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Cortical Neurons Develop Insulin Resistance and Blunted Akt Signaling: A Potential Mechanism Contributing to Enhanced Ischemic Injury in Diabetes

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Abstract

Patients with diabetes are at higher risk of stroke and experience increased morbidity and mortality after stroke. We hypothesized that cortical neurons develop insulin resistance, which decreases neuroprotection *via* circulating insulin and insulin-like growth factor-I (IGF-I). Acute insulin treatment of primary embryonic cortical neurons activated insulin signaling including phosphorylation of the insulin receptor, extracellular signal-regulated kinase (ERK), Akt, p70S6K, and glycogen synthase kinase-3β (GSK-3β). To mimic insulin resistance, cortical neurons were chronically treated with 25 m*M* glucose, 0.2 m*M* palmitic acid (PA), or 20 n*M* insulin before acute exposure to 20 n*M* insulin. Cortical neurons pretreated with insulin, but not glucose or PA, exhibited blunted phosphorylation of Akt, p70S6K, and GSK-3β with no change detected in ERK. Inhibition of the phosphatidylinositol 3-kinase (PI3-K) pathway during insulin pretreatment restored acute insulin-mediated Akt phosphorylation. Cortical neurons in adult BKS-db/db mice exhibited higher basal Akt phosphorylation than BKS-db$^+$ mice and did not respond to insulin. Our results indicate that prolonged hyperinsulinemia leads to insulin resistance in cortical neurons. Decreased sensitivity to neuroprotective ligands may explain the increased neuronal damage reported in both experimental models of diabetes and diabetic patients after ischemia-reperfusion injury. *Antioxid. Redox Signal*. 14, 1829–1839.

Introduction

STROKE IS THE THIRD LEADING CAUSE OF DEATH in the United States behind heart disease and cancer (24). It is clear from epidemiological studies that diabetes exacerbates and/or is a principal cause of both stroke and myocardial infarction (31, 43, 46). Ischemic stroke is a major macrovascular complication of diabetes and diabetic patients consistently exhibit poorer outcomes and prognoses than nondiabetic patients after a stroke (27, 31). Over the last 15 years, our laboratory has extensively explored how peripheral sensory neurons respond to hyperglycemia (16, 38, 62). Recently, we began examining the effects of hyperglycemia on neurons of the central nervous system. These studies arose because of our growing interest in macrovascular disease and diabetes, with an emphasis on stroke.

While neurons are not insulin dependent, they are insulin responsive (5). Autoradiographic studies detect insulin receptors (InsR) in the brain of rodents and humans, and during development, insulin facilitates glucose metabolism during periods of neuronal growth (5). Increases in peripheral insulin lead to parallel increases in the brain *via* InsR-mediated uptake across the blood–brain barrier (12). The role of insulin in the brain is not fully known, but insulin acutely alters brain glucose utilization in a region-specific manner and alters short-term memory (11). Moreover, insulin is a well-documented growth factor for neurons of both the peripheral and central nervous systems (66).

Insulin resistance is a state of decreased responsiveness of target tissues to normal circulating levels of insulin and is a major feature of type 2 diabetes, glucose intolerance, obesity, dyslipidemia, and hypertension; that is, metabolic syndrome (7). Recent epidemiological evidence suggests that the insulin resistance associated with type 2 diabetes is a risk factor for stroke (31, 46). Patients with diabetes show a two- to sixfold increase in the risk of stroke compared to nondiabetic individuals. Population-based cohort studies demonstrate that otherwise healthy individuals with metabolic syndrome demonstrate a significant increase in stroke as well as cardiovascular mortality (43). Insulin resistance also increases

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the risk of stroke recurrence and, cumulatively, a poorer outcome and increased mortality (25). While these studies clearly document the correlation of diabetes and stroke, the underlying mechanism has yet to be identified.

Multiple factors, including hyperglycemic neuronal injury and insulin resistance, may contribute to the reported increase in mortality after stroke in diabetic patients. The contributions of each component of the metabolic syndrome to stroke vary and are controversial. Studies concerning the role of hyperglycemia on stroke demonstrate conflicting results; some conclude there is increased stroke risk with chronic hyperglycemia (1, 37), whereas other work finds no such relationship (45). The UK Prevention in Diabetes Study failed to demonstrate significantly reduced risk of stroke in patients treated with tight glucose control compared to conventional diet therapy (21). Even though hyperlipidemia is a high risk factor for cardiovascular disease (CVD), its contribution to stroke is also unclear, with some studies reporting a beneficial effect of cholesterol reduction, whereas others find no variation by lipid profile among diabetic status (23, 36, 54). In contrast, studies consistently demonstrate the relationship between hyperinsulinemia and stroke, though less than the association with CVD. The Atherosclerosis Risk in Communities Study reported that hyperinsulinemia increased stroke risk by 1.19-fold/50 p*M* increment in fasting insulin level (18). A 22-year follow-up study of healthy Finnish men also demonstrated that hyperinsulinemia carried a 2.1-fold increase in stroke risk after adjustment for age (48). Analysis of the data from the Third National Health and Nutrition Survey demonstrates independent association of insulin resistance with stroke (odds ratio 1.06) after adjustment with age, hypertension, myocardial infarction, claudication, physical activity, and HbA1C (6). Ultimately, our understanding of the impact of individual elements of the metabolic syndrome has the most impact on the increased risk of stroke observed in diabetic patients requiring more investigation at both the clinical and basic science levels.

Experimental studies of stroke and brain ischemia document the contribution of neuronal apoptosis after stroke and brain ischemia, and Akt signaling is a key regulator of these processes (71). Akt, a serine/threonine kinase activated downstream of phosphatidylinositol 3-kinase (PI3-K), is a critical signaling molecule in eukaryotic cells (71). Akt phosphorylation decreases immediately after global brain ischemia, followed by a dramatic increase within 24 h before returning to basal levels within 48 h (47). Preventing the initial decrease of Akt phosphorylation by insulin-like growth factor-I (IGF-I) treatment reduced neuronal death (30), whereas inhibition of Akt and glycogen synthase kinase-3β (GSK-3β) by the PI3-K inhibitor, LY294002, facilitated DNA fragmentation in hippocampal neurons (17). These results suggest that precise regulation of Akt is critical for neuronal survival after brain injury.

