Coupling of Mammalian Target of Rapamycin with Phosphoinositide 3-Kinase Signaling Pathway Regulates Protein Phosphatase 2A- and Glycogen Synthase Kinase-3β-dependent Phosphorylation of Tau* 

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Tau is an important microtubule-stabilizing protein in neurons. In its hyperphosphorylated form, Tau protein loses its ability to bind to microtubules and then accumulate and is part of pathological lesions characterizing tauopathies, e.g. Alzheimer disease. Glycogen synthase kinase-3β (GSK-3β), antagonized by protein phosphatase 2A (PP2A), regulates Tau phosphorylation at many sites. Diabetes mellitus is linked to an increased risk of developing Alzheimer disease. This could be partially caused by dysregulated GSK-3β. In a long term experiment (−16 h) using primary murine neuron cultures, we interfered in the insulin/phosphoinositide 3-kinase (PI3K) (LY294002 treatment and insulin boost) and mammalian target of rapamycin (mTor) (AICAR and rapamycin treatment) signaling pathways and examined consequent changes in the activities of PP2A, GSK-3β, and Tau phosphorylation. We found that the coupling of PI3K with mTor signaling, in conjunction with a regulatory interaction between PP2A and GSK-3β, changed activities of both enzymes always in the same direction. These balanced responses seem to ensure the steady Tau phosphorylation at sites where only one of two enzymes (in this case PP2A) is involved in the regulation.

The main physiological function of the protein Tau is to promote the assembly and stabilization of the microtubule structure in neurons. Tau is a protein with multiple phosphorylation sites. Phosphorylation is essential for its function, influencing the ability of the protein to bind microtubules and to promote polymerization. Tau protein is susceptible to hyperphosphorylation, which commutes it into an inactive form (3–8). Hyperphosphorylated Tau aggregated into paired helical filaments (PHFs), which hallmark the pathohistology of several neurodegenerative diseases, denoted as tauopathies. Of these, Alzheimer disease is the most prominent. Several kinases and phosphatases have been identified to regulate the phosphorylation of Tau.

Among the various Tau kinases glycogen synthase kinase-3β (GSK-3β) is of particular interest. GSK-3β has been shown to phosphorylate Tau at most sites found hyperphosphorylated in PHFs (9). GSK-3β is regulated by phosphorylation at Ser-9, which is the target for several kinases, including Akt (10). Activated Akt maintains GSK-3β in a Ser-9-phosphorylated, inhibited state, whereas decreases in Akt activity lead to dephosphorylation and activation of GSK-3β. This regulatory unit plays an essential role in PI3K-mediated signal transduction, like the insulin-signaling pathway.

Several prospective and retrospective epidemiological findings indicate that type II diabetes mellitus is a significant risk factor for developing Alzheimer disease (11–13). It was hypothesized that the disease-related disturbances in either the insulin-receptor binding or transduction pathway leads to overactivated GSK-3β creating hyperphosphorylated Tau protein in neurons. In vitro and in vivo studies dealing with this subject showed contradictory results (14–19). In a preliminary study we found that not only PI3K but also mTor signaling participates in the regulation of GSK-3β-mediated Tau phosphorylation. This is important insofar as mTor kinase influences the activity of the protein phosphatase 2A (PP2A) (20). PP2A is known to be the major Tau phosphatase, antagonizing the phosphorylation mediated by Tau kinases (21).

To gain more insight into this regulation, we systematically interfered either the insulin/PI3K signaling (LY294002 treatment and insulin boost) or the mTor kinase signaling (AICAR and rapamycin treatment) in cultured murine neurons. We detected sustained changes (up to 16 h) in both the specific activity of PP2A and in the phosphorylation of activity-regulating sites of Akt (Ser-473), GSK-3β (Ser-9), and mTor (Ser-2448). We found that PP2A and GSK-3β were simulta-

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2 The abbreviations used are: PHF, paired helical filaments; AICAR, 5-aminimidazole-4-carboxyamide ribonucleotide; AMPPK, AMP-activated kinase; GSK-3β, glycogen synthase kinase-3β; mTor, mammalian target of rapamycin; P70 S6, 70-kDa ribosomal protein S6 kinase; PI3K, phosphoinositide 3-kinase; PP2A, protein phosphatase 2A; Rheb, ras homologue enriched in brain; TSC, tuberous sclerosis complex; mTor, mammalian target of rapamycin; PBS, phosphate-buffered saline.

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neously activated during an inhibition of PI3K and mTor, whereas they were both repressed during a stimulation of PI3K with insulin. This equilibrium was established by the regulatory coupling of mTor with the PI3K signaling and by a dephosphorylation of GSK-3β at Ser-9 mediated by PP2A. The changes in activities of PP2A and GSK-3β seemed to be well balanced, explaining why the phosphorylation of Tau protein at GSK-3β/PP2A-dependent sites remained unchanged over a long period. The investigation of Tau at Ser-262 showed that interferences in the insulin/PI3K and mTor signaling potentially affects phosphorylation when only one of the enzymes, in this case the PP2A, is involved in the regulation.

