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Tau Is Hyperphosphorylated at Multiple Sites in Mouse Brain In Vivo After Streptozotocin-Induced Insulin Deficiency

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Abstract

Deficient signaling by insulin, as occurs in diabetes, is associated with impaired brain function, and diabetes is associated with an increased prevalence of Alzheimer's disease. One of the hallmark pathological characteristics of Alzheimer's disease is the presence of neurofibrillary tangles containing hyperphosphorylated tau, a microtubule-associated protein. Therefore, we tested the hypothesis that insulin depletion caused by administration of streptozotocin may cause tau hyperphosphorylation in mouse brain by using site-specific phosphorylation-dependent tau antibodies to obtain precise identification of the phosphorylation of tau on individual residues. A massive (fivefold average increase) and widespread at multiple residues (detected with eight different phosphorylation-dependent tau antibodies) increase in the phosphorylation of tau was found in mouse cerebral cortex and hippocampus within 3 days of insulin depletion by streptozotocin treatment. This hyperphosphorylation of tau at some sites was rapidly reversible by peripheral insulin administration. Examination of several kinases that phosphorylate tau indicated that they were unlikely to account for the widespread hyperphosphorylation of tau caused by streptozotocin treatment, but there was a large decrease in mouse brain protein phosphatase 2A activity, which is known to mediate tau phosphorylation. These results show that insulin deficiency causes rapid and large increases in tau phosphorylation, a condition that could prime tau for the neuropathology of Alzheimer's disease, thereby contributing to the increased susceptibility to Alzheimer's disease caused by diabetes.

The regulation of glucose utilization by insulin is one of the most fundamental processes necessary for providing sufficient nutrition to maintain the vitality of mammalian organisms (1,2). Unfortunately, there is a rapidly increasing prevalence of individuals developing insulin resistance, conditions in which the ability of insulin to properly regulate glucose is impaired. For example, the number of people with diabetes in the world was estimated to be 135 million in 1995 and is predicted to reach a total of 300 million in 2025 (3). Not only do insulin resistant conditions like diabetes affect peripheral tissues, such as muscle and fat cells, but brain function also can be significantly impaired (4,5). However, the links between deficient cellular responses to insulin and the ensuing detrimental changes in brain function have not been identified in sufficient enough detail to suggest corrective interventions other than targeting peripheral mechanisms that control insulin and glucose. Recently, this topic has taken on additional importance with the identification of significant links between diabetes and Alzheimer's disease, another illness with a rapidly growing prevalence (6–11).

One of the hallmark pathological characteristics of Alzheimer's disease is the presence of neurofibrillary tangles that contain as their primary component aggregates of the protein tau in a hyperphosphorylated state (12). Tau is a microtubule-binding protein that contributes to the stability of microtubules when it is bound to polymerized tubulin. The binding of tau to

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microtubules is reduced by increases in the phosphorylation state of tau, and hyperphosphorylation of tau "may disrupt microtubules and interfere with intraneuronal organelle transport, ultimately leading to dysfunction of synapses, degeneration of neurons, and cognitive impairment" (13). Insulin normally regulates signaling cascades that contribute to the regulation of tau phosphorylation (14), raising the possibility that one factor linking diabetes and Alzheimer's disease may involve changes in the phosphorylation state of tau consequent to deficient actions of insulin. However, this is a complex interaction because tau can be phosphorylated on multiple serines and threonines by many different kinases (12). Fortunately, because of the involvement of tau in Alzheimer's disease, many site-specific phosphorylation-dependent tau antibodies are available to allow precise identification of the phosphorylation state of tau on individual residues. A panel of these antibodies was used in the present study to test whether insulin depletion caused by administration of streptozotocin altered site-specific tau phosphorylation in mouse brain. The results show that insulin deficiency leads to large increases in the phosphorylation of tau at multiple residues in mouse cerebral cortex and hippocampus, and this is associated with decreased activity of protein phosphatase 2A (PP2A), a phosphatase known to regulate tau phosphorylation at multiple sites.

RESEARCH DESIGN AND METHODS

Adult, male C57BL/6 mice (Frederick Cancer Research, Frederick, MD), 6 to 7 weeks old, were injected once intraperitoneally with streptozotocin (150 mg/kg in citrate buffer, pH 4.6) or vehicle for controls 3 days before death. Where indicated, mice were given an intraperitoneal injection of insulin (5 IU/kg bovine pancreas insulin; Sigma) in PBS after overnight food withdrawal. Blood glucose levels were measured using a glucose monitor (True Track Smart System). Insulin concentrations were measured using an ultra-sensitive mouse insulin ELISA assay (Mercodia, Winston Salem, NC). Body temperatures were measured rectally using a CyQ model 111 thermocouple (CyperSense, Nicholasville, KY). All mice were cared for according to the principles and guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

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 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 0.5% NP-40, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 μg/ml pepstatin, 1 mmol/l phenylmethanesulfonyl fluoride, 1 mmol/l sodium vanadate, 50 mmol/l sodium fluoride, and 100 nmol/l 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 protein assay (15).

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 tau, which are listed in Table 1, or to p35 and p25 subunits of cyclin-dependent kinase-5 (Cdk5) (Santa Cruz Biotechnology, Santa Cruz, CA), phospho–Ser9 – glycogen synthase kinase (GSK) 3β, total GSK3β, phospho–Thr202,Tyr204 – extracellular signal–regulated kinases 1 and 2 (ERK1/2), total ERK1/2, phospho–Thr180,Tyr182-p38, total p38, phospho–Thr183,Tyr185– c-Jun N-terminal kinase (JNK), and total JNK (Cell Signaling Technology, Beverly, MA). Immunoblots were developed using horseradish peroxidase– conjugated goat anti-mouse or goat anti-rabbit IgG (Bio-Rad Laboratories, Hercules, CA), followed by detection with enhanced chemiluminescence, and statistical significance was determined using ANOVA.

