Physiological and Pathological Changes in Glucose Regulate Brain Akt and Glycogen Synthase Kinase-3*

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Insulin regulates the phosphorylation and activities of Akt and glycogen synthase kinase-3 (GSK3) in peripheral tissues, but in the brain it is less clear how this signaling pathway is regulated in vivo and whether it is affected by diabetes. We found that Akt and GSK3 are sensitive to glucose, because fasting decreased and glucose administration increased by severalfold the phosphorylation of Akt and GSK3 in the cerebral cortex and hippocampus of non-diabetic mice. Brain Akt and GSK3 phosphorylation also increased after streptozotocin administration (3 days), which increased blood glucose and depleted blood insulin, indicating regulation by glucose availability even with deficient insulin. Changes in Akt and GSK3 phosphorylation and activities in epididymal fat were opposite to those of brain after streptozotocin treatment. Streptozotocin-induced hyperglycemia and increased brain Akt and GSK3 phosphorylation were reversed by lowering blood glucose with insulin administration. Long term hyperglycemia also increased brain Akt and GSK3 phosphorylation, both 4 weeks after streptozotocin and in db/db insulin-resistant mice. Thus, the Akt–GSK3 signaling pathway is regulated in mouse brain in vivo in response to physiological and pathological changes in insulin and glucose.

Insulin resistance and diabetes represent increasingly prevalent conditions that involve impaired regulation of glucose production and utilization (1). Recently much has been learned about insulin resistance in insulin-sensitive peripheral tissues, such as fat and skeletal muscle (2, 3). In contrast, little is understood about diabetes-induced changes in insulin-linked signaling activities in the brain even though cognition is often impaired in diabetic subjects (4), and the brain accounts for ~20% of the energy utilization in the body of adult humans (5). Glucose is the predominant substrate of the brain, and its consumption is tightly linked to neuronal activity, leaving neuronal function highly dependent on a continual supply of glucose (6). Because of this high demand for glucose, neurons have developed energy-efficient designs (7) and specialized glucose uptake mechanisms (8) to ensure adequate supply even at times when circulating glucose levels are low. Incumbent upon a system relying almost exclusively on glucose are mechanisms to sense and respond to fluctuations in the level of glucose, such as the well known regulation of blood flow by the brain that constricts or dilates arterioles to regulate local blood flow in accordance with demands of neuronal activity (9, 10). However, the brain is largely insulated from changes of insulin in the blood (11), so little is known about how insulin-coupled signaling systems within the brain either sense, or respond to, fluctuations in the circulating level of glucose or if they are buffered from such fluctuations. Insulin receptors in virtually all vertebrate tissues, including the brain, are coupled to the prominent signaling pathway encompassing Akt (also known as protein kinase B) and glycogen synthase kinase-3 (GSK3) (2). In insulin-responsive tissues, insulin signaling activates Akt, which inactivates GSK3 (12). Akt is activated by dual phosphorylation on threonine 308 and serine 473 carried out by 3-phosphoinositide-dependent kinase-1 (PDK1) and an unidentified kinase often called PDK2, respectively (13). Conversely, the activity of GSK3 is inhibited by N-terminal serine phosphorylation of the two GSK3 isoforms, serine 9 in GSK3β and serine 21 in GSK3α. Akt often appears to be the predominant kinase mediating this phosphorylation of GSK3, although this serine phosphorylation of GSK3 can be carried out by several other kinases under certain circumstances (14). This coupling of Akt and GSK3 leads to inverse changes in their activities; when Akt activity is high it maintains GSK3 in a serine-phosphorylated inhibited state, and decreases in Akt activity lead to dephosphorylation and activation of GSK3. Thus insulin causes increases in the phosphorylation states of both Akt and GSK3, and in peripheral tissues such as epididymal fat and skeletal muscle these kinases can become dephosphorylated by insulin resistance and diabetes (2, 3). The brain is considered to be largely buffered from detrimental effects of glucose fluctuations by efficient insulin-independent glucose transporters and utilization mechanisms (8, 15). However, numerous studies have documented changes in higher brain functions, such as memory and mood, associated with fluctuations in the circulating glucose concentration, indicating an influence on neuronal signaling systems (4, 16–18). Here we report that in mouse brain in vivo the Akt–GSK3 signaling pathway senses physiological and pathological variations in circulating glucose levels with respondent changes in the regulatory phosphorylation states of Akt and GSK3.

