective attention. In 2001, Torres-Aleman and his colleagues (16, 68) documented that physical exercise (i.e., treadmill running) in rodents stimulated transport of circulating IGF-I to brain; it was essential for the exercise-induced increases in widespread c-Fos expression in neurons and in the number of newly formed hippocampal neurons and hippocampal brain-derived neurotrophic factor level; these changes were associated with the protection against spatial memory deficit in hippocampas-injured mice, as well as motor coordination impairment in brainstem- and cerebellum-damaged rats. In 2004, they also showed that systemic injection of IGF-I in adult mice increased brain vessel density; physical exercise- or brain injury-induced vascular remodeling in the adult mouse brain was dependent on IGF-I (16, 69).

In humans, insulin sensitivity normally declines with aging, and insulin resistance is an important risk factor affecting morbidity, disability, and mortality among the elderly; one of the striking physiological characteristics recently identified in longevity of centenarians is their greatly enhanced sensitivity to insulin (13). In rodents, number of insulin receptors remarkably decreased in an age-dependent manner between 3 months and 2 years in various brain regions (e.g., hippocampus, cerebral cortex, and choroid plexus) (11). Diabetes mellitus affected 4% of the population in 1995, escalating into over 5% of morbidity by the year 2025. The most common form of type 2 diabetes develops as the result of insulin resistance, with the compensatory increased secretion of insulin from pancreatic β-cells, eventually culminating in the deficit of insulin secretion at the later stage of diabetes. Type 1 diabetes is caused by absolute deficiency of insulin due to the impairment of pancreatic β-cells. In 1950, DeJong published an original article “The nervous system complications in diabetes mellitus with special reference to cerebrovascular changes” and coined the term “diabetic encephalopathy”, as introduced by Reske-Nielsen et al. (15). In 1965, Reske-Nielsen et al. reported neuropathological findings in the brains of 16 juvenile (type 1) diabetic patients, which differed from those seen in other diseases, and justified the term diabetic encephalopathy (15). The multifactorial pathogenesis of diabetic encephalopathy is not yet completely understood, but shares features with brain aging and diabetic neuropathy, including functional and morphological abnormalities (e.g., delayed latency of evoked potential, modest cerebral atrophy, and white matter lesions) (10, 14, 15). Treatment with insulin not only ameliorates diabetic complications in peripheral tissues, but could also improve brain functions in diabetic patients (10, 14).

In addition to diabetes mellitus, defective intracellular signalings of insulin receptor and IGF-I receptor, abnormal levels of insulin and IGF-I in plasma and cerebrospinal fluid, as well as insulin resistance have been documented to be involved in cognitive impairment in normal aging and age-related neurodegenerative diseases (e.g., Alzheimer’s disease, stroke, amyotrophic lateral sclerosis, depression, and vascular dementia) (14, 17 – 22). Insulin- or IGF-I-induced PI3K and ERK signaling pathways are conserved from Caenorhabditis elegans to mammals; and they are pivotal to neuronal excitability/synaptic plasticity, learning/memorY (10, 11, 16 – 22), cell survival (12, 70), and lifespan (12, 13). Insulin- or IGF-I-induced activation of Akt increases phosphorylation of proapoptotic molecules (e.g., Forkhead transcriptional factor and Bad) and causes their subsequent association with cytoplasmic 14-3-3 protein, preventing interaction between these proapoptotic molecules and their target molecules (2, 12). Unfortunately, signaling molecules in PI3K and ERK pathways are down-regulated by pathogenetic molecules (e.g., α-synuclein in Lewy body for Parkinson’s disease and Lewy body dementia), as evidenced in cultured neuronal cells (71). In Alzheimer’s patients, insulin levels in cerebrospinal fluid are low, despite elevated levels of plasma insulin (20, 39). In 1996, Craft et al. (40) provided the first evidence that raising plasma insulin level by intravenous infusion, while keeping plasma glucose level at a fasting baseline level, produced striking memory enhancement in patients suffering from dementia of Alzheimer’s disease.