Immunohistochemistry

Mouse brains were sectioned coronally and immersion fixed in Bouin's fixative overnight at 4°C. The brains were processed in paraffin, and 4-μm thick sections were prepared on a microtome. Paraffin-embedded sections were deparaffinized in serial solutions of Citrisolv (Fisher Scientific, Pittsburgh, PA), isopropyl alcohol, and water, followed by antigen retrieval by steaming in 10 mmol/l citric acid (pH 6.0) for 20 min followed by a 20-min cooling period. Endogenous peroxidase activity was inhibited by incubation in 3% H_2O_2 in PBS for 5 min, followed by three 5-min washes in PBS. Sections were incubated for 30 min in PBS-blocking buffer (1% BSA, 0.2% skim milk, and 0.3% Triton X-100 in PBS) to inhibit nonspecific antibody binding. Sections were incubated overnight with phospho–Thr231-tau (AT180) primary antibody diluted in PBS blocking buffer (1:5,000), followed by PBS washes and by application of horseradish peroxidase– conjugated anti-mouse secondary antibody (Jackson Immuno Research Laboratories, West Grove, PA) for 1 h at room temperature. After three washes in PBS, cyanine 3– conjugated tyramide was deposited according to the manufacturer's protocol to localize sites of antibody binding (TSA Plus; Perkin-Elmer Life Science Products, Boston, MA). Sections were then washed in PBS, counterstained with Hoechst 33,258, coverslipped with PBS:glycerol (1:1), and viewed with a Zeiss-Axioskop microscope equipped with epifluorescence. Digital images were captured with a Zeiss Axiocam and Axiovision software.

Protein phosphatase assay

The activities of PP2A and PP2B were measured using the serine-threonine phosphatase assay system from Promega (Madison, WI) as previously described for mouse brain analysis (16). After decapitation, one-half of the cortex was homogenized in 2 ml ice-cold lysis buffer (10 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 0.5% NP-40, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 μg/ml pepstatin, and 1 mmol/l phenylmethanesulfonyl fluoride). The homogenate was centrifuged for 1 h at 100,000*g* to remove particulate matter, and Sephadex columns were used to remove free phosphate from the supernatant according to the manufacturer's instructions. Protein concentrations were determined in triplicate using the Bradford protein assay (15). PP2A and PP2B activities were measured using PP2A and PP2B assay buffer (formulation provided by manufacturer) in each sample (10 μg protein; 15 min) in triplicate according to the manufacturer's instructions by measuring the release of phosphate from a synthetic peptide substrate. Released phosphate was determined by measuring the absorbance at 595 nm of the molybdate–malachite green–phosphate complex. Statistical significance was determined using Student's *t* test.

RESULTS

Three days after streptozotocin treatment, the serum insulin concentration was reduced to 0.10 ± 0.02 ng/ml from the control level of 0.35 ± 0.02 ng/ml. The site-specific phosphorylation of tau was examined by using eight different phosphorylation-dependent antibodies to specific residues of tau in Western blots of the cerebral cortex and the hippocampus. Three days after streptozotocin treatment, there were large increases in the phosphorylation of tau at all of the epitopes examined in both the cerebral cortex (Fig. 1) and the hippocampus (Fig. 2). These included increased phosphorylation of tau on threonine-181, serine-199, serine-202, threonine-212, threonine-231, serine-262, and serine-396/404, which altogether showed an average fivefold increase in tau phosphorylation. Thus, streptozotocin-induced insulin deficiency led to massive increases in the phosphorylation of mouse brain tau at multiple residues.

Immunohistochemical analysis with antibody AT180, which recognizes phospho–Thr231-tau, was used to examine the localization of the streptozotocin-induced hyper-phosphorylation of

tau (Fig. 3). AT180 staining was clearly evident throughout neurons in the cerebral cortex (Fig. 3*A*) and hippocampus (Fig. 3*B*) of control mice. AT180 staining was greatly increased after streptozotocin treatment, especially in layer three of the cortex and in the CA4 region of the hippocampus.

The increased phosphorylation of tau on many residues after streptozotocin treatment could be caused by activation of multiple kinases and/or by inhibition of a phosphatase that dephosphorylates these sites. Examination of five different kinases that are known to phosphorylate tau suggested that their activation could contribute to tau hyperphosphorylation but was unlikely to account for the multisite hyperphosphorylation of tau (Fig. 4). GSK3 was inhibited after streptozotocin treatment as indicated by increased phosphorylation on serine-9, which inhibits its activity, as previously reported (17). No changes were found in the Cdk5 catalytic p35 subunit or its truncated p25 subunit, which was undetectable. Streptozotocin treatment also did not change the activation-associated phosphorylation of ERK1/2. However, streptozotocin treatment increased the phosphorylation of both p38 and JNK, indicating activation of these two kinases. These results indicate that streptozotocin treatment activates some kinases that are known to phosphorylate tau, but this limited kinase activation may not be sufficient to cause increases in all of the sites of tau that were hyperphosphorylated.