MATERIALS AND METHODS

Animals and Treatments—Adult, male C57BL/6 mice (Frederick Cancer Research, Frederick, MD), 6–7 weeks old, were injected intraperitoneally (ip) with streptozotocin (150 mg/kg in citrate buffer, pH 4.6) or vehicle for controls, either 3 days or 4 weeks before sacrifice. Adult male BKS.Cg-m/+/+Leprdb/rd) mice (Jackson Laboratories), 7–8 weeks old, were used for a type II diabetic model animal. All mice were allowed to drink ad libitum and kept on a 12 h light/dark cycle. Where indicated, mice were given intraperitoneal injections of d-glucose (2 g/kg) or insulin (5 international units/kg bovine pancreas insulin; Sigma) in phosphate-buffered saline after overnight food withdrawal. Blood glucose levels were measured using a glucose monitor (True Track Smart System). Insulin concentrations were measured using an ultrasensitive mouse insulin enzyme-linked immunosorbent assay
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(ELISA) assay (Mercodia, Winston Salem, NC). Body temperatures were measured rectally using a CyQ model 111 thermocouple (CyberSense Inc, Nicholasville, KY).

**Tissue Preparation**—Mice were decapitated, and brains were rapidly dissected in ice-cold saline. Brain regions were homogenized in ice-cold lysis buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 5 µg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 50 mM sodium fluoride, and 100 mM okadaic acid. The lysates were centrifuged at 20,800 × g for 10 min to remove insoluble debris. Protein concentrations in the supernatants were determined in triplicate using the Bradford (19) protein assay.

**Immunoblotting**—Extracts were mixed with Laemmli sample buffer (2% SDS) and placed in a boiling water bath for 5 min. Proteins were resolved in SDS-polyacrylamide gels, and transferred to nitrocellulose. Blots were probed with antibodies to phospho-Ser473-Akt, phospho-Thr308-Akt, and total Akt (Cell Signaling Technology, Beverly, MA). Immunoblots were developed using horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG (Bio-Rad), followed by detection with enhanced chemiluminescence, and statistical significance was determined using analysis of variance.