The BKS.Cg-$m^{+/+}Lepr^{db}$/J mouse (BKS-db/db) is a spontaneous model of type 2 diabetes. Due to the expression of a mutant leptin receptor, these mice are hyperphagic, which results in severe obesity hyperinsulinemia and insulin resistance beginning at $\sim$4 weeks of age (http://jaxmice.jax.org/strain/000642.htm). These animals exhibit increased serum lipids and hyperglycemia similar to that seen in human patients and are the most commonly used model of type 2 diabetes (58).

In the current study, we examined possible molecular mechanisms regarding chronic insulin stimulation-mediated blunting of Akt and its downstream effectors. We demonstrate evidence of insulin resistance in an *in vitro* model of hyperglycemia and cortical neurons from a mouse model of type 2 diabetes. We conclude that chronic stimulation of Akt may contribute to blunted insulin signaling in cortical neurons and underlie, in part, the poor prognosis seen in diabetic patients with stroke.

Materials and Methods

Antibodies and chemicals

All antibodies were purchased from Cell Signaling (Beverly, MA) except Tau5 (Biosource International, Camarillo, CA), anti-glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH; Chemicon, Temecula, CA), and anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA). Inhibitors (LY294002 and U0126) were purchased from Calbiochem (La Jolla, CA). PA was purchased from Nu-Chek Prep, Inc. (Elysian, MN). All other chemicals were purchased from either Sigma (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ).

Cortical neuron preparation

Cortical neurons were harvested from E15 embryos of Sprague Dawley rats. The cortex was dissected and dissociated using trypsin and plated in 12-well tissue culture plates coated with poly-L-lysine (PLL). For immunohistochemistry (IHC), cells were plated on glass coverslips coated with PLL in 24-well culture plates. Cells were maintained in the feed medium (Neurobasal medium; Invitrogen, Grand Island, NY) supplemented with 1$\times$B27 without antioxidant (Invitrogen), antibiotics (penicillin, streptomycin, and neomycin; Sigma), 2.5 μg/ml albumin, 10 μg/ml apo-transferrin, 0.1 μg/ml biotin, 15 μg/ml D-galactose, 7 ng/ml progesterone, 16 μg/ml putrescine, 4 ng/ml selenium, 3 ng/ml μ-estradiol, 4 ng/ml hydrocortisone, 3 μg/ml catalase, and 2.5 μg/ml superoxide dismutase. Neurobasal medium contains 25 m*M* of glucose. The medium was replaced with a fresh feed medium on days 1 and 3. On day 6 (24 h before treatment), to exclude the effect of insulin contained in B27, the culture medium was changed to treatment medium (feed medium without B27). Insulin, glucose, or palmitate was added on day 6 to the treatment medium for 24 h treatment.

Mouse brain preparation

BKS-db/db and db$^+$ (BKS.Cg-m$^{+/+}$ Lepr^{db}/J, JAX Mice stock No. 000642) were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were euthanized at 24 weeks of age (20 week of diabetes). All mice were housed in a pathogen-free environment, and cared for following the University of Michigan Committee on the Care and Use of Animals guidelines.

The mice were euthanized per our published protocols with an overdose of sodium pentobarbital (38, 62). Brains were cut in half and the cortex minced, and divided equally into microcentrifuge tubes in the cortical neuron treatment medium. The tubes were left at 37°C for 45 min to stabilize before stimulation with insulin or IGF-I.

Western immunoblotting

Cortical neuron cultures were lysed in RIPA buffer (Pierce, Rockford, IL) containing protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Mouse cortex was homoge-

nized using a plastic pestle in a microcentrifuge tube in T-PER tissue protein extraction reagent (Pierce) containing protease inhibitor cocktail (Roche Diagnostics). Lysates were collected, briefly sonicated, and centrifuged at 13,000 rpm for 15 min at 4°C. Western immunoblotting was performed as described previously (33). TBS with 0.01% Tween-20 and 5% fat-free milk was used for blocking and antibody dilution. The incubations with primary and secondary antibodies were carried out either at RT for 2 h or at 4°C overnight. The signal was observed using enhanced chemiluminescence reagents (ECL; Amersham Bioscience, Piscataway, NJ) or SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). Images were captured using the Chemidoc XRS system and analyzed by Quantity One software (Bio-Rad Laboratory, Hercules, CA) (33) and the statistical analysis was performed by Prism software (GraphPad, Inc., La Jolla, CA). All experiments were repeated at least 3 times and representative results are presented in the figures. In some experiments, the nitrocellulose membranes were incubated at 60°C for 15 min in stripping solution (2% SDS, 100 m*M* dithiothreitol and 100 m*M* Tris, pH 6.8) whereupon they were utilized for immunoblotting with another antibody.

Immunohistochemistry

The cortical neurons cultured on PLL-coated coverslips were permeabilized with phosphate-buffered saline (PBS) containing 3% bovine serum albumin, 0.1% Triton X-100, and 1% normal goat serum (IHC buffer). The cells were incubated with Tau5 antibody diluted in IHC buffer in a humidified chamber at 4°C overnight. After rinsing with PBS three times for 5 min each, the cells were incubated with the anti-mouse IgG secondary antibody conjugated with AlexaFluor 488 (Molecular Probes, Eugene, OR) for 2 h at RT. After rinsing with PBS, the coverslips were mounted with the ProLong antifade mounting medium containing DAPI (Molecular Probes). The digital images were captured using a Spot-RT camera (Diagnostic Instrument Inc., Sterling Heights, MI) attached to Nikon Microphot-FXA microscope.

Statistical analysis

All experiments were repeated at least 3 times and presented as the mean ± SEM. Statistical analysis was performed by one-way analysis of variance with Tukey's post analysis or Student's *t*-test using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). Statistical significance was defined as $p < 0.05$.