These variable changes in tau kinases accompanied by the increased phosphorylation of tau on multiple sites suggested that impaired activity of a protein phosphatase capable of dephosphorylating many sites on tau may contribute to tau hyperphosphorylation caused by streptozotocin treatment. The most well-characterized protein phosphatase that acts on phosphorylated tau is protein phosphatase 2A (PP2A), an enzyme capable of dephosphorylating multiple residues of tau and previously implicated in the tau hyperphosphorylation pathology associated with Alzheimer's disease (18). Therefore, we tested whether PP2A activity in mouse brain was reduced from control levels by streptozotocin treatment. In confirmation of this hypothesis, the activity of PP2A was reduced by 44% in the cerebral cortex and by 55% in the hippocampus 3 days after streptozotocin treatment (Fig. 5*A*). Immunoblots of the total level of the PP2A catalytic subunit demonstrated that the reduction in activity was not due to changes in the protein level after streptozotocin treatment. In contrast to PP2A, the activity of PP2B was not altered in either brain region by streptozotocin treatment (Fig. 5*B*). Thus, streptozotocin treatment caused a large, selective decrease in PP2A activity, but not PP2B activity, and this was associated with tau hyperphosphorylation on multiple residues.

To test whether the tau hyperphosphorylation present 3 days after streptozotocin treatment was reversible, insulin was administered acutely and tau phosphorylation measured 0 to 30 min after treatment. After insulin administration, the streptozotocin-induced hyperphosphorylation of tau at Thr231 (AT180) and Ser396/404 (PHF-1) in the cerebral cortex was decreased within 15 min (Fig. 6*A*), but tau hyperphosphorylation at other sites remained unchanged. This demonstrates that tau hyperphosphorylation at selective sites was reversible after short-term insulin deficiency. The mechanism for this reversal of tau hyperphosphorylation remains to be identified, as acute insulin treatment did not reactivate cortical PP2A that was inhibited by streptozotocin treatment as would be expected because insulin only caused limited site-specific tau dephosphorylation (Fig. 6*B*).

DISCUSSION

A massive and widespread increase in the phosphorylation of the microtubule-binding protein tau was found in mouse brain after depletion of insulin by administration of streptozotocin. Tau phosphorylation is a key regulator of the ability of tau to bind and stabilize microtubules (12), suggesting that this function is impaired by insulin insufficiency. Additionally, tau

hyperphosphorylation is a key early event in the pathogenesis of Alzheimer's disease, raising the possibility that the reported association between diabetes and Alzheimer's disease (6–11) could in part be due to increased phosphorylation of tau caused by insulin deficiency. Although tau hyperphosphorylation alone is not sufficient to cause the neuropathological changes that occur in Alzheimer's disease, preexisting tau hyperphosphorylation may sensitize neurons to subsequent or concomitant insults associated with Alzheimer's disease to promote progressive neurodegeneration.

Our finding that mouse brain tau is hyperphosphorylated on multiple residues in this streptozotocin model of type 1 insulin-dependent diabetes extends previous reports of increases in tau phosphorylation in mouse brain in other conditions where insulin signaling was experimentally depressed. The many site-specific phosphorylation-dependent tau antibodies that are available allow precise measurements of the phosphorylation state of tau on individual residues, providing the means for identification of individual and multisite phosphorylation changes in tau.

These antibodies have previously been used in studies of mouse models of deficient insulin signaling, and the findings are all indicative that hyperphosphorylation of tau results from deficient insulin signaling in the brain. Increased brain tau phosphorylation specifically on threonine-231 (AT180 antibody) was found in adult mice with neuron-specific knockout of the insulin receptor, and this was attributed to increased activity of GSK3, which is known to phosphorylate this site on tau (19). Similarly, Thr-231-tau was selectively hyperphosphorylated in the brains of 36-h-old insulin receptor knockout mice (20). In insulin receptor substrate-2 (IRS-2) knockout mice, brain tau phosphorylation was increased on serine-202 (AT8 antibody), and it was suggested that this may result from a decrease in PP2A activity (21). These previous findings indicated that eliminating insulin receptors or IRS-2 in the brain caused increased site-specific phosphorylation of tau. Our results support and extend this conclusion, showing that modeling diabetes by streptozotocin treatment causes large increases (fivefold average increase) in the phosphorylation of tau at multiple residues in both the cerebral cortex and the hippocampus. Clearly, streptozotocin-induced insulin deficiency has a more profound effect on tau phosphorylation than elimination of insulin receptors or IRS-2, suggesting that more than just deficient insulin receptor–mediated signaling is involved in the diabetic outcome of tau hyperphosphorylation on multiple residues. Thus, insulin deficiency has a much greater effect on tau phosphorylation than was previously known.

The phosphorylation state of tau results from a coordinated balance between kinase-mediated phosphorylations of tau and dephosphorylation by protein phosphatases (22,23). Many kinases have been shown to phosphorylate tau but invariably on only a limited number of residues (12). Considering that streptozotocin treatment–increased tau phosphorylation was detected with eight different phosphorylation-dependent tau antibodies, we postulated that this multisite tau hyperphosphorylation may be due to deficient protein phosphatase activity. We particularly focused on PP2A because previous studies have shown that it is the major protein phosphatase acting on tau (18,24–27) and PP2A is decreased in Alzheimer's disease (22,28–32). Specifically, the activity of PP2A and PP2B toward tau was decreased ~30% in Alzheimer's disease brain compared with matched controls (33). In mouse brain after streptozotocin treatment, the activity of PP2A was decreased to an even greater extent, by 44% in the cerebral cortex and by 55% in the hippocampus. This was a specific effect because the activity of PP2B was not changed by streptozotocin treatment. These results support the hypothesis that PP2A, rather than PP2B, is a major regulator of tau phosphorylation in the cerebral cortex and hippocampus of type 1 insulin-dependent diabetic mice in accordance with previous reports that PP2A is the major tau phosphatase in the brain (18,24–27). Thus, the large decrease in PP2A activity is likely to account for a majority of the large multisite increases in tau phosphorylation on multiple residues caused by streptozotocin treatment. However, these

experiments do not rule out the possibility that one or more kinases may contribute to the increased tau phosphorylation because increases in the phosphorylation of p38 and JNK were detected concurrently with the decreased PP2A activity.