**EXPERIMENTAL PROCEDURES**

**Primary Neuronal Cell Culture**—Neuron cultures were prepared from cortices of embryonic mice (E16, C57B6). Cells were plated (50,000 cells/cm²) in poly-d-lysine (Sigma)-coated culture flasks (BD Falcon). Cells were grown in Neurobasal medium, containing the supplement B-27, 50 µg/ml gentamycin, and 2 mM l-glutamine (all Invitrogen). Cultures were kept at 37 °C in a moist atmosphere (5% CO₂). Half of the medium was replaced by fresh medium every 3 days. For experiments 7-day-old cultures were used.

**Culture Conditions for Insulin Treatment**—Insulin is part of the B-27 supplement. The supplement contains several other components that are essential for neuronal survival and hence could not be completely omitted. To lower the insulin concentration in cultures, we used medium without the B-27 supplement for the last feeding 24 h before the experiments were started.

**Treatments of Cell Cultures**—The part of the study in which we interfered either in the insulin/PI3Kor mTor signaling pathway was designed as a long term experiment. Samples were taken after 0.5, 1, 6, and 16 h. Experiments in which we specifically inhibited the GSK-3β or PP2A were performed within 2 h, and samples were taken at regular intervals of 20 or 30 min. Substances were applied to the cells at concentrations that are listed as follows: okadaic acid (Sigma) at 20, 40, 60, and 100 nM, from a 100 mM stock solution in 0.05N HCl; lithium chloride (Sigma) at 15 mM, from a 1 M stock solution in PBS; AICAR (Calbiochem) at 1 mM, from 0.1 M stock solution in PBS; insulin (Sigma) at 200 nM, from a 200 µM stock solution in 0.05 N HCl; LY294002 (Calbiochem) at 15 µM, from a 5 mM stock solution in Me₃SO; and rapamycin (Calbiochem) at 20 ng/ml, from a ready to use stock solution.

**Sample Preparation**—Cells grown in 1 flask were washed twice with 4 ml of cold (4 °C) PBS and subsequently scraped into 300 µl of 4 °C cold extraction buffer: 30 mM Tris/ HCl, pH 7.4, protease/inhibitor mixture (Roche Applied Science), 0.5% (v/v) Triton X-100 (Sigma), 1 mM activated sodium orthovana- date (Sigma), 20 mM sodium fluoride (Sigma), and 100 mM okadaic acid (Sigma) from a 100 µM stock solution in Me₃SO. After transferring into chilled Eppendorf tubes, neurons were disintegrated with 20 pulses (100 watts, intervals of 0.5-s pulses and 0.5-s pause), using a UP100H sonifier equipped with a MS1 sonotrode (Dr. Hielscher) on ice. Extracts were centrifuged for 10 min, 10,000 × g (Eppendorf centrifuge 5804 R), at 4 °C. From the supernatant an aliquot was taken for the protein quantification (BCA, Amersham Biosciences). The remaining cell extract was supplemented with SDS sample buffer, heated at 60 °C for 5 min, and centrifuged (10,000 × g, 5 min, room temperature). The supernatants were stored at −20 °C until further use.

**SDS-PAGE/Immunoblotting**—Equal amounts of protein from control and treated cells were separated on 10 and 6% (v/v) SDS-PAGE and blotted semi-dry (Bio-Rad, minigel and transfer unit) on polyvinylidene difluoride membranes (Milli- pore). After blocking for 1 h in 10% (w/v) skimmed milk (Merck) in TBST (TBS containing 0.1% (v/v) Tween 20), the antibodies, diluted in TBST containing 3% (w/v) dried skimmed milk, were applied and incubated overnight at 4 °C. The concentrations of the individual antibodies are listed below. After washings with TBST, horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Vector Laboratories), diluted 1:4000 in TBST, was incubated for 1 h at room temperature. After washings (TBST), bands were visualized by chemiluminescence (ECLPlus detection kit, Amersham Biosciences). Hyperfilms (Amersham Biosciences) were used for the detection. The films were scanned with an Epson 1680 professional scanner. The optical densities and the areas of bands were measured using the NIH-image software. Calculation of volumes (area × density) and statistics were performed in EXCEL (Microsoft).