β-Amyloid is normally a soluble protein, and its conversion to less soluble forms accounts for the generation and deposition of extracellular neuritic plaques, which is characteristic of Alzheimer’s patient brains; however, β-amyloid deposition is also found in brains of normal aged humans, but to a lesser extent, as compared to Alzheimer’s patients (18). Insulin or IGF-I has the capacity to inhibit production of β-amyloid by causing Akt-induced phosphorylation/inactivation of GSK-3; LiCl, an inhibitor of GSK-3, inhibited production of β-amyloid in brains of mice that overproduced amyloid precursor protein (72). In addition, insulin inhibits degradation of β-amyloid by insulin-degrading enzyme that degrades both insulin and β-amyloid (14, 18, 19). The activity and protein level of insulin-degrading enzyme were decreased in Alzheimer’s patients, consis-
tent with the increased level of β-amyloid in mice deficient in insulin-degrading enzyme (18, 19). An in vitro study showed that β-amyloid competitively inhibited 125I-insulin binding to insulin receptor and attenuated insulin-induced autophosphorylation of insulin receptor (73).

Recently, intracellular rather than extracellular accumulation of β-amyloid has been proposed as an initiating factor in the pathogenesis of Alzheimer’s disease, because intracellular accumulation of β-amyloid and extensive neuronal degeneration occurred in the absence of extracellular neuritic plaques in an animal model of Alzheimer’s disease (14, 17–21, 74). Insulin- or IGF-I-induced activation of PI3K and ERK pathways prevents abnormal intracellular accumulation of β-amyloid by increasing extracellular secretion of β-amyloid in brain (17, 18, 20, 21). In addition, IGF-I promotes transport of β-amyloid-binding carrier proteins (e.g., albumin and transthyretin) into brain via the choroid plexus, thus facilitating clearance of β-amyloid out of the brain into the circulation (17, 18, 20, 21); also, insulin increased transport of albumin into the brain in humans (75). In consecutive autopsy studies on 292 cases of Alzheimer’s patient brains, Miklossy et al. unveiled that β-amyloid deposition and τ-tangle-like inclusions (known as Biondi inclusions) developed in the ependyma and choroid plexus, prior to the occurrence of similar pathological changes in the cerebral cortex (76). Transthyretin levels in cerebrospinal fluid were lower in Alzheimer’s patients, compared with normal aged subjects (18), implicating the decreased excretion capacity of β-amyloid in Alzheimer’s disease brains. In 16 healthy humans aged 70 or older, intravenous infusion of insulin under euglycemic condition increased insulin concentration in cerebrospinal fluid, which was associated with elevation of β-amyloid level in cerebrospinal fluid and facilitation of declarative memory (77); these observations may implicate the enhancement of in vivo β-amyloid secretion by insulin and/or the reduced degradation of β-amyloid due to the possible competition between insulin and β-amyloid for insulin-degrading enzyme that degrades both insulin and β-amyloid (14, 18). Based on these findings, one laboratory proposed that insulin resistance/IGF-I resistance in choroid plexus may be one of the early pathogenetic changes precipitating Alzheimer’s disease (18).

Abnormal hyperphosphorylation of microtubule-binding protein tau is the major component of intracellular neurofibrillary tangles, which is associated with cognitive dysfunction in normal aging, as well as Alzheimer’s disease and tauopathies (78). Insulin or IGF-I inhibits abnormal hyperphosphorylation of tau by stimulating Akt-induced phosphorylation/inactivation of GSK-3 (tau kinase), thus preventing formation of neurofibrillary tangles (14, 17–22). In knockout mice lacking brain/neuron-specific insulin receptor (NIRKO mice) or IRS-2, tau is hyperphosphorylated in brain, leading to its intracellular deposition during aging (2, 27). More importantly, β-amyloid may promote formation of neurofibrillary tangles; in mice that overexpressed a mutant form of tau, as occurring in frontotemporal dementia and Parkinsonism, injection of β-amyloid into the hippocampus increased tau phosphorylation and filament formation in the amygdala, one of the brain regions affected in Alzheimer’s disease (17). Taken together, these correlative findings suggest that a single pathogenetic event (i.e., more pronounced dysfunction of insulin/IGF-I in brain, compared to those of normal aged brain) may account for neuronal atrophy/death, neurofibrillary tangles, and neuritic plaques characteristic of Alzheimer’s disease brain. It still remains, however, unclear whether dysfunction of insulin/IGF-I axis triggers onset of Alzheimer’s disease or merely contributes to the progression of Alzheimer’s symptoms.