**Enzyme Activities**—Akt activity in the cerebral cortex was measured after immunoprecipitation of Akt from 100 µg of protein with 3.5 µg of monoclonal Akt antibody. Immobilized immune complexes were washed twice with lysis buffer and twice with kinase buffer (10 mM MOPS, pH 7.4, 1 mM EDTA, 10 mM magnesium acetate, 20 mM magnesium chloride, 1 mM dithiothreitol, 1 µg/ml aprotinin, and 1 µg/ml pepstatin). Kinase activity was measured by mixing immunoprecipitates with 30 µl of kinase buffer containing 125 µM ATP, 1.4 µCi of [γ-32P]ATP (Amersham Biosciences), and 100 µM crocrodile peptide substrate (Upstate Biotechnology, Inc., Lake Placid, NY). The samples were incubated at 30 °C for 20 min, centrifuged, and triplicates were spotted onto P81 filter paper. The filter papers were washed four times in 0.5% phosphoric acid for a total time of 1 h, rinsed in 95% ethanol, air-dried for 30 min, and counted in a liquid scintillation counter. Akt activity in epidymal fat was measured after immunoprecipitation from 200 µg of protein using a nonradioactive Akt activity assay kit (Cell Signaling Technology). GSK3α activity in cerebral cortex and epidymal fat was measured after immunoprecipitation of GSK3α from 100 µg of protein with 3 µg of polyclonal GSK3α antibody. Immobilized immune complexes were washed twice with lysis buffer and twice with kinase buffer. Kinase activity was measured by mixing immunoprecipitates with 30 µl of kinase buffer containing 125 µM ATP, 1.4 µCi of [γ-32P]ATP, and 100 µM phosphoglyceron synthase-2 substrate (Upstate Biotechnology, Inc.). The samples were incubated at 30 °C for 20 min, centrifuged, triplicates were spotted onto P81 filter paper, and treated as in the Akt activity assay. GSK3β activity in cerebral cortex and epidymal fat was measured as described previously (20) after immunoprecipitation of GSK3β from 100 µg or 200 µg of protein, respectively, with 1.5 µg of monoclonal GSK3β antibody. Immobilized immune complexes were washed as for GSK3α. Kinase activity was measured by mixing immunoprecipitates with 30 µl of kinase buffer containing 125 µM ATP, 1.4 µCi of [γ-32P]ATP, and 0.1 µg/µl recombinant Tau protein (Panvera, Madison, WI). The samples were incubated at 30 °C for 15 min, and 25 µl of Laemmli sample buffer (2% SDS) was added to each sample to stop the reaction. Samples were placed in a boiling water bath for 5 min, and proteins were separated in 7.5% SDS-polyacrylamide gels. The gels were vacuum-dried, exposed to a phosphoimage overnight, and quantitated using a PhosphoImager (Molecular Dynamics, Sunnyvale, CA). The efficiencies of immunoprecipitations were determined by immunoblotting with appropriate antibodies.

**RESULTS**

**Brain Akt and GSK3 Are Dephosphorylated during Fasting**—To test if the Akt–GSK3 coupled signaling pathway in mammalian brain is sensitive to peripheral glucose availability, we first examined if food withdrawal for 24 h affected the phosphorylation levels of Akt and GSK3 in mouse brain. These measurements used immunoblot analyses with phoshospecific antibodies to Akt or each of the two isoforms of GSK3 in samples of cerebral cortex and hippocampus obtained from adult, male C57BL/6 mice, with four mice per group. Food withdrawal significantly reduced blood glucose concentrations to 66 ± 4% of control levels, reduced blood insulin levels to 51 ± 3% of controls, but did not significantly change body weight or temperature (TABLE ONE). It has been reported that hypothermia during hypoglycemia caused by three days of starvation increased phospho-Ser5/-GSK3B levels in mouse brain (21); thus, in the present study, temperature was monitored to ensure that hypothermia did not occur to obscure the effects of changes in glucose. In both brain regions food withdrawal resulted in large decreases of the dual phosphorylation of Akt, phospho-Thr308-Akt (in the cortex to 24 ± 6% of control, means ± S.E.; n = 4) and phospho-Ser473-Akt (in the cortex to 41 ± 8% of control), but the total level of Akt was unaffected (Fig. 1A). There were also large decreases in the serine

| Table One |
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| **Body weight, blood glucose, serum insulin, and body temperature of control and 24 h fasted mice** |
| **Body weight** | **Blood glucose** | **Serum insulin** | **Temperature** |
| gm | mg/dl | ng/ml | °C |
| Control | 21.4 ± 0.6 | 126.5 ± 2.2 | 0.39 ± 0.02 | 34.0 ± 0.1 |
| Fasted | 19.9 ± 0.4 | 83.1 ± 5.6 | 0.20 ± 0.01 | 32.9 ± 0.2 |

*Data are means ± S.E. for four mice per group.*

* p < 0.05.
phosphorylation of both isoforms of GSK3, phospho-Ser9-GSK3β (in the cortex to 38 ± 9% of control) and phospho-Ser21-GSK3α (in the cortex to 30 ± 5% of control), whereas phosphotyrosine and total levels of GSK3α and GSK3β were unaltered (Fig. 1B). Thus, decreased blood glucose and insulin concentrations caused by food withdrawal for 24 h were associated with decreases in the phosphorylation of Akt and GSK3 in two regions of mouse brain.