Results

Cortical neuron cultures were maintained for 3 and 6 days in the standard feeding medium (see Materials and Methods section). IHC for Tau5, a neuron-specific marker, confirmed the cell's neuronal phenotype (Fig. 1A). From day 3 to 6 *in vitro*, cortical neurons extended neurites and appeared more mature (Fig. 1A). Treatment of the cortical neurons with insulin or IGF-I after 6 days *in vitro* did not result in any noticeable effects on cell size or neurite extensions (data not shown). It is possible that the cells already have so many neurites that we could not detect any measurable changes. Both insulin and IGF-I influence the development of cortical and hippocampal neurons by promoting neurogenesis, survival, and differentiation (3, 14, 52). Intracellular signaling cascades that mediate the effect of insulin and IGF-I include the mitogen-activated protein kinase (MAPK) and PI3-K/Akt

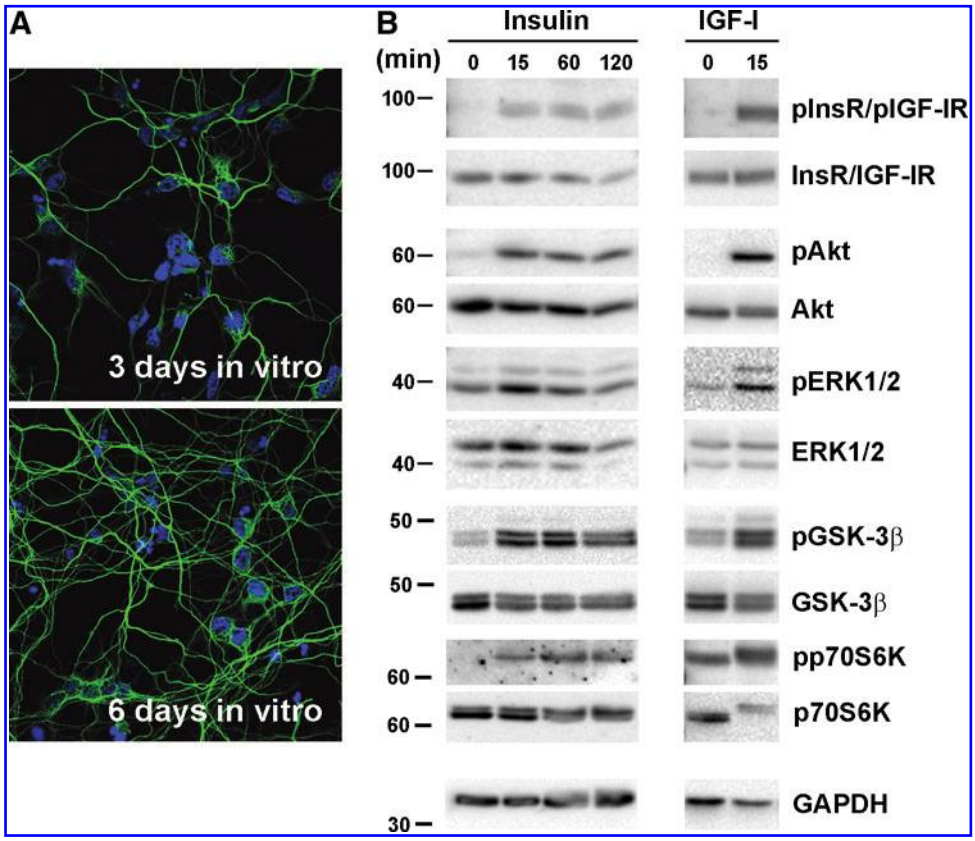

FIG. 1. Insulin and insulin-like growth factor-I (IGF-I) signaling in cultured cortical neurons. (A) Cortical neurons were harvested from E15 rat embryos. The cells were cultured for 3 or 6 days and immunolabeled for tau, a neuron-specific microtubule associated protein (green, detected by Tau5 antibody). Nuclei were observed with DAPI (blue). **(B)** Cortical neurons cultured for 7 days were stimulated with 20 n*M* insulin or IGF-I for 0–2 h. Cell lysates were prepared in RIPA buffer and immunoblotted with the indicated antibodies. These experiments were repeated at least 3 times and a representative result is shown. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

pathways (5). To examine insulin and IGF-I signaling in our system, cortical neurons were treated with 20 n*M* insulin or IGF-I for 0–2 h followed by Western immunoblotting. Insulin stimulation induced a time-dependent increase in the phosphorylation (*i.e.,* activation) of intracellular signaling molecules, including InsR, and its downstream targets, Akt, GSK-3β, and p70S6K (Fig. 1B). Phosphorylation of extracellular signal-regulated kinase (ERK) was not prominent and, in many cases, not different from the control (untreated neurons). Each blot was stripped and reprobed with antibodies against total protein as well as GAPDH to confirm equal protein loading. IGF-I treatment also resulted in a similar response; however, activation of Akt and p70S6K was generally stronger compared to insulin stimulation.

Hyperglycemia, dyslipidemia, and hyperinsulinemia are characteristic of type 2 diabetes and the metabolic syndrome, all of which may contribute to insulin resistance (7). We next examined the contribution of each of these factors to insulin resistance in cortical neurons. To create an *in vitro* model of the metabolic syndrome and assess insulin resistance, cortical neurons were treated with 25 m*M* added glucose, 0.2 m*M* PA, or 20 n*M* insulin for 24 h. The medium was then replaced with a fresh treatment medium for 30 min and the cells were exposed to 20 n*M* insulin for 15 min. Twenty-four hour insulin treatment resulted in reduced InsR phosphorylation, whereas 24 h glucose and PA treatment resulted in an increase in InsR phosphorylation (Fig. 2A). In untreated cortical neurons, Akt is activated after 15 min of insulin treatment. Twenty-four hour insulin treatment reduced this response to subsequent short-term insulin treatment. In contrast, 24 h of glucose or PA had no effect on the activation of Akt in response to short-term insulin treatment. An increase in basal Akt phosphorylation was noted after 24 h insulin treatment (Fig. 2A). Densitometric analysis confirmed that only insulin pretreatment resulted in a statistically significant decrease in insulin-stimulated Akt phosphorylation (Fig. 2B).

Full activation of Akt requires sequential phosphorylation of Thr308 and Ser473 by PDK1 and mammalian target of rapamycin complex 2 (mTORC2), respectively (71). The 24 h insulin treatment decreased the phosphorylation at both of these sites (Fig. 3A). Densitometric analysis demonstrates a statistically significant suppression of Akt phosphorylation after 24 h insulin pretreatment (Fig. 3B). p70S6K and GSK-3β are two important signaling molecules mediating Akt's role in survival, neuronal differentiation, and metabolism (71). In parallel with the effect on Akt phosphorylation, 24 h insulin treatment also reduced short-term insulin-stimulated p70S6K and GSK-3β phosphorylation (*i.e.,* activation of p70S6K and inactivation of GSK-3β). When p70S6K is phosphorylated, it displays slower migration (34). We observed a slower migrating band of p70S6K in insulin-stimulated samples without pretreatment but not in samples pretreated with insulin for 24 h (Fig. 3A). Total protein levels of p70S6K and GSK-3β were not affected (Fig. 3A). In contrast, IGF-I-stimulated Akt phosphorylation was less affected by insulin pretreatment even up to 48 h without a reduction in total Akt protein (Fig. 3C). Of the three treatments tested, these results suggest that hyperinsulinemia, rather than hyperglycemia or hyperlipidemia, is the main factor responsible for the development of insulin resistance in cortical neurons.