Relevance to tauopathies and α-synucleinopathies

Pathological aggregation of tau associated with brain degeneration in the absence of β-amyloid deposition are the defining pathologies of neurodegenerative tauopathies such as frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) (78). In patients with Parkinson’s disease, insulin receptor mRNA or protein level was markedly depressed or lost in the substantia nigra (79, 80). It has become increasingly evident that clinical symptoms and pathological findings of tauopathies (e.g., Alzheimer’s disease) could be overlapped with those of α-synucleinopathies (e.g., Parkinson’s disease and Lewy body dementia), accounting for most late-onset age-related human neurodegenerative diseases (81). α-Synuclein pathology was originally identified in Alzheimer’s disease, prior to the discovery of its involvement in Parkinson’s disease (81). In addition, α-synuclein aggregation is a potent inducer of tau aggregation and tau phosphorylation (81). These correlative findings may raise the question of whether insulin and IGF-I signaling pathways display crucial roles in a wide variety of neurodegenerative diseases.

Therapeutic implication for intranasal administration of insulin and IGF-I

Insulin following its intranasal administration is considered to enter the cerebrospinal fluid from the nasal mucosa via intercellular clefts along the nervus olfactorius and the bulbus olfactorius. In 38 healthy humans, intranasal administration of human regular
insulin (4 × 40 IU/day) for 8 weeks significantly promoted declarative memory processing and enhanced mood (e.g., enhanced self-confidence and reduced anger), with no effect on plasma glucose and insulin levels (23). HallSchmid et al. (24) documented that intranasal administration of human regular insulin (4 × 40 IU/day) for 8 weeks decreased body weight by 1.28 kg, body fat by 1.38 kg, and waist circumference by 1.63 cm in 12 healthy normal-weight men mainly from reduced everyday food intake; in addition, these differences between the insulin and placebo groups had vanished during 4–5 months after the cessation of treatment. In 8 healthy women, the same insulin treatment rather increased body weight by 1.04 kg due to a rise in extracellular water (24), presumably reflecting the gender-dependent catabolic effect of insulin in energy homeostasis (47).

In rats, intranasal administration of IGF-I exerted a neuroprotective effect, even when initiated 25 min after the onset of middle cerebral artery occlusion; IGF-I at 150 µg reduced the infarct volume by 63% and improved the neurologic deficit tests of motor, sensory, reflex, and vestibulomotor functions (82).

**Physiological roles of insulin receptor and IGF-I receptor in adrenal chromaffin cell**

**Insulin-induced up-regulation of cell surface functional voltage-dependent Na⁺ channel: enhancement of voltage-dependent Ca²⁺ channel opening and exocytic secretion of catecholamines**

The complexity of cellular mechanisms whereby insulin regulates formation, maintenance, and repair of neuronal network is only now starting to be appreciated. In neuronal cell systems, several laboratories have reported that insulin regulated cell surface targeting and endocytic internalization of glutamate receptor and γ-aminobutyric acid receptor, determining cell surface density of these receptors to modulate synaptic plasticity (11). Density and activity of voltage-dependent Na⁺ channel are pivotal in regulating phenotypic and genomic responses of neurons; different patterns of action potentials are decoded via not-fully defined mechanisms, and translated into other ionic and metabolic signals in a spatiotemporal-specific manner, regulating specific short- and long-term cellular events (83). To our knowledge, however, there is no report examining whether insulin or IGF-I could regulate cell surface expression of Na⁺ channel in any given tissue.

In cultured bovine adrenal chromaffin cells, we found that chronic (≥12 h) treatment with insulin up-regulated density of Na⁺ channels in a time (t₁/₂ = 26 h)- and concentration (EC₅₀ = 3 nM)-dependent manner, as evidenced by the cell surface [³H]saxitoxin binding assay and ²²Na⁺ influx measurement (28). Allosteric potentiation of ²²Na⁺ influx caused by veratridine, α- or β-scorpion venom, or Ptychodiscus brevis toxin-3 was similar between nontreated and insulin-treated cells, thus pharmacological properties of up-regulated Na⁺ channel by insulin being comparable to those of native Na⁺ channels. The increasing effects of insulin on [³H]saxitoxin binding and ²²Na⁺ influx were prevented by RNA synthesis inhibitor or protein synthesis inhibitor; however, insulin did not appreciably increase Na⁺ channel α-subunit mRNA level. Up-regulation of Na⁺ channel and the resultant increased influx of ²²Na⁺ enhanced ⁴⁵Ca²⁺ influx via voltage-dependent Ca²⁺ channel and exocytic secretion of catecholamines.