**Determination of Total Tau Protein**—For measuring total Tau protein, protein blots were treated with alkaline phosphatase followed by probing Tau with Tau-1 antibody for dephosphorylated sites Ser-199/Ser-202. The efficiency of dephosphorylation was tested with the AT8 antibody. The procedure described below allows a reliable measurement of total Tau protein. After blocking, blots were incubated with alkaline phosphatase (Sigma) diluted to 20 units/ml in alkaline reaction buffer (50 mM Tris, pH 9.5, 10 mM MgCl₂) for 30 min at 37 °C. After three washings with TBST, the antibody Tau-1 (Roche Applied Science) was applied, diluted 1:3000 in TBST, 3% (w/v) dried skimmed milk, and incubated overnight at 4 °C. Treatments with secondary antibody, ECL-based detection, and volumetric measurements were performed as described above.

**Antibodies Used for Analysis**—For Tau analysis, AT8 (Innogenetics) recognizing phosphorylation at Ser-202/Thr-205 was diluted 1:2000. For Tau-1 recognizing dephosphorylation at Ser-199/Ser-202 (Roche Applied Science) was diluted 1:3000. PHF1 (generous gift from Peter Davies) specific for phosphorylated Ser-396/Ser-404 was diluted 1:2000. 12E8 (Athena Neurosciences) binding at phosphorylated Ser-262 was diluted 1:2000. For signal transduction, all antibodies were purchased from Cell Signaling Technology and were diluted 1:1000 prior to use. Phosphorylation-independent antibodies for measuring total protein were as follows: Akt (9272), GSK-3β (9315), and mTor (2972). Phosphorylation-dependent antibodies specific for activity-regulating sites were as follows: phospho-Akt Ser-473 (9271), phospho-GSK-3β Ser-9 (9336), and phospho-mTOR Ser-2448 (2971).

**Phosphatase 2A Activity**—We used a phosphatase activity assay from Upstate for measuring the PP2A activity in cell extracts. The procedures of sample preparation, immune precipitation, and substrate incubation were performed according to the manufacturer’s protocol. The color development of liberated phosphate was performed in 96-well plastic-plates (Fal-
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FIGURE 1. Immunoblots of Tau phosphorylation after inhibition of PP2A (okadaic acid; 100 nm, 1 h) and GSK-3β (lithium chloride; 15 mM, 2 h), $n = 3$, for each treatment. Mean values ± S.D. of band volumes (optical density $(OD) \times$ area) were determined and depicted in percent (mean values of control set to 100%). Loaded protein is 1 μg/lane. Phosphorylation of Tau-1 (Ser-199/202), AT8 (Ser-202/Thr-205) and PHF1 (Ser-396/Ser-404) epitopes depend on GSK3β and PP2A. All p values are $< 0.005$ for Tau-1, AT8, and PHF1 after treatments with LiCl, and okadaic acid; whereas the phosphorylation of the 12E8 epitope (Ser-262) is independent from GSK3β (p value okadaic acid, $p < 0.005$; LiCl, $p = 0.43$).

Statistics—Mean values, analysis of variance, and significance (Student’s $t$ test) were calculated in EXCEL (Microsoft).

RESULTS

Significance of GSK-3β and PP2A for the Phosphorylation of Tau Epitopes—To test the involvement of the PP2A and the GSK-3β on the Tau phosphorylation at certain phosphorylation sites, we treated cell cultures for 1 h with okadaic acid (100 nm), an inhibitor of the PP2A, and in a second experiment with lithium chloride (15 mM), an inhibitor of the GSK-3β. Phosphorylation at the epitopes recognized by the antibodies AT8, PHF1, and Tau-1 showed strong dependence on both the PP2A and the GSK-3β activity (Fig. 1). The inhibition of PP2A led to a strong increase of PHF1 and AT8-positive Tau, whereas the Tau fraction positive for Tau-1 antibody nearly vanished after 1 h. In this context we can confirm data published by Lovestone et al. (22), showing inverse binding of Tau-1 and AT8 antibodies. Both antibodies share the phosphorylation site Ser-202 recognized by AT8 in its phosphorylated form and by Tau-1 only in its dephosphorylated form. Inhibition of the GSK-3β with lithium chloride led to a massive decline of phosphorylated Tau at AT8 and PHF1 epitope accompanied by the increase of Tau protein positive for Tau-1 antibody (Fig. 1).

Inhibition of the PI3K with LY294002—For effects on total protein levels, the administration of the PI3K inhibitor reduced the protein levels of total GSK and Tau protein after an incubation period of 16 h (Fig. 2A). The protein load of the respective protein samples had to be adjusted for the subsequent determination of the phosphorylated form of the proteins. The levels of total Akt and mTor were not affected (Fig. 2A).