These findings show that streptozotocin-induced insulin deficiency shares with Alzheimer's disease two common outcomes: reduced PP2A activity and increased tau phosphorylation. Tau hyperphosphorylation is an early event in the pathogenesis of Alzheimer's disease, eventually aggregating into filamentous polymers and neurofibrillary tangles that parallel the progression of neuronal loss in Alzheimer's disease. Thus, the decreased PP2A activity and tau hyperphosphorylation associated with insulin deficiency may increase the susceptibility of diabetic brain to insults associated with Alzheimer's disease, thereby contributing to the recently recognized association between diabetes and heightened susceptibility to Alzheimer's disease.

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Glossary

Cdk5
: cyclin-dependent kinase-5

ERK1/2
: extracellular signal–regulated kinases 1 and 2

GSK
: glycogen synthase kinase

JNK
: c-Jun N-terminal kinase

PP2A
: protein phosphatase 2A

PP2B
: protein phosphatase 2B

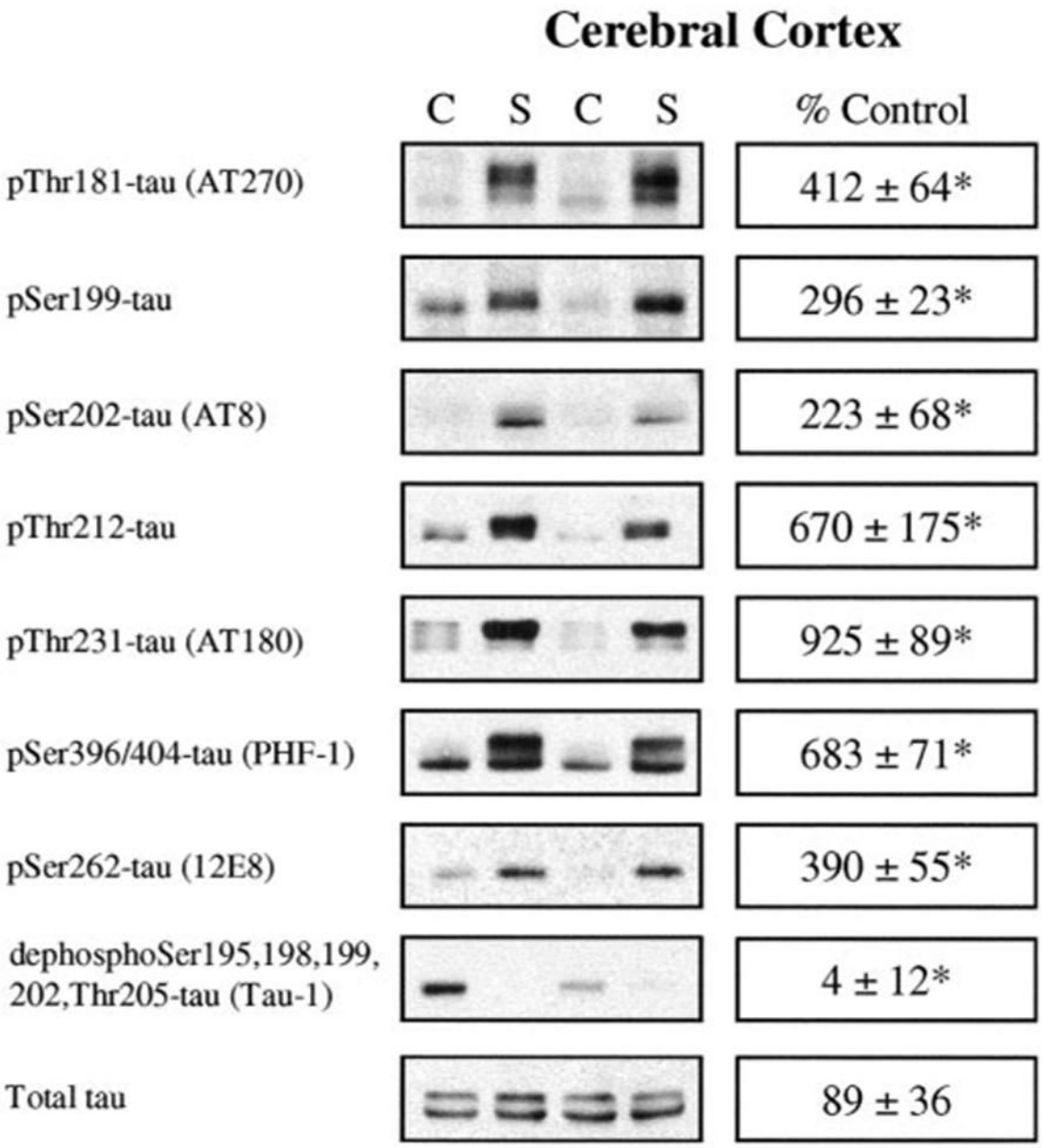

FIG. 1.

Streptozotocin administration caused multisite hyperphosphorylation of tau in mouse cerebral cortex. Mice were treated with streptozotocin, and after 3 days, protein extracts from the cerebral cortex were immunoblotted for phospho–Thr181-tau (AT270), phospho–Ser199-tau, phospho–Ser202-tau (AT8), phospho–Thr212-tau, phospho–Thr231-tau (AT180), phospho–Ser396/404-tau (PHF-1), phospho–Ser262-tau (12E8), dephospho–Ser195,198,199,202,Thr205-tau (tau-1; immunoreactivity increases as tau is dephosphorylated), and total tau. C, control; S, streptozotocin treated. Quantitative values were obtained by densitometric measurements of immunoblots and are means ± SE from four mice per group. *$P < 0.05$ compared with control values.