Brain Akt and GSK3 Are Phosphorylated after Glucose Administration—Because food withdrawal indicated there was an inverse relationship between blood glucose and/or insulin levels and the phosphorylation of brain Akt and GSK3, the converse experiment was carried out in which a bolus of glucose was administered to mice. To provide a consistent baseline blood glucose level in all of the mice, the glucose was administered after a 14-h period of food withdrawal. Following treatment with 2 g/kg glucose, the blood glucose and insulin levels rapidly increased and reached peak levels after 10 and 5 min, respectively, (Fig. 2A) whereas body temperature was not changed. Glucose administration caused rapid and substantial increases in the levels of phospho-Thr308-Akt, phospho-Ser473-Akt, phospho-Ser21-GSK3α, and phospho-Ser9-GSK3β in both the cerebral cortex and the hippocampus (Fig. 2, B and C). Taken together, the results of food deprivation and of glucose administration show that the phosphorylation states of Akt and GSK3 in two regions of mouse brain are regulated by both decreases and increases in the circulating levels of glucose and insulin.

Brain Akt and GSK3 Are Transiently Phosphorylated after Insulin Administration—To test if increased insulin was sufficient to trigger changes in brain Akt and GSK3 phosphorylation, insulin was administered to mice. Administration of 5 international units/kg insulin caused a large increase in blood insulin and a subsequent decrease in blood glucose (Fig. 3A). The rapid increase in insulin concentration caused a rapid but transient increase in the dual phosphorylation of Akt in cerebral cortex and hippocampus (Fig. 3B). Akt phosphorylation reached a peak 5 min after insulin administration, which rapidly reverted to basal levels as the glucose concentration plummeted, although the insulin concentration remained elevated. Similar patterns of changes in the phosphorylation of both GSK3 isoforms occurred in both brain regions (Fig. 3C). These results suggest that both glucose and insulin contribute to regulating the phosphorylation states of Akt and GSK3 in vivo.

Overall, these experiments demonstrate that the regulatory phosphorylation states of Akt and GSK3 in mouse cerebral cortex and hippocampus are modulated in an inverse manner by fluctuations in the blood glucose concentration, such that the kinases are dephosphorylated when blood glucose levels are below normal, and are phosphorylated when blood glucose levels are elevated, and that circulating insulin is also able to modulate Akt and GSK3 in the brain. This raised the question of whether changes in circulating glucose in the absence of insulin is sufficient to regulate these kinases in the brain, which can occur in pathological states of hyperglycemia associated with diabetes. 

Diabetes Increases Brain Akt and GSK3 Phosphorylation—Hyperglycemic conditions in mouse models of diabetes were used to test if short and long term pathological increases in circulating glucose levels regulate the phosphorylation of brain Akt and GSK3. The first group of experiments employed streptozotocin treatment to induce hyperglycemia resulting from insulin depletion. Streptozotocin administered to mice causes a rapid degeneration of insulin-producing β-cells of the
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![Graph A](image1)

**FIGURE 3.** Insulin transiently increases the phosphorylation of Akt and GSK3 in mouse brain. Food was withdrawn overnight and after an ip injection of 5 international units/kg insulin, blood glucose and serum insulin concentrations were measured (A), and protein extracts from the cerebral cortex and hippocampus were immunoblotted for Phospho-Thr308-Akt, Phospho-Ser473-Akt, and Total Akt (B), and Phospho-Ser21-GSK3\(\alpha\), Phospho-Tyr279/216-GSK3\(\beta\), Total GSK3\(\alpha\)/\(\beta\), and Total GSK3\(\alpha\)/\(\beta\) (C). Quantitative values were obtained by densitometric measurements of immunoblots and are means ± S.E. from three mice per group.