We next explored the possible mechanism behind the reduction in Akt activation after chronic insulin treatment.

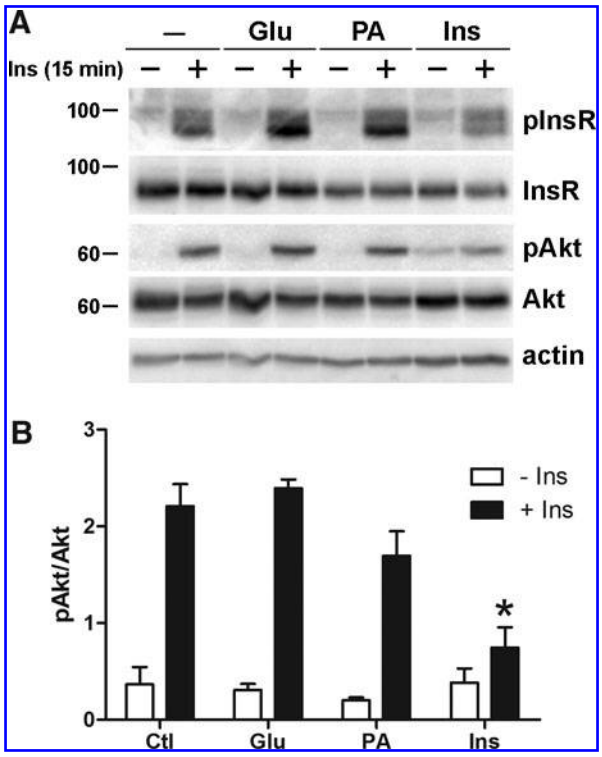

FIG. 2. Chronic insulin stimulation mimicking hyperinsulinemia downregulates the ability of cortical neurons to respond to acute insulin stimulation. (A) Cortical neurons were incubated with 25 m*M* glucose (Glu), 0.2 m*M* palmitic acid (PA), or 20 n*M* insulin (Ins) for 24 h and then stimulated with 20 n*M* insulin for 15 min. **(B)** Densitometric analysis of Akt phosphorylation. Cell lysates were prepared in RIPA buffer and immunoblotted with antibodies against phosphorylated insulin receptor (pInsR) or Akt (pAkt). The blots were stripped and reprobed with antibodies against total InsR or Akt. Anti-actin immunoblot demonstrate the equal loading of the proteins. $^*p < 0.05$ compared to insulin-stimulated control cells using one-way analysis of variance (ANOVA). Akt phosphorylation in Glu and PA pretreated cells were not statistically significant compared to control cells.

During 24 h insulin treatment, cortical neuron cultures were incubated without or with inhibitors of Akt (LY294002) or ERK (U0126) signaling pathways. After 24 h the cells were washed with HBSS and incubated in a fresh treatment medium (without insulin or inhibitors) for 30 min. The cultures were then treated with 20 n*M* insulin for 15 min. As in the previous experiments, cortical neurons chronically pretreated with insulin followed by acute insulin treatment exhibited reduced Akt phosphorylation (Fig. 4A). Combined treatment with insulin and the PI3-K inhibitor, LY294002, restored Akt phosphorylation by acute insulin treatment to control levels. The MAPKK inhibitor, U0126, had no effect on Akt phosphorylation. Densitometric analysis (Fig. 4B) confirms that LY294002 treatment (lane 5) restored Akt phosphorylation, which was significantly higher than without inhibitor (lane 4) or U0126 (lane 6) treatment and displayed no statistically significant difference compared to untreated neurons (lane 2). As in the previous experiments, ERK phosphorylation was not significantly affected by 24 h insulin treatment or by either inhibitor. These results suggest that chronic hyperactivation of Akt by insulin prevents further activation by acute insulin