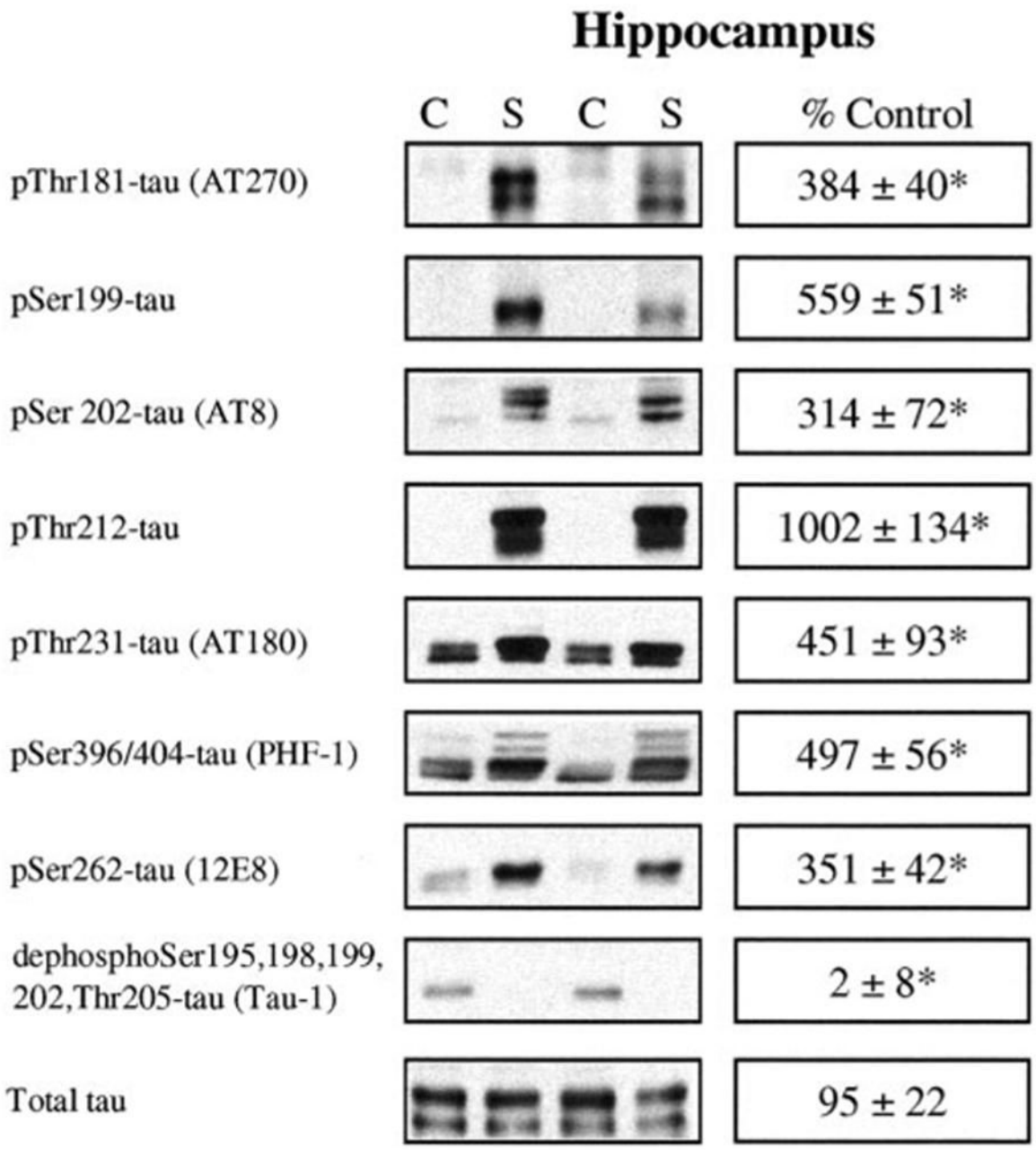

FIG. 2.

Streptozotocin administration caused multisite hyperphosphorylation of tau in mouse hippocampus. Mice were treated with streptozotocin, and after 3 days, protein extracts from the hippocampus were immunoblotted for phospho–Thr181-tau (AT270), phospho–Ser199-tau, phospho–Ser202-tau (AT8), phospho–Thr212-tau, phospho–Thr231-tau (AT180), phospho–Ser396/404-tau (PHF-1), phospho–Ser262-tau (12E8), dephospho–Ser195,198,199,202,Thr205-tau (tau-1), and total tau. C, control; S, streptozotocin treated. Quantitative values were obtained by densitometric measurements of immunoblots and are means ± SE from four mice per group. *$P < 0.05$ compared with control values.