![Graph B](image2)

To ensure that streptozotocin-induced insulin depletion decreased signaling by insulin receptors, epididymal fat samples were examined as a control tissue. As expected, and in marked contrast to brain tissue, 3 days after streptozotocin treatment epididymal fat displayed large decreases in the levels of phospho-Thr\(308\)-Akt and phospho-Ser\(473\)-Akt, whereas the total levels of Akt remained unchanged (Fig. 5A). There were also large decreases in phospho-Ser\(21\)-GSK3\(\alpha\) and phospho-Ser\(9\)-GSK3\(\beta\) in fat from treated mice compared with controls, but no changes in the tyrosine-phosphorylated or total levels of GSK3 (Fig. 5B). Thus, the phosphorylation of Akt and GSK3 in fat exhibited the large decreases predicted to occur following insulin depletion.

The activities of Akt and GSK3\(\beta\) were measured in cerebral cortex and epididymal fat 3 days after streptozotocin treatment to ensure that changes in phosphorylation levels reflected altered kinase activities, as has been well documented previously for each enzyme. Three days after streptozotocin treatment, Akt activity was increased in the cerebral cortex by 2.5-fold concurrently with the increased levels of phospho-Thr\(308\)-Akt and phospho-Ser\(473\)-Akt in the same samples (Fig. 6A). Conversely, in the same animals epididymal fat exhibited a greater than 50% decrease in Akt activity which correlated with the decreased dual phosphorylation of Akt evident in immunoblots. In accordance with the well known inhibitory effect of N-terminal serine phosphorylation of GSK3 activity, after streptozotocin treatment, the activities of GSK3\(\alpha\) and GSK3\(\beta\) were increased in fat where serine phosphorylation was decreased. The activities were decreased in the cerebral cortex where serine phosphorylation of GSK3 was increased (Fig. 6B). These results confirm that the phosphorylation levels of Akt and GSK3 reflect their enzymatic activities in both fat and brain tissue.

The effects of sustained streptozotocin-induced hyperglycemia were measured by examining phosphorylation levels of Akt and GSK3 in the pancreas, resulting in greatly diminished production of insulin and a resultant elevation of blood glucose levels. Three days after streptozotocin treatment, insulin levels were reduced to 28 ± 2% of control levels (TABLE TWO). Concurrently, blood glucose levels were elevated to 304 ± 6% of control levels. The body weights but not temperatures of streptozotocin-treated mice were significantly different from controls.

Three days after streptozotocin administration the phosphorylation of Akt at both Thr\(308\) and Ser\(473\) was significantly increased above the control levels in both the cerebral cortex and the hippocampus (Fig. 4A). There were no differences in the total protein level of Akt in either brain region. There were similarly large increases in the serine phosphorylation levels of GSK3\(\alpha\) and GSK3\(\beta\) 3 days following streptozotocin treatment in both the cerebral cortex and hippocampus (Fig. 4B). There were no changes in tyrosine phosphorylation, and total levels of GSK3 in either brain region 3 days after administration of streptozotocin. These results further underscore the conclusion that the circulating glucose concentration regulates brain Akt and GSK3 phosphorylation because insulin was reduced but glucose was elevated in association with the increases in Akt and GSK3 phosphorylation. Thus, these results show that short term pathological hyperglycemia caused large increases in the regulatory phosphorylation states of brain Akt and GSK3.

### TABLE TWO

| Body weight | Blood glucose | Serum insulin | Temperature |
|-------------|---------------|---------------|-------------|
| gm          | mg/dl         | ng/ml         | °C          |
| Control     | 25.5 ± 0.5    | 135.4 ± 4.9   | 33.0 ± 0.3  |
| STZ 3 day   | 19.5 ± 0.5    | 412.0 ± 8.3   | 32.2 ± 0.2  |

*\(a\) Data are means ± S.E. for four mice per group.

