Peripheral Hyperinsulinemia Promotes Tau Phosphorylation In Vivo

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Cerebral insulin receptors play an important role in regulation of energy homeostasis and development of neurodegeneration. Accordingly, type 2 diabetes characterized by insulin resistance is associated with an increased risk of developing Alzheimer’s disease. Formation of neurofibrillary tangles, which contain hyperphosphorylated tau, represents a key step in the pathogenesis of neurodegenerative diseases. Here, we directly addressed whether peripheral hyperinsulinemia as one feature of type 2 diabetes can alter in vivo cerebral insulin signaling and tau phosphorylation. Peripheral insulin stimulation rapidly increased insulin receptor tyrosine phosphorylation, mitogen-activated protein kinase and phosphatidylinositol (PI) 3-kinase cascade (20), including the extracellular regulated kinase (Erk) family (21,22) and glycogen synthase kinase (GSK)-3β (23–25). In insulin receptor substrate-2–disrupted mice (13), peripherally injected insulin enters the cerebrospinal fluid (CSF) rapidly, within 5 min. However, in rodents, <1% of the applied insulin reaches the CSF (14), although this may vary among other species.

In Alzheimer patients (15) and healthy subjects (16), hyperinsulinemic-euglycemic clamp studies revealed an improving effect of insulin on cognitive function. Furthermore, in healthy subjects under hyperinsulinemic-euglycemic clamp conditions, a pronounced negative shift in the transcortical direct current potential occurred (17), indicating that circulating insulin can rapidly act on brain activity independent from its systemic effects. In healthy humans, intranasally administered insulin directly entered the CSF and improved memory function without influencing peripheral blood glucose levels (18). Intranasally administered insulin not only affects memory function but also acts on food intake and energy homeostasis. Hallschmid et al. (19) showed that in male normal weight subjects, intranasally administered insulin directly entered the CSF and improved memory function without influencing peripheral blood glucose levels (18). Intranasally administered insulin not only affects memory function but also acts on food intake and energy homeostasis. Hallschmid et al. (19) showed that in male normal weight subjects, intranasal insulin exerts catabolic effects on body weight and obesity.

Neurofibrillary tangles mainly composed of hyperphosphorylated tau are a hallmark of neurodegenerative disorders, e.g., Alzheimer’s disease. Appearance of tau hyperphosphorylation in brain represents an early stage of Alzheimer’s disease. In neuronal cell culture models, tau hyperphosphorylation is promoted by several protein kinases that are also activated by the insulin receptor signaling cascade (20), including the extracellular regulated kinase (Erk) family (21,22) and glycogen synthase kinase (GSK)-3β (23–25). In insulin receptor substrate-2–disrupted mice, as a model of human type 2 diabetes, an accumulation of hyperphosphorylated tau was detected in the CNS (26). Furthermore, neuronal/brain-specific deletion of insulin receptor (neuronal/brain-specific insulin receptor knockout [NIRKO]) in mice led to tau hyperphosphorylation in vivo (27). Surprisingly, the pattern of phosphorylation in NIRKO mice (27) compared with insulin
receptor substrate-2 knockout mice (26) is different, suggesting that not only insulin resistance, but also other factors, e.g., hyperinsulinemia, might contribute to sitespecific, differential tau phosphorylation.

Because the role of hyperinsulinemia in type 2 diabetes and neurodegenerative disorders is largely unknown, we addressed the impact of peripherally administered insulin on central insulin receptor signaling and tau phosphorylation in vivo. Our results show that the brain is a direct target of peripheral circulating insulin where it leads to intraneuronal insulin receptor signaling inducing presumably unfavorable tau phosphorylation, predisposing for tangle formation (rev. in 25).

RESEARCH DESIGN AND METHODS

Animals and genotyping. C57BL/6 mice were bred under standard laboratorystations. NIKOR mice were generated as described previously (28) and backcrossed onto a C57BL/6J background for six generations. Animals were housed in a 12-h light/dark cycle (0700 on/1900 off) and were fed a standard rodent diet. All animal procedures were performed in accordance with the German Laws for Animal Protection and were approved by the local animal care committee and the Bezirksregierung Koeln.