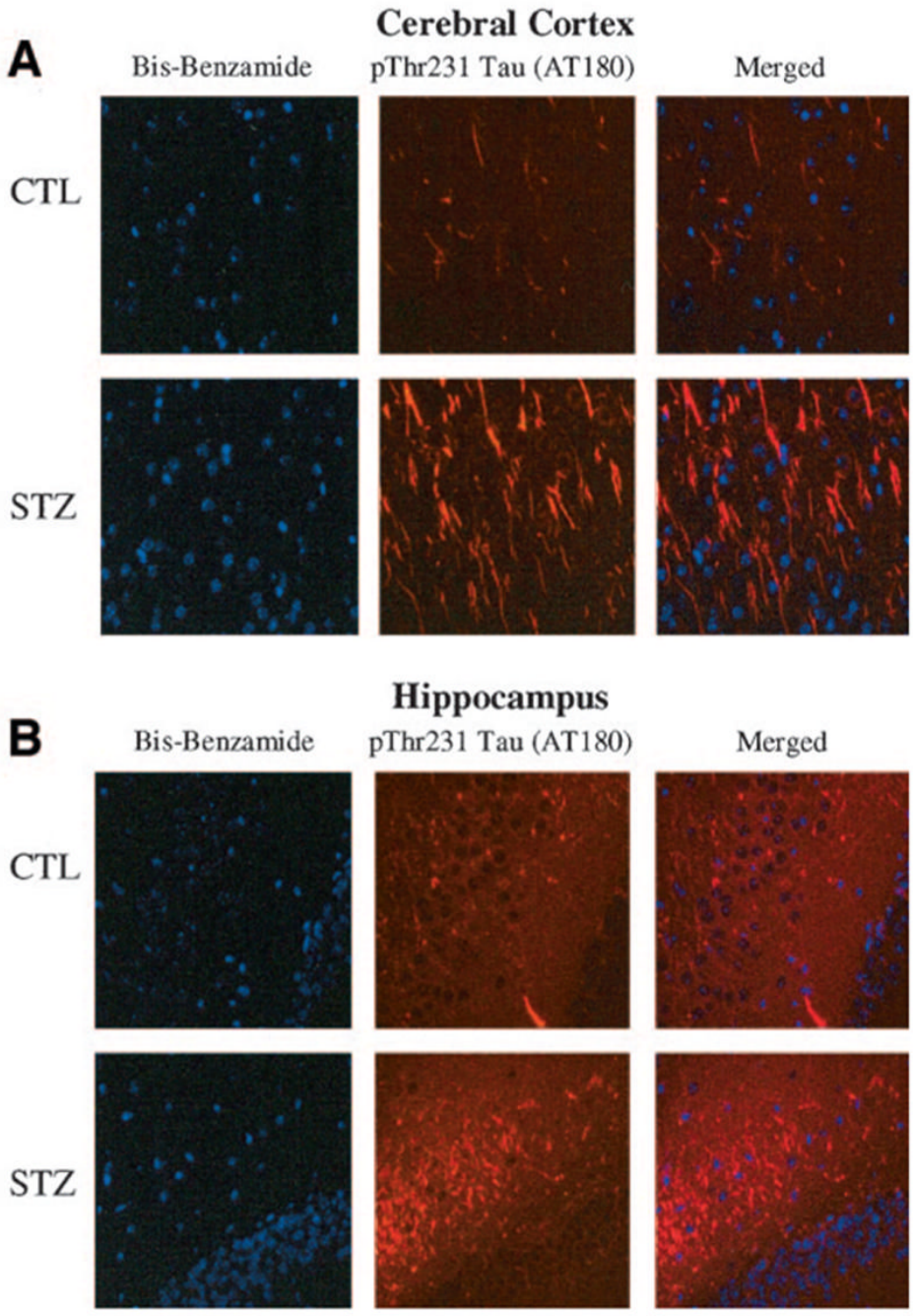

FIG. 3.

Streptozotocin treatment increased phospho–Thr231-tau immunoreactivity in mouse brain. Immunohistochemical detection of phospho–Thr231-tau (AT180) in control mouse brain showed extensive neuronal labeling in cerebral cortical (*A*) and in hippocampal neurons (*B*). Identically processed, stained, and imaged sections from streptozotocin-treated mice showed a marked increase in phospho–Thr231-tau (AT180) immunoreactivity in both of these regions. Each group consisted of four animals, and the illustrated immunohistochemical findings were consistent among all group members.