In peripheral efferent and afferent neurons, previous studies documented that insulin receptors exist in the perikarya and axolemma, including the node of Ranvier at the axon (4, 7, 9), where Na⁺ channels are concentrated in myelinated neurons. In experimental animal and human type 1 insulin-dependent diabetes mellitus, insulin deficiency culminated in the disruption of paranodal myelination, independent of hyperglycemia; the resultant abnormal dislocation of Na⁺ channel from nodal to paranodal axolemma caused down-regulation of nodal Na⁺ channel density and defective nerve conduction (4, 7, 9). In streptozotocin-induced diabetic rats, administration of low doses of insulin close to neuron or into subarachnoideal space reversed defective conduction velocity and atrophy of motor and sensory neurons; in contrast, subarachnoideal administration of insulin antibody generated nerve conduction slowing and atrophy resembling those in diabetes (9). In auditory and visual circuits in brain, axonal conduction velocity was decreased in streptozotocin-induced diabetic rats; insulin treatment with its subcutaneous implants, initiated 6 months after streptozotocin-induced diabetes mellitus, almost completely reversed the existing abnormalities in brain, whereas normalization of blood glucose levels had no effect (10). Because Na⁺ channel is essential to generation and conduction of action potential, up-regulation of cell surface Na⁺ channel by insulin demonstrated in our study (28) may be instructive in understanding the regenerative effect of insulin in diabetic neuropathy. More surprisingly, the axon guidance role of insulin receptor was evidenced by Song et al. (84) in the photoreceptor of the fruit fly Drosophila; insulin receptor signaling was required for the extending neuronal axon to find its way from the retina to the brain during development of visual system.
IGF-I-induced up-regulation of voltage-dependent Na' channel gene expression: involvement of GSK-3β

IGF-I acts as an endocrine and paracrine/autocrine regulator to promote neuronal development and differentiation in developing brain, as well as neuroprotection in adult brain (16–18, 20, 21, 82). Degenerative neuronal diseases are characterized by the defective formation or excessive degradation of myelin along the axon. IGF-I promotes proliferation of oligodendrocyte and stimulates myelin synthesis in oligodendrocyte, inducing remyelination and limiting demyelination following nerve injury (85).

In cultured bovine adrenal chromaffin cells, IGF-I treatment elevated cell surface [3H]saxitoxin binding by up to 40% (t1/2 = 24 h, EC50 = 45 nM), without altering the Kd value; it was prevented by LY294002 or wortmannin, an inhibitor of PI3K (the authors’ unpublished observations). Treatment with LiCl, valproic acid, or SB216763, an inhibitor of GSK-3, increased [3H]saxitoxin binding by up to 40%, whereas concurrent treatment of IGF-I with LiCl, valproic acid, or SB216763 did not produce any additive increasing effect on [3H]saxitoxin binding. IGF-I (100 nM) reduced GSK-3 activity by 52% and increased Ser3-phosphorylation of GSK-3β (a hallmark of GSK-3 catalytic activity inhibition) by 90% within 1 min. Either treatment raised Na’ channel α-subunit protein level; the increasing effect of IGF-I was prevented by LY294002 or wortmannin. Either treatment increased Na’ channel α-subunit mRNA level by up to 50% between 3 and 48 h and accelerated α-subunit gene transcription by up to 50% without altering α-subunit mRNA stability. Thus, IGF-I-induced suppression of GSK-3β up-regulated Na’ channel gene expression via mechanisms distinct from those of insulin.

Quality control and up- and down-regulations of insulin receptor in adrenal chromaffin cells

Essential role of Hsp90 family in conformational maturation of insulin receptor

With regard to a variety of receptor tyrosine kinases, G protein-coupled receptors and ion channel-associated receptors, their subunit compositions, amino acid sequences, and membrane topologies, as well as post-translational modifications (e.g., phosphorylation, ubiquitination, and partner protein recruitments) have been documented. In contrast, the quality control mechanisms in the endoplasmic reticulum (ER) ensuring cell surface expression of only a correct folded /assembled-receptor/ion channel remain largely unknown. Insulin receptor consists of two extracellular α-subunits (approximately 135 kDa) and two transmembrane β-subunits (approximately 95 kDa) that are encoded by the same gene and derived from the single-chain insulin receptor precursor molecule. Insulin receptor precursor undergoes cotranslational glycosylation, intrachain disulfide-linked homodimerization at the ER. The homodimeric insulin receptor precursor is processed at the trans-Golgi network by proproteinase furin into the disulfide-linked αβ2 complex, which is targeted to plasma membrane via as yet unidentified mechanisms. In spite of this general framework of insulin receptor synthesis, little is known about the quality control mechanisms ensuring the conformational maturation of monomeric insulin receptor precursor into αβ2 complex in any given tissue.