*\(b\) Data are means ± S.E. for three mice per group.

\(p < 0.05\).
brain 4 weeks after streptozotocin treatment. Large increases in the levels of phospho-Ser473-Akt and phospho-Thr308-Akt were present in both the cerebral cortex and hippocampus of mice 4 weeks after treatment with streptozotocin (Fig. 7A). These mice also displayed increased levels of phospho-Ser21-GSK3α and phospho-Ser9-GSK3β in both brain regions (Fig. 7B). Quantitation of the phosphorylation levels in four mice per group revealed statistically significant increases ranging from 2–10-fold in samples from streptozotocin-treated mice compared with control mice. In contrast to the large increases in serine phosphorylation of GSK3, tyrosine phosphorylation and total levels of both GSK3α and GSK3β were not affected by long term streptozotocin treatment. Thus, hyperglycemia caused by streptozotocin treatment caused both short and long term large increases in the phosphorylation states of Akt and GSK3 in both brain regions.

To determine if the phosphorylation levels Akt and GSK3 in the brain were increased in a second mouse model of diabetes associated with hyperglycemia, male db/db mice that develop an insulin resistance phenotype (22), were examined. The db/db mice were hyperglycemic and insulinopenic, with blood glucose concentrations 390% ± 7% of control levels and insulin concentrations 280 ± 12% of controls, and their body weights were significantly increased (TABLE THREE). In db/db mice, compared with control mice, there were large increases in phospho-Thr308-Akt and phospho-Ser473-Akt (Fig. 8A) and in phospho-Ser21-GSK3α and phospho-Ser9-GSK3β (Fig. 8B) in the cerebral cortex and hippocampus. There were no differences in total protein levels of Akt or GSK3 in db/db brain regions compared with controls. These results show that in two mouse models of diabetes, hyperglycemia was associated with large chronic increases in the phosphorylation states of Akt and GSK3 in both brain regions.

To test if the hyperglycemic-induced increases in phosphorylation of brain Akt and GSK3 could be reversed by lowering the glucose level, and to confirm that the glucose was regulatory, 3 days after streptozotocin treatment, blood glucose levels were reduced by acute insulin treatment. Administration of 5 international units/kg insulin caused a rapid increase in blood insulin and a corresponding decrease in blood glucose (Fig. 9A). Insulin treatment resulted in decreases in the dual phosphorylation of Akt (Fig. 9A) and in the serine phosphorylation of both GSK3 isoforms (Fig. 9B). These results show that the increased phosphorylation of Akt and GSK3 in the brain caused by streptozotocin-induced
DISCUSSION

This investigation found a surprisingly strong role for physiological and pathological changes in the circulating concentration of glucose, in addition to insulin, in regulating the Akt-GSK3 signaling pathway in mouse brain. In both the cerebral cortex and the hippocampus, the phosphorylation states of Akt and GSK3 were found to be influenced by physiological fluctuations in the blood glucose concentration, by acutely administered glucose or insulin, by short term (3 days) and long term (4 weeks) hyperglycemia caused by pathological insulin depletion after streptozotocin administration, and by hyperglycemia linked to insulin resistance in type 2 diabetic db/db mice. Hyperglycemia in the presence of normal or deficient insulin levels caused large increases in the phosphorylation levels of Akt and GSK3 in the brain, as did acute insulin administration, whereas they were reduced by fasting-induced hypoglycemia or by lowering hyperglycemic glucose levels by insulin administration to streptozotocin-treated mice. Taken together, these results show that glucose as well as insulin contributes to the in vivo regulation of the phosphorylation of Akt and GSK3 in two regions of mouse brain, indicating that a glucose-sensing mechanism regulates brain Akt and GSK3.