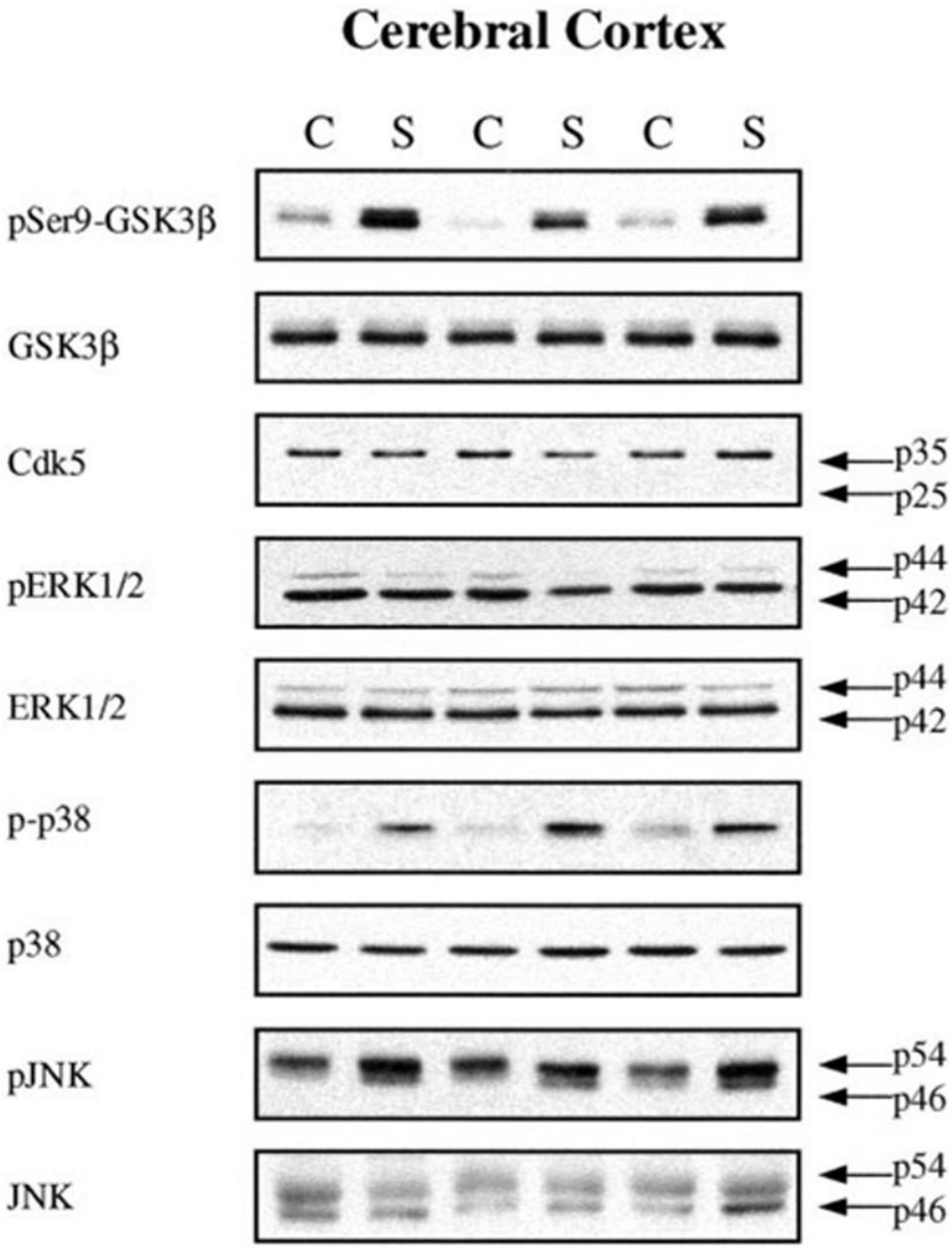

FIG. 4.

Effects of streptozotocin treatment on several kinases that can phosphorylate tau. Mice were treated with streptozotocin (S), and after 3 days, cerebral cortex protein extracts were immunoblotted for phospho–Ser9-GSK3β, total GSK3β, Cdk5 (p35 and p25 catalytic subunits), phospho–Thr202,Tyr204-ERK1/2, total ERK1/2, phospho–Thr180,Tyr182-p38, total *p*-38, phospho–Thr183,Tyr185-JNK, and total JNK. C, control; S, streptozotocin treated.

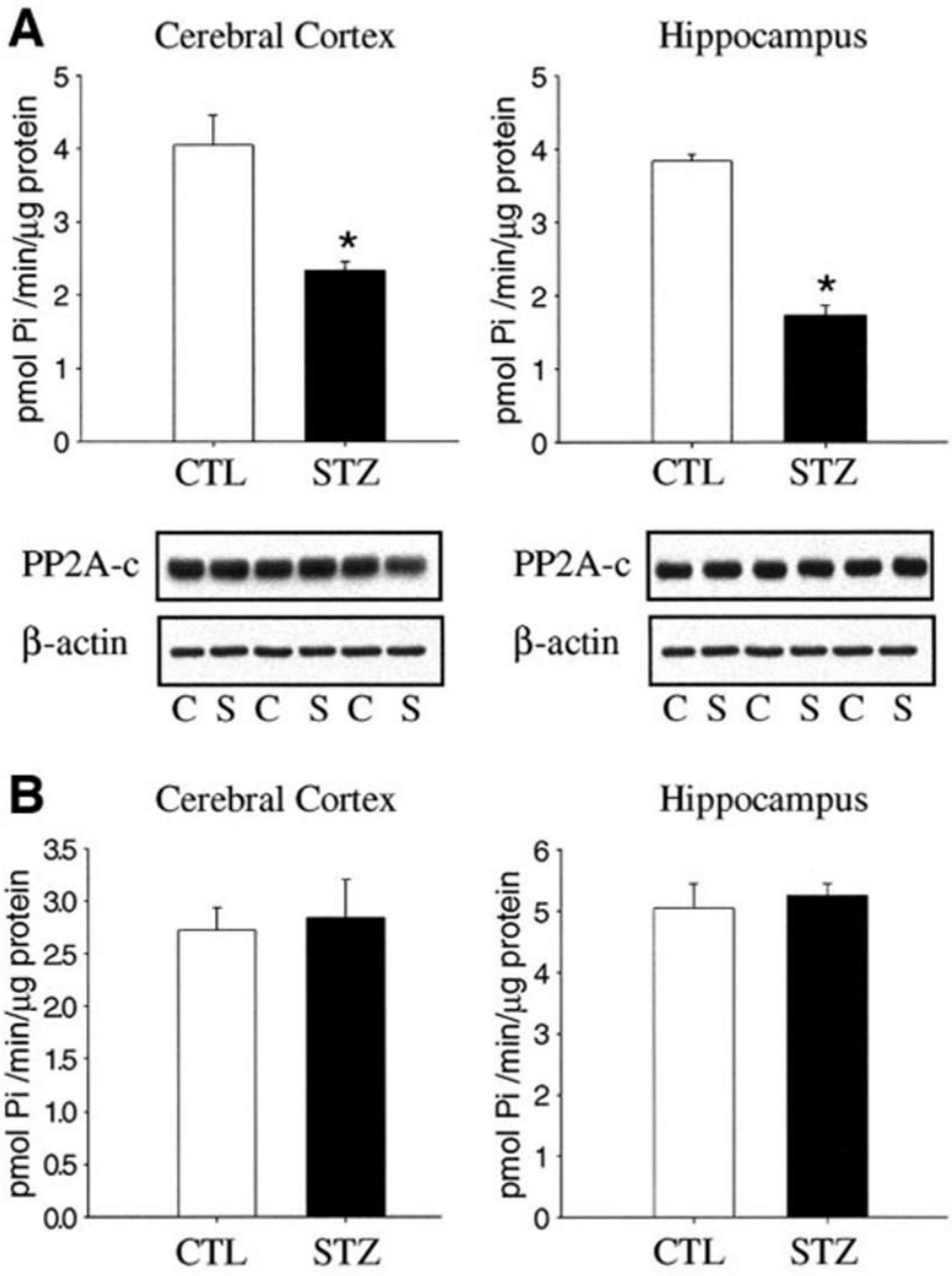

FIG. 5.