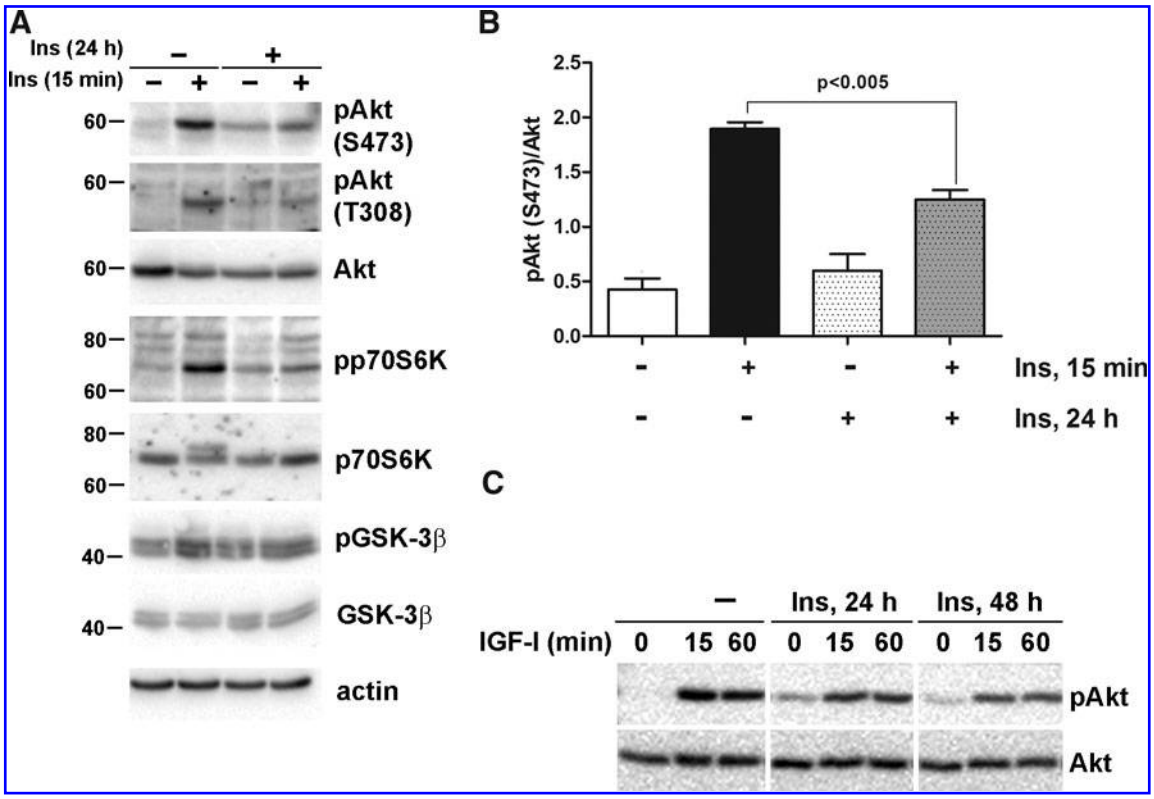

FIG. 3. Chronic insulin treatment reduces insulin stimulated Akt phosphorylation and downstream signaling molecules. (A) The cells were treated without or with 20 n*M* insulin for 24 h and stimulated with 20 n*M* insulin for 15 min. **(B)** Densitometric analysis of Akt phosphorylation (Ser473). Statistical significance was confirmed by Student's *t*-test. **(C)** The cells were pretreated with insulin for 24 or 48 h and then stimulated with 20 n*M* IGF-I for 0, 15, or 60 min. Cell lysates were prepared in RIPA buffer and immunoblotted with the indicated antibodies.

treatment. Insulin treatment-induced InsR phosphorylation was not affected by the chronic insulin, LY294002, or U0126 treatments (Fig. 5). The phosphorylation pattern of p70S6K and GSK-3β paralleled that of Akt phosphorylation (Fig. 5). p70S6K displayed decreased mobility when phosphorylated (lanes 2 and 5) (34). These results confirm the upstream requirement of Akt for the activation of these signaling molecules.

These observations were confirmed in an animal model of type 2 diabetes, the BKS-db/db mouse. The BKS.Cg-m$^{+/+}$ Lepr^{db}/J, commonly known as BKS-db/db, expresses a homozygous mutation of the leptin receptor and demonstrates typical characteristics of type 2 diabetes, including obesity, hyperinsulinemia, and hyperglycemia (http://jaxmice.jax.org/strain/000642.html). BKS-db$^+$ mice are heterozygous for this mutation and serve as a control. At 24 weeks of age (20 weeks of diabetes), cortex was harvested from BKS-db/db and db$^+$ and treated with 20 n*M* insulin for 0–15 min. Insulin stimulation resulted in a time-dependent increase in Akt phosphorylation in BKS-db$^+$ cortex (Fig. 6A). In contrast, db/db cortex displayed higher basal Akt phosphorylation, and insulin treatment did not increase this phosphorylation. Densitometric analysis demonstrates statistically significant increases in Akt phosphorylation in BKS-db$^+$ cortex but no such changes from db/db cortex (Fig. 6B). These results confirm our *in vitro* observations and demonstrate that hyperinsulinemia may induce insulin resistance *in vivo* by the same mechanism, that is, reduced Akt activation.

Discussion

Hyperinsulinemia resulting from systemic insulin resistance is characteristic of type 2 diabetes and is also part of the constellation of symptoms associated with the metabolic syndrome (7). Because insulin crosses the blood–brain barrier, its levels are also increased within the central nervous system (15, 57). We hypothesized that increased insulin signaling would result in insulin resistance within the central nervous system, contributing to the increased neuronal damage seen in patients with type 2 diabetes after ischemia-reperfusion injury. In neurons, the PI-3K/Akt pathway is normally activated by insulin and IGF-I and is critical for translating the protective effects of these ligands (14, 52). In the current study, we found that long-term (24 h) exposure to insulin increased Akt activation and severely blunted this response after subsequent short-term insulin treatment. Acute Akt signaling was restored when the PI3-K inhibitor, LY294002, was added with 24 h insulin, indicating that hyperstimulation of this pathway is involved in insulin resistance. These observations were confirmed in an animal model of type 2 diabetes, the BKS-db/db mouse. Cortical neurons harvested from these animals exhibited the same blunted Akt activation after a 15 min exposure to insulin as observed under chronic insulin stimulation *in vitro*.

Cortical neurons *in vitro* extend neurites and express neuronal markers, including tau. These neurons also express both insulin and IGF-I receptors and respond to physiologic levels