Insulin stimulation. Eight-week-old O/N fasted male/female C57BL/6 mice and eight-week-old male/female NIKOR mice were anesthetized. After loss of pedal and corneal reflexes were assurred, the abdominal cavity of the mice was opened, and 100-μl samples containing 1 μU (low-dose group) or 4 units (high-dose group) regular human insulin (Actrapid; Novo Nordisk, Bagsværd, Denmark) were drawn at 5-min intervals to measure blood glucose (GlucoMen; A. Menarini Diagnostics, Berlin-Chemie, Neuss, Germany). After 0, 5, 10, 15, and 20 min, brain tissue was harvested, and protein was extracted for Western blot analysis.

Histology and immunostaining. Animals were anesthetized and their vascular system rinsed for 60 s with 0.9% NaCl and fixed for 30 min by transcardial perfusion with a minimum of 4% paraformaldehyde in 0.1 mol/l phosphate buffer, pH 7.4. The brains were dissected from perfused mice and postfixed for 4 h in 4% paraformaldehyde (in 0.1 mol/l phosphate buffer, pH 7.4). Then the tissue was embedded in paraffin and cut in 10 μm-thick sections. After paraffin removal, the following incubation protocol was used: 1) 0.25% (wt/vol) Triton-X 100 in Tris-buffered saline (TBS) (50 mmol/l Tris/HCl buffer plus 0.9% NaCl, pH 7.6) for 10 min, 2) initial block with 5% (wt/vol) normal goat (sheep) serum, 3) incubation with 1:400 of the corresponding Cy3-labeled secondary antibody in 5% (wt/vol) normal goat (sheep) serum in TBS for 15 min, and 4) incubation with the appropriate antisera. Tau phosphorylation at Ser202 and Thr 231.

RESULTS

Activation of insulin receptor signaling in the murine CNS. To study the effect of peripherally injected insulin on cerebral insulin receptor signaling, we investigated the phosphorylation of downstream insulin receptor signaling proteins in total brain lysates of insulin- and saline-injected mice using Western blot analysis. After insulin injection, physiological blood glucose values were detected for the whole observation period of 20 min (Fig. 1A). Injection of 1 μU insulin (low-dose group) rapidly increased insulin receptor tyrosine phosphorylation within 5 min, which remained stable for 20 min (Fig. 1B). Protein kinase B/Akt was transiently phosphorylated at Ser473 with a maximum at 10 min (Fig. 1C). To directly determine the activity of Akt kinase, assays were performed. Akt activity increased 5 min after injection of 1 μU insulin with a maximum at 10 min (Fig. 1D). GSK-3β is a downstream target of Akt and a known tau kinase. Its phosphorylation at Ser9 increased within 10 min after injection (Fig. 1E). Furthermore, Erk-1/2 was transiently phosphorylated with a maximum at 10 min (Fig. 1F). Thus, even mild peripheral hyperinsulinemia promotes activation of the PI-3-kinase and the mitogen-activated protein kinase pathway in the CNS in vivo.

Tau phosphorylation at Ser202 and Thr231. Immunoblots of tau1/2 revealed a clear gel shift, suggesting tau phosphorylation due to insulin stimulation (Fig. 2A). In vitro studies show that tau is phosphorylated at Ser202 by Erk-2 (21,22) and at Thr231 by GSK-3β (23). Site-specific tau phosphorylation can be detected by the antibodies AT8 and AT180 (Fig. 2B). The low-dose group showed increased tau phosphorylation at Ser202 5 min after injection of 1 μU insulin, reaching significance after 15 min (Fig. 2C). Injecting 4 units insulin (high-dose group) revealed a further increase of Ser202 tau phosphorylation after 15 min (Fig. 2C), indicating a correlation between tau phosphorylation and peripheral insulin levels. In both groups (low dose and high dose) tau phosphorylation at Thr231 remained unchanged (Fig. 2D).

Euglycemic-hyperinsulinemic clamp studies. After applying an insulin bolus of 200 mU/kg via the central venous catheter, blood glucose levels were measured every 10 min from the tail vein and kept stable in physiological range by infusing 50% glucose. All animals showed euglycemic blood glucose values at any time of the study (Fig. 3A). However, using whole-brain lysates, we might pick up signaling exclusively from regions having a leaky or no BBB, e.g., area postrema and circumventricular organs. Therefore these regions were removed, after which protein was extracted from the remaining brain parts. To exclude artifacts due to anesthesia, anesthesia-induced hypothermia, or insulin-induced hypoglycemia, we only tested animals that were wide awake and able to move glucose was measured from the tail tip. After basal sampling, regular human insulin (Actrapid; Novo Nordisk) diluted in saline with 0.1% BSA (Sigma) was infused (200 mU/kg bolus) at the time point 0 min. To keep the animals euglycemic, 50% glucose was infused adapted to the measured blood glucose values. Blood samples were drawn at 10-min intervals to determine blood glucose levels (Glucose Analyzer; Bayer, Elkhart, IN). To maintain constant body temperature, animals were irradiated with a red light. Brains were harvested 10, 20, 30, and 60 min after insulin administration and prepared for Western blot analysis.