As the growing polypeptide of glycoprotein enters into the lumen of ER via Sec61 translocon, the oligosaccharide core unit, Glc-Man2GlcNAc2 (Glucose2Mannose2N-Acetylgalactosamine2), is cotranslationally transferred to the N-linked glycosylation site of the polypeptide intermediate by oligosaccharyltransferase, and sequentially trimmed by glycosidases I and II (86, 87). Calnexin, a lectin chaperone of the ER transmembrane protein, and calreticulin, a calnexin homologue within the ER lumen, bind to monoglycosylated glycoprotein intermediate bearing Glc-Man2GlcNAc2, thus enhancing correct glycoprotein folding/assembly and tethering incompletely folded/assembled glycoprotein in the ER. Also, calnexin functions as a molecular chaperone by recognizing the exposed hydrophobic polypeptide segments that are normally buried inside the native mature glycoprotein. Glucosidase II-catalyzed trimming of final glucose residue from Glc-Man2GlcNAc2 causes dissociation of glycoprotein from calnexin/calreticulin; correctly folded/assembled glycoprotein is exported to its destination, whereas incompletely folded/assembled glycoprotein is reglucosylated by UDP-glucose/glycoprotein glucosyltransferase, a folding sensor, and reassociates with calnexin/calreticulin, multiple rounds of this association-dissociation cycle being postulated to occur. When one or more mannose residues of finally misfolded glycoprotein are cleaved by the ER α-mannosidase I, its calnexin cycle is slowed down; ER degradation enhancing α-mannosidase-like protein (EDEM), an enzymatically inactive mannosidase-like protein, interacts with calnexin and extracts misfolded glycoprotein from the calnexin cycle (88, 89), which is retro-translocated via Sec61 translocon into the cytoplasm, being proteolytically degraded by the multiple mechanisms, including the ubiquitin-proteasome system.

Hsp90, a molecular chaperone in the cytoplasm, and the 94-kDa glucose-regulated protein (Grp94), a Hsp90
homologue in the ER lumen, ensure correct conformational maturation and translocation of signaling molecules (e.g., steroid hormone receptors, Src-tyrosine kinases, growth factor receptors, and cystic fibrosis transmembrane conductance regulator [CFTR]), as evidenced by using geldanamycin or herbimycin A, an inhibitor of Hsp90 family (90). Geldanamycin binds to the adenosine nucleotide binding site of the N-terminal domain of Hsp90 with affinity higher than that of ATP and inhibits the ATPase activity/chaperone function of Hsp90.

In cultured bovine adrenal chromaffin cells, long-term (≥26 h) treatment with geldanamycin lowered cell surface 125I-insulin binding capacity by up to 87% in a time (t₁/2 = 8.5 h)- and concentration (EC₅₀ = 84 nM)-dependent manner, with no effect on the Kᵣ value (29). Treatment with herbimycin A decreased 125I-insulin binding capacity approximately 10-fold less effectively, compared with geldanamycin, as shown in the case of v-Src-induced, Hsp90-mediated oncogenic morphological transformation of fibroblasts (90). Western blot analysis with insulin receptor β-subunit antibody revealed that geldanamycin treatment decreased insulin receptor β-subunit level by up to 83% (t₁/₂ = 7.4 h, EC₅₀ = 74 nM), while increasing insulin receptor precursor level by up to 100% (t₁/₂ = 7.9 h, EC₅₀ = 300 nM). Pulse-label followed by reducing and nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis demonstrated that monomeric insulin receptor precursor (approximately 190 kDa) underwent homodimerization (approximately 380 kDa) at 30 min and developed into the mature αβ₂ insulin receptor (approximately 410 kDa) after 1 h in geldanamycin-nontreated cells, whereas monomeric insulin receptor precursor failed to undergo homodimerization in geldanamycin-treated cells; the homodimerization-incompetent form of insulin receptor precursor in geldanamycin-treated cells was degraded via ER-associated protein degradation. Immunoprecipitation followed by immunoblot analysis showed that insulin receptor precursor was associated with calnexin to a greater extent in geldanamycin-treated cells, compared with nontreated cells. Geldanamycin did not alter insulin receptor mRNA levels and endocytic internalization of cell surface insulin receptor. In geldanamycin (1 µM for 24 h)-treated cells, subsequent insulin (100 nM for 10 min)-induced tyrosine-phosphorylation of IRS-1 was attenuated by 77%, with no change in the cellular level of IRS-1. Thus, inhibition of the chaperone function of Hsp90 family impairs homodimerization of monomeric insulin receptor precursor molecule in the ER, in which monomeric insulin receptor precursor was retained with calnexin, leading to the retardation of cell surface expression of insulin receptor and the attenuation of insulin-induced activation of IRS-1.