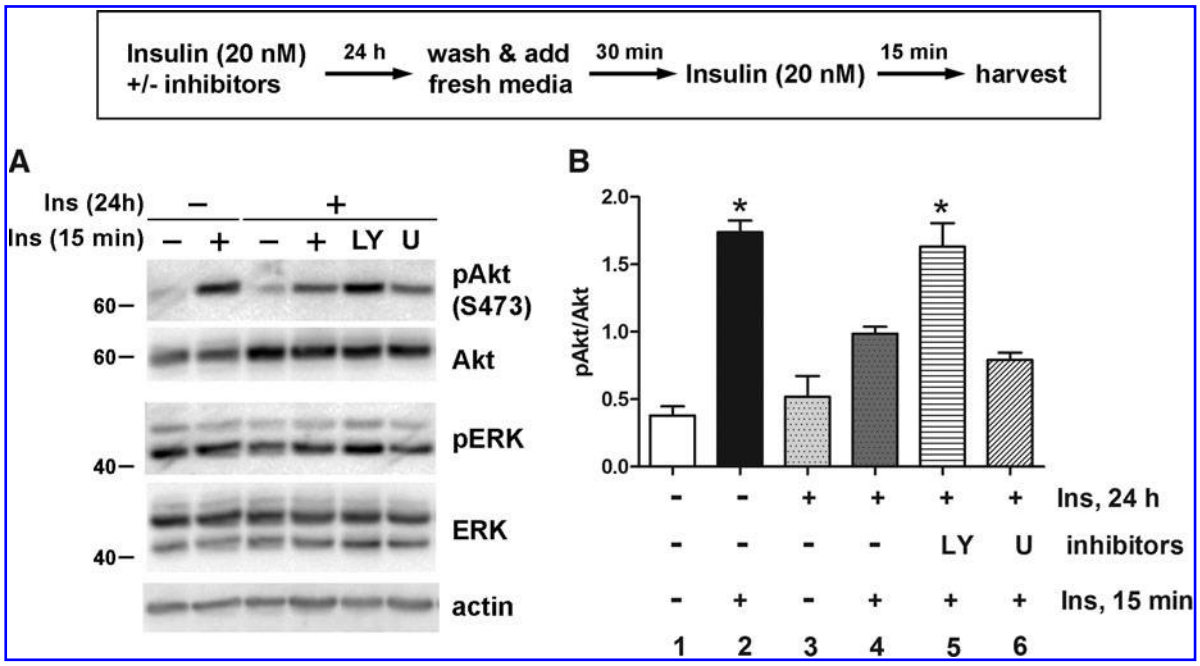

FIG. 4. LY294002 pretreatment prevents hyperinsulinemia induced downregulation of Akt stimulation. (A) Cortical neurons were incubated with insulin along with 20 μM LY294002 (LY) or U0126 (U) for 24 h. The cells were washed and incubated in a fresh treatment medium for 30 min before acute insulin stimulation for 15 min. Immunoblotting was performed using the antibodies against phosphorylated Akt or extracellular signal-regulated kinase (ERK) and then stripped and reprobed for total protein as well as for actin. **(B)** Densitometric analysis of Akt phosphorylation. $^*p < 0.05$ by one-way ANOVA compared to other treatment groups.

of these ligands as demonstrated by activation of Akt and ERK1/2 and downstream targets of Akt, GSK-3β, and p70S6K. These downstream effectors are activated within 15 min of treatment. Insulin signaling was followed for 2 h at which time both the total levels of InsR and its activated form are diminished. This may be due to InsR recycling (10) or to saturation of intracellular substrates.

Once a normal signaling pattern was established, we examined the impact of long term insulin exposure on insulin signaling. Epidemiological data reveal that diabetic patients have an increased risk for stroke and poorer outcomes after stroke including increased infarct size and increased hemorrhage after initial ischemia (31, 43, 46). Many of these studies cite insulin resistance as the major risk factor; therefore, we examined cortical neuron cultures after chronic insulin treatment as a model of neuronal insulin resistance. As discussed above, cortical neurons respond to insulin and IGF-I stimulation with activation of Akt within 15 min. To examine the factors associated with insulin resistance, cortical neurons were treated with insulin, glucose, or PA to mimic hyperinsulinemia, hyperglycemia, and hyperlipidemia, respectively.

There are few direct reports regarding insulin resistance in neurons of the central nervous system; however, several studies document increased neuronal damage when insulin signaling is blocked. Neuron-specific InsR knock-out mice exhibit decreased Akt and GSK-3β activation and increased tau phosphorylation, but with no memory dysfunction or neuronal loss (53). In contrast, diet-induced insulin resistant mice exhibited similar decreases in Akt signaling coupled with increased amyloid plaques and decreased spatial performance (26). Both the BKS-db/db and ob/ob mice are insulin resistant and sustain greater infarct areas and increased behavioral deficits than their nondiabetic littermates (60). Although these studies did not specifically address insulin signaling, they confirm data collected from human patients regarding the negative impact of diabetes on ischemia-reperfusion injury.

Neurons preferentially use glucose as their main energy source. The neurobasal (NB^+) medium contains 25 mM, which is optimal for maintaining neuronal health and axonal outgrowth (51). In our previous work examining the effects of diabetes on peripheral sensory neurons, we demonstrated that 20–25 mM (total 50 mM, 280 mg/dl) added glucose induces oxidative stress, mitochondrial depolarization, and programmed cell death (38, 62). These levels are within the range of that detected 2 h postprandial (200 mg/dl, 11.1 mM) in a patient with poorly regulated diabetes (42). With regard to the central nervous system, brain glucose levels are two- to threefold lower than the plasma glucose levels. Silver and Erecinska (57) demonstrated that brain glucose levels are 3-fold lower both under control (2.4 mM brain *vs.* 7.6 mM blood) and hyperglycemic (4.5 mM brain *vs.* 15.2 mM blood) conditions. Most importantly, however, brain glucose levels exhibit a linear correlation with peripheral blood glucose levels (15, 57); that is, an increase in peripheral blood glucose directly increases brain glucose. Although it is almost impossible to create primary culture conditions that exactly replicate *in vivo* conditions, we believe that our *in vitro* experiments accurately reflect diabetic conditions within the central nervous system as long as we maintain the ratio of glucose increase. Our findings demonstrate that hyperglycemia had no significant effect on insulin signaling.

To examine the effects of hyperlipidemia, cortical neurons were treated with PA for 24 h. Our recent results in peripheral

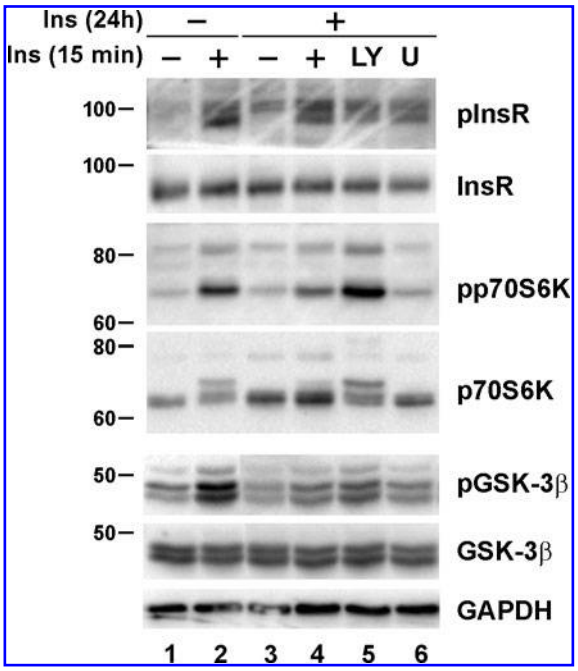

FIG. 5. Chronic insulin treatment downregulates p70S6K and glycogen synthase kinase-3β (GSK-3β) phosphorylation, which is reversed by phosphatidylinositol 3-kinase (PI3-K) inhibitor pretreatment. Cortical neurons were incubated with insulin along with 20 μM LY294002 (LY) or U0126 (U) for 24 h. The cells were washed and incubated in a fresh treatment medium for 30 min before acute insulin stimulation for 15 min. Immunoblotting was performed using the antibodies against phosphorylated InsR, p70S6K, or GSK-3β and then stripped and reprobed for total protein as well as for glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