The present results extend previous studies of the effects of diabetes-related conditions on the phosphorylation of Akt or GSK3 in mouse brain. The IRS-2-deficient mouse model of diabetes previously was found to have increased brain levels of phospho-Ser9-GSK3β (23), and a prior study had reported that these mice are hyperglycemic (24). Although the link was not noted previously, these results are consistent with our conclusion that with deficient insulin signaling, the phosphorylation levels of Akt and GSK3 in the brain can increase in response to elevated blood glucose levels. Other studies showed that in the absence of elevated glucose levels, deficient insulin signaling causes decreased phosphorylation of Akt and GSK3 in mouse brain. Neuron-specific insulin receptor knock-out (NIRKO) mice with unaltered cerebral glucose metabolism had 50–60% decreases in the phosphorylation of Akt and GSK3β in brain, demonstrating that insulin receptor-linked signaling can influence the phosphorylation levels of these two kinases in the brain (25). This relationship also was observed in mice with diet-in-

hyperglycemia could be rapidly reversed by lowering the blood glucose concentration.
phospho-Ser\(^2\)-GSK3\(\beta\) increased in mouse brain. Taken together with our results, these findings indicate that insulin has a significant influence on the phosphorylation levels of Akt and GSK3 in the brain, which decrease with deficient insulin signaling if glucose levels remain normal. However, if hyperglycemia is associated with insulin deficiency, the elevated glucose levels cause large increases in the phosphorylation levels of brain Akt and GSK3. Thus, both insulin and glucose contribute to regulating the phosphorylation levels of these kinases in the brain, indicating that a glucose-sensing mechanism operates in conjunction with insulin signaling to regulate the phosphorylation of brain Akt and GSK3.

The glucose-sensing regulation of brain Akt and GSK3 differentiates the responses of these enzymes in the brain from changes in peripheral tissues under insulin-resistant conditions. In our study, Akt and GSK3 in epididymal fat were dephosphorylated following streptozotocin treatment while the phosphorylation levels of Akt and GSK3 in the brain were dramatically increased in the same animals. There have been only a limited number of studies of the in vivo phosphorylation states of Akt and GSK3 in peripheral tissues in insulin-resistant animal models, but these previous studies have reported results consistent with decreased or unchanged phosphorylation of GSK3 and/or Akt in contrast to the large increases in brain-phosphorylated Akt and GSK3. In epididymal fat of mice with high fat diet-induced diabetes, the activity of GSK3\(\beta\) was increased 2-fold, likely indicative of decreased phosphorylation, whereas the activity of Akt was not changed (27). In these same mice there was a small decrease of GSK3\(\beta\) activity in liver and no change in skeletal muscle, demonstrating that regulation differs among tissues even in the periphery. Studies of streptozotocin-treated rats and Zucker diabetic fatty (ZDF) rats also reported no differences in skeletal muscle GSK3\(\beta\) activity (28, 29). However, in human patients with type 2 diabetes, the GSK3 activity was elevated 2-fold in skeletal muscle, consistent with a decrease in the inhibitory serine phosphorylation of GSK3\(\beta\) (30). The myocardium of streptozotocin-treated rats displayed decreased basal phosphorylation of Akt on Thr\(^308\), but not on Ser\(^473\), and decreased insulin-induced serine phosphorylation of both GSK3 isoforms (31). Overall, these studies indicate that the effects of insulin resistance on Akt and GSK3 vary considerably among different tissues and that changes in the brain are relatively large, as well as opposite to, changes in peripheral tissues.