Peptidyl prolyl cis-trans isomerase activity of immunophilins: cell surface targeting of insulin receptor from trans-Golgi network

Immunophilins (e.g., cyclophilin and FK506-binding protein [FKBP] families) are more enriched in the nervous system than the immune system, and the peptidyl prolyl cis-trans isomerase activity of immunophilin plays a growing number of diverse biological roles, as evidenced by using cyclosporin A, an inhibitor of cyclophilin, or FK506 or rapamycin, an inhibitor of FKBP. Previous expression studies in Xenopus oocytes documented that cyclosporin A or FK506 decreased cell surface expression of homo-oligomeric (but not hetero-oligomeric) α- neuronal nicotinic receptor, type 3 serotonin receptor, and Kir₂.1 K⁺ channel, whereas it remains to be clarified whether cell surface expression of receptor tyrosine kinase family may require the chaperone function of immunophilin in any given tissue (30).

In cultured bovine adrenal chromaffin cells, we observed that chronic (≥23 h) treatment with cyclosporin A lowered cell surface 125I-insulin binding capacity by 62% (IC₅₀ = 18 µM, t₁/₂ = 16 h), with no effect on the Kᵣ value (30). FK506 (1 µM for 24 h) or rapamycin (3 µM for 24 h) decreased 125I-insulin binding by 25 or 32%; in addition, 24-h concurrent treatment with FK506 and rapamycin lowered 125I-insulin binding by 52% in an additive manner. Western blot analysis showed that cyclosporin A treatment decreased insulin receptor level (t₁/₂ = 15 h) in membrane fraction, but did not alter total cellular levels of insulin receptor precursor molecule and insulin receptor. Cyclosporin A did not accelerate the internalization rate of cell surface insulin receptor. Thus, inhibition of peptidyl prolyl cis-trans isomerase activity of cyclophilin or FKBP down-regulates cell surface expression of insulin receptor presumably by reducing externalization of insulin receptor from the trans-Golgi network.

SERCA activity: cell surface targeting of insulin receptor from trans-Golgi network

Defective increase of cytoplasmic concentration of Ca²⁺ ([Ca²⁺]) is assumed to account for the impaired glucose tolerance via as yet unidentified mechanisms in various peripheral and central tissues of non-insulin-dependent (type 2) diabetes mellitus, essential hypertension, and obesity (91). In human and streptozotocin- or alloxan-induced animal model of diabetes mellitus, active uptake of cytoplasmic Ca²⁺ into the ER catalyzed by SERCA was defective in pancreatic β-cells (92) and cardiac myocytes (93), impairing glucose-induced
[Ca\(^{2+}\)] oscillation, insulin secretion, and cardiac diastolic relaxation. Similar abnormalities of glucose-induced [Ca\(^{2+}\)] oscillation and insulin secretion were induced by chronic treatment of normal Langerhans islet with thapsigargin, an inhibitor of SERCA (92).

In cultured bovine adrenal chromaffin cells, long-term (≥12 h) treatment with thapsigargin caused a time (t\(_{1/2} = 16 h\))- and concentration (IC\(_{50} = 37\) nM)-dependent reduction of cell surface 125I-insulin binding capacity by 35%, with no change in the K\(_d\) value (31). Thapsigargin caused a rapid (up to 10 min) monophasic increase of [Ca\(^{2+}\)] followed by sustained (at least 48 h) plateau increase of [Ca\(^{2+}\)] (31, 94), and the effect of thapsigargin on 125I-insulin binding was abolished by BAPTA-AM (31), a cell membrane-permeable Ca\(^{2+}\) chelator. Thapsigargin lowered insulin receptor protein level in membrane (but not in cell lysate) and did not promote endocytic internalization of cell surface insulin receptor. Thus, SERCA activity is indispensable to cell surface targeting of insulin receptor presumably from the trans-Golgi network.