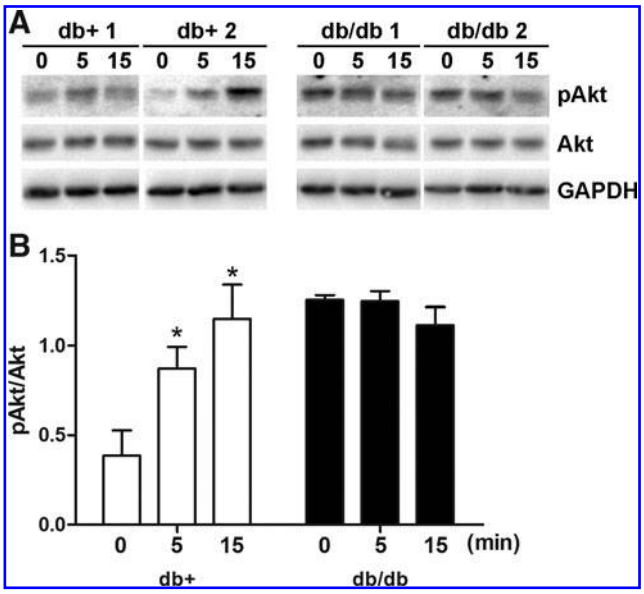

FIG. 6. Insulin cannot stimulate Akt phosphorylation in db/db cortex. (A) Cortical slices were harvested from BKS-db$^+$ and db/db mice and stimulated with 20 nM insulin as described in the Materials and Methods section. Typical results from two out of five animals tested from each group are shown. **(B)** Densitometry analysis of Akt phosphorylation. *$p < 0.05$ by one-way ANOVA compared to nonstimulated db$^+$ cortex.

sensory neurons suggest that dyslipidemia contributes to diabetic neuropathy by increasing plasma-oxidized low-density lipoprotein, which directly leads to oxidative stress and injury in dorsal root ganglion neurons *via* LOX-1 (61). In the current study, exposure to PA had no effect on insulin signaling.

Excessive exposure to insulin, rather than hyperglycemia, has been suggested as the primary instigator of insulin resistance in a type 1 diabetes mouse model (40). Of these parameters tested in the current study (high glucose, PA, or insulin), only chronic insulin treatment prevented activation of Akt. Specifically, phosphorylation of Akt at Thr308 and Ser 473 was blocked. In addition to reduced Akt activation, the phosphorylation of downstream effectors, GSK-3β and p70S6K, were diminished after chronic insulin treatment. This observation is specific for insulin signaling, as acute IGF-I activation of Akt was not affected by chronic insulin treatment.

Based on our *in vitro* findings and the reports outlined above in diabetic mice, neuronal insulin resistance was examined in the BKS-db/db mouse. These mice are obese, become hyperinsulinemic at 10–14 days of age and similar to human diabetic patients, experience more damage after ischemia-reperfusion injury to the brain (60). Because of their extreme insulin resistance, it is very difficult to separate the effects of hyperglycemia from chronic insulin exposure in these animal models. Our observation that neurons dissected from BKS-db/db mice at 24 weeks of age (20 week of diabetes) are unable to phosphorylate Akt in response to acute insulin treatment argues for hyperinsulinemia. Further examination of these animals or other type 2 models of diabetes is warranted.

The absence of leptin signaling in the db/db mouse is responsible for hyperphagy; the dramatic obesity that follows nonstop eating results in insulin resistance and diabetes in this model. Type 2 diabetes may also be induced by feeding mice a Western style high-fat diet (65). While our data clearly support a role for insulin resistance in decreased Akt signaling, leptin also decreases Akt activation in the rat brain (8). In our studies, the lack of leptin receptor should minimize the effects of leptin signaling in this regard. Leptin receptors are expressed in the hypothalamus, cerebellum, and cerebral cortex (8) and are neuroprotective after ischemic injury (69). Further studies of diet-induced type 2 models with intact leptin signaling are an important step toward examining the combined role of insulin resistance and leptin signaling with regard to neuronal survival after ischemia-reperfusion injuries.

The beneficial effects of insulin treatment after ischemic injury have been demonstrated in *in vitro* and *in vivo* models, and in all cases reported depend on Akt activation. *In vitro* induction of oxidative stress decreases constitutive tyrosine phosphorylation of the insulin and IGF-I receptors and would normally result in neuronal apoptosis. Insulin treatment restores baseline activation of these receptors and increases activation of Akt and subsequent maintenance of Bcl-2 and inhibition of caspase 3 (14). *In vivo*, a single bolus of insulin given at the onset of re-perfusion activated Akt, protected hippocampal neurons from apoptosis, and spared learning and memory (52). Akt also plays a key role in neuroprotection

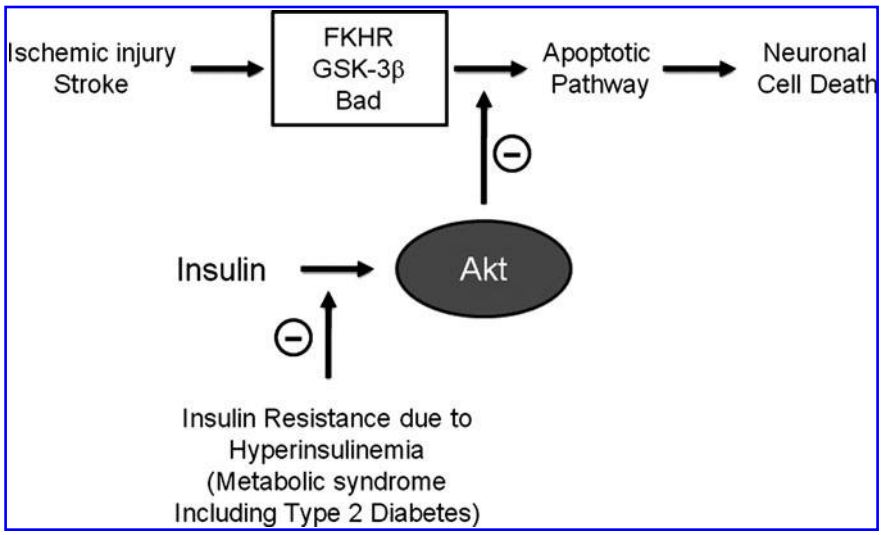

FIG. 7. Model for increased neuronal injury due to insulin resistance during metabolic syndrome including type 2 diabetes. Ischemic insult induces cell death through proapoptotic signaling molecules, including forkhead transcription factor (FKHR), GSK-3β, and Bad, which can be prevented by Akt activation. Insulin resistance state induced by hyperinsulinemia prevents Akt activation and inhibits cell survival.