Statistical analysis. To quantify the changes in optical density, we used the software AIDA (Version 4.90.027; Raytest, Straubenhardt, Germany). For statistical analysis of the different study groups, unpaired Student’s t test was performed. Statistical significance was defined as P < 0.05.
their limbs freely. Under such euglycemic-hyperinsuline-mic clamp conditions, Erk-1/2 was transiently phosphory-lated with a maximum at 10 min (Fig. 3B), thus serving as a control for an accurate insulin stimulation. As already shown in whole-brain lysates of anesthetized mice (Fig. 2C) under clamp conditions, tau phosphorylation at Ser\(^{202}\) increased with a maximum at 20 min and was still present 60 min after insulin stimulation (Fig. 3C).

These results show that the above-described insulin-induced tau phosphorylation at Ser\(^{202}\) is detectable until 60 min after insulin administration and occurs independently of hypothermia, hypo- or hyperglycemia, anesthesia, or an untight BBB.

**Immunohistochemistry of insulin-stimulated brains.**

To evaluate whether the detected insulin receptor signal transduction occurred intraneuronally, we performed immunostainings of insulin-stimulated brain sections. Using anti-PIP3 antibody, insulin-stimulated brains showed distinct immunoreactivity at the neuronal plasma membrane (Fig. 4A). FoxO1, a downstream target of the insulin receptor signaling cascade, is a nuclear protein that translocates to the cytoplasm upon phosphorylation. Phospho-FoxO1 immunostainings of insulin-stimulated brains (5 units for 10 min) revealed cytoplasmic staining in hypothalamus, thalamus, cortex, and hippocampus (Fig. 4B). Additionally performed immunoblot analyses showed a transient phosphorylation of FoxO1 with a maximum at 5–10 min after insulin stimulation (Fig. 4C), confirming our previously described results. However, the strongest immunoreactivity was seen in the hippocampus and hypothalamus. Not all neurons reacted to insulin stimulation (Fig. 4A, arrowheads), suggesting that only a subpopulation of neurons is susceptible to insulin action.

**Insulin receptor signaling and tau phosphorylation in NIRKO mice.**

To reassess whether the detected insulin receptor signal transduction is mediated via the insulin...
receptor and not via any other receptor like the IGF-1 receptor, we investigated mice lacking the neuronal insulin receptor (NIRKO) (Fig. 5A) under hyperinsulinemic conditions after intravenous injection of 4 units insulin. Downstream insulin receptor signaling with phosphorylation of Akt, Erk, and GSK-3β was completely abolished in NIRKO mice after insulin stimulation for the whole surveillance period of 20 min (Fig. 5B). Compared with wild-type mice (high-dose group; Fig. 2C), tau phosphorylation at Ser202 was completely eliminated in NIRKO mice after insulin stimulation (Fig. 5C). Thus, the cerebral effect of peripherally administered insulin is mediated via the insulin receptor.

DISCUSSION
Since the early 1960s, insulin action in the CNS has been under investigation (29). Although insulin is unable to regulate glucose uptake in neuronal tissue, it directly affects the CNS: Both insulin and its receptor are expressed throughout the mammalian brain (30,31). High concentrations of insulin in brain extracts led to the assumption that insulin was synthesized and released locally in the CNS (32). However, recent investigations have shown that only a little insulin is produced in the CNS, supporting the notion that the majority of insulin acting in the CNS is of pancreatic origin (33).

Later on, it could be demonstrated that insulin crosses the BBB by a receptor-mediated transport (8). Furthermore, in dogs, it was shown that peripherally administered insulin passes the BBB rapidly, but <1% is transported into the CSF (14).

In the present study, we demonstrate that peripherally applied insulin stimulates brain insulin receptor tyrosine phosphorylation within 5 min. Furthermore, the mitogen-activated protein kinase and PI 3-kinase pathways as downstream targets of the insulin receptor are activated within 10 min after peripheral insulin injection. These findings clearly indicate that circulating insulin is capable of rapidly activating the cerebral insulin receptor signaling cascade.