**GSK-3β:** homologous positive feedback-regulation maintaining steady-state level of cell surface insulin receptor

Evidence has accumulated that activities of the insulin receptor and IRS are subjected to homologous negative or positive feedback-regulation in the physiological state; Ser/Thr-phosphorylation of IRS-1 caused by downstream signals of the PI3K pathway (e.g., mammalian target of rapamycin) results in the self-attenuation of IRS-1 activity, whereas Akt-catalyzed Ser/Thr-phosphorylation of IRS-1 causes positive feedback activation of IRS-1 (95). In addition, agents inducing insulin resistance (e.g., tumor necrosis factor-α) increase Ser/Thr-phosphorylation of IRS-1 via activating protein kinases (e.g., c-Jun N-terminal kinase), thus providing heterologous negative feedback inhibition for insulin receptor signaling in pathological states (95). Catalytic activity of GSK-3 is constitutively active in non-stimulated cells and catalyzes phosphorylation of glycogen synthase, transcription factors (e.g., β-catenin and NF-κB), and translation initiation factor eIF-2B, thereby keeping these protein substrates in an inactive state or promoting their degradation. In contrast, stimulation of insulin receptor, IGF-I receptor, other growth factor receptors, G protein-coupled receptors, or Wnt receptor leads to Ser\(^{372}/\)Ser\(^{373}\)-phosphorylation of GSK-3α/3β and turns off the catalytic activity of GSK-3, turning on the signaling pathways that are constitutively suppressed by GSK-3 in nonstimulated cells and initiating multiple physiological effects. Therefore, a cellular event positively regulated by the basal constitutive activity of GSK-3 has not been specified in any given nonstimulated cells.

In cultured bovine adrenal chromaffin cells, treatment with GSK-3 inhibitor (e.g., LiCl or SB216763) or insulin lowered cell surface 125I-insulin binding capacity (no effect on the K\(_d\) value), insulin receptor protein, and insulin receptor mRNA levels; in contrast, the same treatment did not change cell surface 125I-IGF-I binding capacity and IGF-I protein level (the authors’ unpublished observations). LiCl-induced time-dependent decrease of 125I-insulin binding was preceded by the time-dependent increase of Ser\(^{372}\)-phosphorylation of GSK-3β caused by LiCl. These two effects of LiCl were reversed by the washout of LiCl-treated cells; 125I-insulin binding capacity and Ser\(^{372}\)-phosphorylation level of GSK-3β restored to those of LiCl-nontreated cells. Thus, GSK-3β constitutively maintains a steady-state level of cell surface insulin receptor in nonstimulated cells, whereas suppression of GSK-3β activity down-regulates cell surface expression of insulin receptor in insulin-stimulated cells.

cPKC-α: transcriptional and translational up-regulation of cell surface insulin receptor

In liver, heart, vascular myocyte, and endothelial cell of hyperglycemic human and rat diabetes mellitus, formation of diacylglycerol was increased, and cPKC-α underwent translative activation from cytoplasm to membrane to a greater extent, compared with the normoglycemic controls (96). In streptozotocin-induced diabetic animals, the level of cell surface insulin receptor increased in hepatocyte, adipocyte, and brain; insulin receptor density was also elevated in human retinas of diabetic patients (97). However, the biological consequences of cPKC-α activation and the mechanisms underlying the up-regulation of insulin receptor in diabetes mellitus remain elusive.