by baicalein (39), simvastatin (9), and humanin (67), which are all implicated in neuronal survival after ischemic injury. Therefore, suppression of Akt phosphorylation (*i.e.,* activation) due to insulin resistance can profoundly affect neuronal survival after ischemic injury and stroke. The specificity of the PI-3K/Akt pathway in the induction of insulin resistance in cortical neurons is underscored by blocking this pathway with LY294002. When cortical neurons were exposed to insulin and LY294002, Akt activation was prevented and acute insulin signaling activated the expected range of downstream proteins. Similar results are reported in mice fed with a high-fat diet. These mice develop insulin resistance with increased basal Akt phosphorylation and display increased oxidative stress (41). When the mice were treated with LY294002 during the day time (when mice usually do not eat), all these changes were reversed with increased insulin sensitivity. Therefore, Akt may be the key factor connecting increased risk of ischemia and stroke as well as poor prognosis after stroke in patients with diabetes and other metabolic syndromes. Several signaling molecules regulated by Akt, including Bad, forkhead transcription factor (FKHR), and GSK-3β, are implicated in ischemia/reperfusion-mediated neuronal injury (71) and are discussed below.

Bad is a proapoptotic member of the Bcl family of proteins (13). After cerebral ischemia, Bad translocates to the outer membrane of the mitochondria and dimerizes with Bcl-xL (2). This process triggers cytochrome c release and caspase activation. Akt-induced phosphorylation inactivates Bad and prevents cell death (13). Peroxisome proliferator-activated receptor (PPAR)-γ is a ligand-modulated transcription factor activated by insulin-sensitizing thiazolidinediones and a therapeutic target for treating type 2 diabetes (22). Rosiglitazone, troglitazone, and pioglitazone have all demonstrated protective effects against ischemia-reperfusion–induced myocardial damage and cerebral infarction in animal studies (55, 59, 68). These effects were mediated by preventing ischemia-induced degradation of Akt, Bcl-2, and Bcl-xL and subsequent phosphorylation and inactivation of Bad (19).

In response to apoptotic stimuli (including ischemia) FKHR translocates to the nucleus and initiates the expression of proapototic proteins such as FAS. Akt phosphorylates FKHR, leading to suppression of its apoptotic activity and promoting cell survival (50). The vanadyl compound bis(1-oxy-2-pyridinethiolato)oxovanadium(IV) [VO(OPT)] is neuroprotective in a mouse ischemic model *via* Akt activation and subsequent phosphorylation of FKHR (56).

Active (*i.e.,* dephosphorylated) GSK-3β is increased after ischemic injury and GSK-3β inhibitor reduces ischemic infarction (35). Akt phosphorylates and thus inactivates GSK-3β (63). Lithium, traditionally used as a mood stabilizer, displays neuroprotective effects after stroke by increased expression of brain-derived neurotrophic factor and Bcl-2 and inhibition of GSK-3β (64). Induction of focal ischemic stroke by middle cerebral artery occlusion in diabetic mice exacerbated ischemia-induced cognitive deficits and brain infarction (70). These changes were accompanied with increased tau phosphorylation and decreased GSK-3β phosphorylation.

Even though less studied, p70S6K is also involved in preventing neuronal cell death after ischemic brain injury. Phosphorylation of p70S6K was decreased during transient focal ischemia (29) and the protective effect of thrombin against focal cerebral ischemia was accompanied with increased phosphorylation of p70S6K (28). Our current report demonstrates the regulation of GSK-3β and p70S6K phosphorylation by Akt in cortical neurons. Impairment of Akt activity by hyperinsulinemia-induced insulin resistance affects the downstream signaling molecules involved in ischemic/stroke injury and increases neuronal cell death. We are currently investigating the signaling downstream of Akt in both cortical neurons *in vitro* and in the BKS-db/db mice. Our hypothesis is summarized in Figure 7.

In summary, our experiments reveal that chronic insulin stimulation *in vitro* and *in vivo* results in a decrease in acute insulin-stimulated Akt activation, a form of insulin resistance in cortical neurons. Insulin resistance (or metabolic syndrome) is a risk factor for stroke (31) and its recurrence (25). Six out of nine recent epidemiological studies provide evidence that insulin resistance is associated with stroke (31). Insulin resistance was observed in up to 50% of the patients with transient ischemic attack or stroke (32). The prevalence of metabolic syndrome was almost twice as high (43.5% *vs.* 22.8%) in people with a self-reported history of stroke

compared to those without a history of vascular disease (46). Individuals with metabolic syndrome, even without diabetes or CVD, are at increased risk of long-term cardiovascular outcomes, suggesting the importance of identifying metabolic syndrome early to intervene in the possible development of CVD and stroke (43). Precise regulation of Akt phosphorylation is critical for neuronal survival during brain ischemia (20, 47, 71). Our results suggest for the first time that decreased responsiveness of Akt phosphorylation due to hyperinsulinemia may explain the increased neuronal damage reported in both experimental models of diabetes and diabetic patients (4, 44, 49). Experiments are ongoing to determine the role(s) of InsR turnover and the signaling capacity of intracellular substrates responsible for insulin signal transduction. The inability of neurons to respond to circulating growth factors, including insulin or IGF-I, provides a potential explanation for the increased neuronal damage observed in diabetic human patients after stroke.

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Author Disclosure Statement

The authors state that no competing financial interests exist.

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Abbreviations Used

ANOVA = analysis of variance
CVD = cardiovascular disease
ERK = extracellular-signal regulated kinase
FKHR = forkhead transcription factor
GAPDH = glyceraldehyde 3-phosphate dehydrogenase
GSK-3β = glycogen synthase kinase-3β
IGF-I = insulin-like growth factor-I
IHC = immunohistochemistry
InsR = insulin receptor
MAPK = mitogen-activated protein kinase
mTORC2 = mammalian target of rapamycin complex 2
PA = palmitic acid
PBS = phosphate-buffered saline
PDK1 = pyruvate dehydrogenase [lipoamide] kinase isozyme 1, mitochondrial
PI3-K = phosphatidylinositol 3-kinase
PLL = poly-L-lysine
PPAR = peroxisome proliferator-activated receptor

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