Moreover, we demonstrate that insulin signaling in the CNS occurs predominantly in neurons and not in other brain cells such as glia or endothelial cells: Immunohistochemistry of different brain regions in mice revealed insulin-stimulated PIP3 formation predominantly in neurons. Accordingly, phosho-FoxO1 immunostaining in insulin-stimulated mice showed cytoplasmic neuronal staining with maximum intensity in the hippocampus formation and the hypothalamus.

In addition, we show that only a subset of neurons responds to insulin stimulation. This is consistent with findings in rat brains in which insulin receptor densities and the insulin content were found to be unevenly distributed with a distinct regional pattern (rev. in 34). Highest insulin receptor and insulin concentrations were seen in hypothalamus, olfactory bulb, cerebellum, cerebral cortex, and brain stem (34). It is still unclear whether regional different responses to insulin emerge from different neuronal insulin receptor density, variable cerebral blood circulation, diverse capillary density in the brain, or other factors. Whatever the mechanism, certain neuronal populations are a direct unique target of peripheral circulating insulin, where it rapidly induces insulin receptor signal transduction. It is important to state that the regions that
and time point. **

Insulin treatment resulted in a significant increase of tau phosphorylation. However, in cultured human neurons (NT2 cells), GSK-3β was inhibited associated with reduced tau phosphorylation after insulin stimulation (39). Similar findings were seen in insulin-stimulated skeletal muscle cells and adipocytes (40).

We show significantly increased tau phosphorylation at Ser202 in the brain within 10 min after 1-mU insulin injection and an even further increase after injection of 4 units insulin, indicating a correlation in vivo between tau phosphorylation and peripheral insulin levels. However, Thr231 tau phosphorylation remained unchanged in both groups (low dose and high dose).

Our initial studies have been performed in anesthetized mice and have been shown that in mice, hypothermia triggered by 3-day starvation or hypoglycemia was associated with cerebral tau phosphorylation (41). Even hypothermia in hibernating squirrels (42) and in mice treated with cold water stress (43) led to cerebral tau phosphorylation (rev. in 44). To exclude any effects arising from anesthesia, we performed hyperinsulinemic-euglycemic clamp studies in wide-awake nonanesthetized normothermic animals. Moreover, in this setup, blood glucose levels were kept stable in the physiological range, avoiding the development of hypo- or hyperglycemia. Because tau hyperphosphorylation was detectable under these experimental conditions, this clearly demonstrates that peripheral hyperinsulinemia per se is responsible for tau hyperphosphorylation.

To examine whether insulin signal transduction is mediated directly via the insulin receptor in the brain and not by any other receptors, such as the IGF-1 receptor, we tested mice lacking the brain insulin receptor (NIRKO) under hyperinsulinemic conditions. Insulin receptor signaling and tau phosphorylation were completely abolished in the brains of these mice, indicating that the cerebral insulin receptors are a direct target of peripheral administered insulin.

Taken together, our data reveal several novel findings with respect to insulin signaling and tau phosphorylation in the brain: 1) insulin rapidly activates signaling in the brain, even in areas separated from the circulation by a tight BBB, e.g., the hippocampus; 2) peripherally administered insulin evokes site-specific tau phosphorylation at Ser202; 3) this occurs rapidly after acute insulin injection, but also upon prolonged stimulation during clamp studies; and 4) insulin action and insulin-stimulated tau phosphorylation at Ser202 depend on neuronal insulin receptors because lack of insulin receptors cannot be compensated for by other receptors, such as the IGF-1 receptor. Thus, our study suggests that insulin action in the brain is directly linked to neurodegeneration through activation of tau phosphorylation.

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**FIG. 5. Insulin receptor signaling and tau phosphorylation in NIRKO mice.**

A: Immunoblot analysis of insulin receptor expression in wild-type and NIRKO brains. B: Western blot analyses of insulin (IN)-stimulated NIRKO brains with antibodies against pAkt, Akt, pErk, GSK-3β, and pGSK-3β. C: Cerebral tau phosphorylation at Ser202 in NIRKO (●) and wild-type (□) mice after stimulation with 4 units insulin. Data represent means ± SE of two to five animals per genotype and time point. **P < 0.01.
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REFERENCES