During the last few years increasing attention has been focused on GSK3 as a potential therapeutic target in diabetes because inhibition of GSK3 facilitates control of glucose levels in animal models of insulin resistance (32). Although as noted above there is limited data indicating that GSK3 in peripheral tissues is abnormally activated in diabetes, this is not a requirement for inhibitors to contribute to the control of glucose levels. However, overexpression of GSK3\(\beta\) in skeletal muscle was recently found to be sufficient to cause glucose intolerance (33). Administration of a peptide inhibitor of GSK3, L803-mts, increased glucose tolerance in diabetic mice (34). Also, administration of rosiglitazone, a peroxisome proliferator-activated receptor agonist that also inhibits GSK3, reduced circulating blood glucose levels in ZDF rats and in patients with type 2 diabetes (35, 36). Ring et al. (37) found that in several models of diabetes, ob/ob mice, db/db mice, and ZDF rats, two

![TABLE THREE](image)

| Body weight, blood glucose, and serum insulin of control and db/db mice | Body weight | Blood glucose | Serum insulin |
|---------------------------------------------------------------|-------------|----------------|----------------|
| **gm** | **mg/dL** | **ng/ml** |
| Control | 24.1 ± 0.4\(^a\) | 127.2 ± 3.7 | 0.50 ± 0.02 |
| db/db | 35.4 ± 0.5\(^p\) | 496.7 ± 8.8\(^b\) | 1.40 ± 0.06\(^b\) |

\(^a\) Data are means ± S.E. from three mice per group.

\(^p\) \(p < 0.05\).
GSK3 inhibitors, CHIR98014 and CHIR99021, lowered plasma glucose levels by as much as 50% in a dose-dependent manner without changing insulin concentrations. Two reports also showed that the GSK3 inhibitor CHIR98023 lowered glucose concentrations in ZDF rats during a glucose tolerance test (29, 38). These results indicate that inhibition of GSK3 in the periphery improves glucose regulation in diabetic animals. However, our finding of increased serine-phosphorylated GSK3 in the brain of streptozotocin-treated mice, which was reflected in reduced GSK3 activity, raises the question of whether further inhibition of brain

FIGURE 8. Akt and GSK3 are highly phosphorylated in db/db mouse brain. Protein extracts from the cerebral cortex and hippocampus were immunoblotted in 8-week-old db/db mice for Phospho-Thr308-Akt, Phospho-Ser473-Akt, and Total Akt (A), and Phospho-Ser21-GSK3α, Phospho-Ser9-GSK3β, Phospho-Tyr279/216-GSK3α/β, and Total GSK3α/β (B). Quantitative values were obtained by densitometric measurements of immunoblots and are means ± S.E. from three mice per group. *, p < 0.05 compared with control values.

FIGURE 9. Increased brain Akt and GSK3 phosphorylation in streptozotocin-treated mice is reversed by insulin administration. Mice were treated with streptozotocin and after 3 days, food was withdrawn overnight. After an ip injection of 5 international units/kg of insulin, blood glucose and serum insulin concentrations were measured (A). Insulin values were divided by ten to show the changes in insulin and glucose levels on the same graph. Protein extracts from the cerebral cortex and hippocampus were immunoblotted for Phospho-Thr308-Akt, Phospho-Ser473-Akt, and Total Akt (B), and Phospho-Ser21-GSK3α, Phospho-Ser9-GSK3β, Phospho-Tyr279/216-GSK3α/β, and Total GSK3α/β (C). Quantitative values were obtained by densitometric measurements of immunoblots and are means ± S.E. from three mice per group.
GSK3 by the administration of GSK3 inhibitors might be detrimental to brain function. Thus, it may be preferable to consider GSK3 inhibitors with limited ability to penetrate the blood brain barrier to limit GSK3 inhibitory effects to the periphery for the possible treatment of diabetes.

Considering the central roles of Akt and GSK3 in many cellular processes, and the critical role that glucose plays in neuronal function, one of our goals in this investigation was to test if changes in circulating levels of glucose affect the regulatory phosphorylation states of these kinases. The coupling of these kinases is an interesting, somewhat novel, signal pathway because phosphorylation increases Akt activity but decreases GSK3 activity, so the signal strength has opposite effects on the GSK3 signaling pathway to act as a cellular glucose sensor and integrator with limited ability to penetrate the blood brain barrier to limit GSK3 availability.

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