Our Western blot analysis showed that among 11 isoforms of the PKC family, adrenal chromaffin cells contain only three PKC isoforms (i.e., cPKC-α, novel PKC-ε [nPKC-ε], and atypical PKC-ζ [aPKC-ζ]) (98). Treatment with phorbol 12,13-dibutyrate (PDBu) or 12-O-tetradecanoylphorbol 13-acetate (TPA) caused a rapid (<15 min) and persistent (>15 h) translocation of both cPKC-α and nPKC-ε (but not aPKC-ζ) from the cytoplasm to membranes, whereas thymeleatoxin increased the similar but selective membrane association of only cPKC-α; even the long-term thymeleatoxin treatment (100 nM for up to 48 h) lowered cPKC-α protein level by only 18% in whole cell lysate (98). Chronic (≥12 h) treatment with PDBu elevated cell surface 125I-insulin binding by up to 115% (t\(_{1/2} = 14.6 h\), EC\(_{50} = 1.9\) nM), with no effect on the K\(_d\) value (33). TPA (30 nM) or
thymeleatoxin (EC$_{50}$ = 6.4 nM) increased $^{125}$I-insulin binding by 97% or up to 88%, whereas the biologically inactive 4α-TPA had no effect. The increasing effect of PDBu was blocked, even when H7, an inhibitor of PKC family, was added at 8 h after the initiation of PDBu treatment. Concurrent treatment with brefeldin A, an inhibitor of vesicular transport from the trans-Golgi network, cycloheximide, an inhibitor of protein synthesis, or 5,6-dichlorobenzimidazole riboside, an inhibitor of RNA synthesis, abolished the PDBu-induced increment of $^{125}$I-insulin binding. PDBu (30 nM) or thymeleatoxin (EC$_{50}$ = 2.3 nM) increased cellular levels of insulin receptor precursor (approximately 190 kDa, t$_{1/2}$ = 7.1 h) and insulin receptor β-subunit (t$_{1/2}$ = 15.4 h) by 52% or up to 59%. PDBu or thymeleatoxin increased insulin receptor mRNA levels by up to 35% as soon as 3 h, peaking at the maximum of 76% increases between 24 and 48 h. All of these increasing effects of PDBu and thymeleatoxin were abrogated by Gö6976, an inhibitor of cPKC-α (but not nPKC-ε and aPKC-ζ).

**Ketone body acetoacetate-induced down-regulation of insulin receptor signaling: no effect of β-hydroxybutyrate and acetone**

Physiological hyperketonemia occurs quite readily via Randle’s glucose-fatty acid cycle as a compensatory defensive response against fasting, particularly in the neonate and pregnancy, occasionally developing into frank ketoacidosis. In addition, hyperketonemia is induced during prolonged physical exercise and high-fat diet in normal humans. In patients with congenital enzyme defects unable to catalyze hepatic mitochondrial synthesis of ketone bodies, even a short-term fasting causes hypoketotic hypoglycemia, increased levels of plasma free fatty acids (FFA), and childhood sudden death due to their inability to oxidize FFA into ketone bodies (99). Ketogenic diet has been successfully employed to treat epilepsy and is proposed for the treatment of various neurodegenerative diseases (e.g., Alzheimer’s disease and Parkinson’s disease) (100). In contrast, diabetic ketoacidosis due to the defective insulin secretion and insulin resistance is a life-threatening state. In patients with diabetes mellitus, obesity, and atherosclerotic vascular diseases, the increased levels of plasma FFA are linked to the insulin-resistant state in these diseases, because of the interference of insulin receptor signalings by FFA.

In cultured bovine adrenal chromaffin cells, treatment (≥24 h) with ketoacidosis-related concentrations (≥3 mM) of acetoacetate (but not β-hydroxybutyrate, acetone, and acidic medium of pH 6.9) caused a time- and concentration-dependent reduction of cell surface $^{125}$I-insulin binding capacity by up to 38%, with no change in the K$_d$ value (32). Acetoacetate (10 mM for 24 h) lowered cellular levels of insulin receptor precursor and insulin receptor by 22% and 28%, respectively; in contrast, acetoacetate did not increase internalization rate of cell surface insulin receptor. Acetoacetate decreased insulin receptor mRNA levels by up to 23% as early as 6 h, producing their plateau of 30% reduction between 12 and 24 h; the half-life of insulin receptor mRNA was shortened by 10 mM acetoacetate from 13.6 to 9.5 h. Insulin (100 nM for 10 min)-induced tyrosine-phosphorylation of IRS-1 was attenuated by 56% in acetoacetate-treated cells, with no change in IRS-1 level.

**Conclusions**

Multiple lines of experiments in the last decade have accumulated compelling evidence that brain insulin receptor signalings play pivotal roles in regulating brain region-specific pleiotropic functions, which is exquisitely linked to the peripheral tissue functions regulated by circulating insulin. At present, however, we are faced with a great deal of critical questions about the precise molecular mechanisms of insulin’s pleiotropic effects which are not readily answered; our knowledge remains still fragmentary and circumstantial, being far from a comprehensive view. Apart from these important fundamental problems, intranasal administration of insulin and IGF-I is expected to be promising in the treatment of various pathological states (e.g., obesity, stroke, and neurodegenerative diseases). In addition, much remains to be elucidated about the molecular mechanisms whereby only the correctly folded insulin receptor is expressed on the cell surface at the appropriate level to meet the requirements for development, differentiation, and neuroprotection. Thus, insulin research in the basic and clinical sciences has developed into the new era toward the comprehensive resolution of many challenging themes.

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