Streptozotocin treatment decreased PP2A activity. Mice were treated with streptozotocin, and after 3 days, cerebral cortex and hippocampal protein extracts were assayed for PP2A (*A*) and PP2B (*B*) activity. Immunoblot analysis of total PP2A catalytic subunit from the same control (CTL) and streptozotocin-treated (STZ) samples revealed no changes in protein levels. C, control; S, streptozotocin treated. Quantitative values are means ± SE from three mice per group. **P* < 0.05 compared with control values.

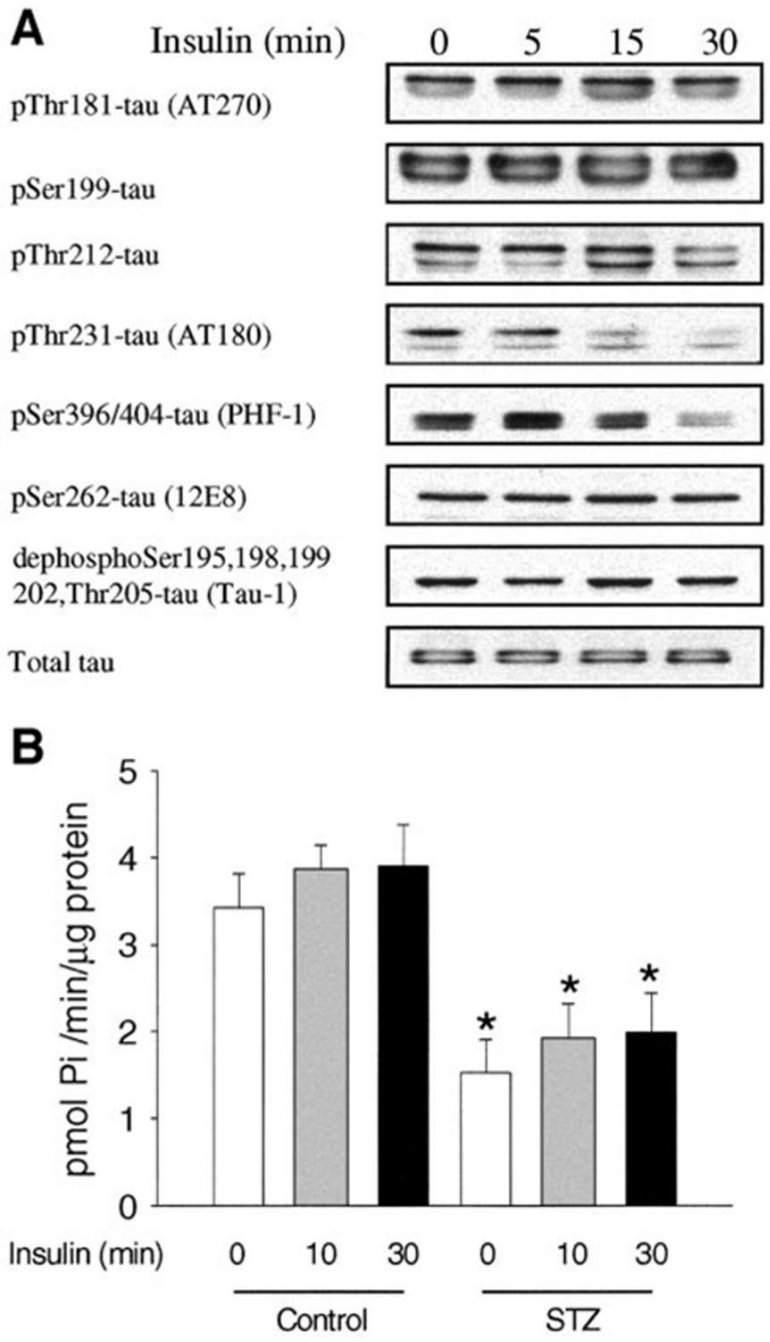

FIG. 6.

Effects of insulin administration in streptozotocin-treated mice. *A*: Mice were treated with streptozotocin, and after 3 days, insulin was administered (5 IU/kg i.p.; 0, 5, 15, and 30 min), and protein extracts of the cerebral cortex were immunoblotted for site-selective tau phosphorylation and total tau. *B*: Insulin was administered to control or streptozotocin-treated mice, and PP2A activity was measured. Quantitative values are means ± SE from four mice per group. *$P < 0.05$ compared with control values.

TABLE 1

Tau antibodies

Antibody	Type	Specificity	Phosphorylation sites	Source
AT270	Monoclonal	Phospho-tau	Thr181	Biosurce
pS199	Polyclonal	Phospho-tau	Ser199	Biosource
AT8	Monoclonal	Phospho-tau	Ser202	Innogenetics
pT212	Polyclonal	Phospho-tau	Thr212	Biosource
AT180	Monoclonal	Phospho-tau	Thr231	Pierce/Endogen
PHF-1	Monoclonal	Phospho-tau	Ser396,404	Dr. P. Davies
12E8	Monoclonal	Phospho-tau	Ser262	Dr. P. Seubert
Tau-1	Monoclonal	Dephospho-tau	Ser195,198,199,202,Thr205	Dr. L. Binder
Total tau	Polyclonal	Total tau		DAKO