Effects on Phosphorylated Protein and PP2A Activity—As expected the inhibition of the PI3K with LY294002 evoked a rapid and strong decline in the phosphorylation of Akt at Ser-473 and GSK at Ser-9 (Fig. 3A). This reduction persisted during the whole period of observation. From the state of GSK-3β phosphorylation, we predicted an increase in the phosphorylation of Tau protein at GSK-3β relevant epitopes Ser-199/Ser-202, Ser-202/Thr-205, and Ser-396/Ser-404. Surprisingly, the phosphorylation at these epitopes was not elevated; on the contrary, the phosphorylation values fell significantly below those of the control after 16 h (Fig. 4A), concomitant with the reduced levels of total GSK protein (Fig. 2A). The antagonist of GSK-3β in Tau phosphorylation is the phosphatase PP2A. The activity of this enzyme is negatively regulated by mTor raptor. We determined the activity of mTor by analyzing its phosphorylation at Ser-2448. We detected a rapid and strong decline in the phosphorylation of this site, persisting over the entire observation period (Fig. 3A). Additionally, we measured the PP2A activity in extracts of neurons treated for 1 and 16 h with LY294002. In both extracts the specific activity of the PP2A was strongly increased over control extracts (Fig. 5A). Similar results were obtained with wortmannin (data not shown).

Effects of the Insulin Stimulus—As already mentioned under “Experimental Procedures,” we could not cultivate the neurons without insulin, which is part of the supplement B-27. But the strategy to omit B-27 in the last medium used for feeding 1 day before the experiments with insulin started ensured that the neurons responded transiently to the boost of insulin.

Effects on Total Protein Levels—The levels of the proteins examined in this study were unaffected by the administration of insulin (Fig. 2B).

Effects on the Phosphorylation of Proteins and PP2A Activity—The phosphorylation of Akt at Ser-473 and GSK-3β at Ser-9 was significantly elevated in extracts obtained from insulin-treated neurons. This increase was transient and vanished during prolonged incubation (16 h) (Fig. 3B). On the basis of these data we expected a reduced phosphorylation of the Tau protein at GSK-3β-dependent epitopes, at least for the first three points of measurement. But the phosphorylation at the Tau-1, AT8, and PHF1 epitopes was largely stable (Fig. 4B). Already the experiments with blocked PI3K showed that the phosphorylation of mTor at Ser-2448 is influenced by Akt. We detected a significant increase in phosphorylation of mTor for a period that was also marked by an elevated phosphorylation of Akt (0.5, 1, and 6 h) (Fig. 3B). The measurement of the PP2A activity at two points (1 and 6 h) revealed that the specific activity of this phosphatase was significantly reduced in extracts from insulin-treated neurons (Fig. 5B).

Activation of the AMPK with AICAR—We used AICAR for the stimulation of the neuronal AMPK; alternatively Metformin (2.5 mM) was used which elicited similar effects (data not shown).

Effects on Total Protein Levels—A prolonged administration of the AMPK activator (16 h) reduced the protein levels of total
GSK and Tau protein (Fig. 2C). The reduction in Tau protein was not significant, whereas the decrease in total GSK was. However, the protein load for the subsequent determination of phosphorylated protein was adapted not only for GSK-3β but also for Tau protein, to compensate for the reduced protein concentrations. The levels of total Akt and mTor were not affected (Fig. 2C).

Effects on the Phosphorylation of Proteins and PP2A Activity—

It is described that activation of AMPK stimulates the activity of Akt, indicated by an increased phosphorylation at Ser-473. Extracts of AICAR-treated neurons also showed significant increases in Akt phosphorylation during the whole period of surveillance (Fig. 3C). As a consequence of the activated Akt, we expected an increase of GSK-3β phosphorylation at Ser-9. Surprisingly, the phosphorylation of GSK-3β was significantly reduced at any point (Fig. 3C). The phosphorylation of Tau protein at the GSK3-β-dependent sites was stable after incubations that lasted for 0.5, 1, and 6 h. After 16 h we noticed a significant reduction (Fig. 4C), an effect that was also observed during the inhibition of PI3K (see above) and

FIGURE 2. Changes in the specific concentrations of proteins. GSK-3β, Akt, mTor, and Tau during treatments with LY294002 (15 μM) (A), insulin (200 nM boost) (B), and AICAR (1 mM) for 0.5, 1, 6, and 16 h; n = 3, for each treatment and time point (C). The means ± S.D. of band volumes (OD × area) were calculated and depicted in percent (mean value of controls set to 100%). Loaded protein is 1 μg/lane. A, LY294002 induced a significant decline of total GSK and Tau after 16 h (p < 0.005 for GSK and <0.05 for Tau). B, insulin treatment did not significantly affect specific amounts of protein. C, treatment with AICAR significantly reduced the amount of total GSK after 16 h (p < 0.005).
that was also accompanied with reduced amounts of total GSK3-β. The AMPK phosphorylates TSC2 and thereby inactivates the mTor. We investigated the phosphorylation of the activity-regulating site Ser-2448. We detected reduced phosphorylation of this site after 1 h, an effect that lasted for at least 16 h (Fig. 3C). The dephosphorylation at Ser-2448 indicates inhibited mTor. This should have consequences for the activity of the PP2A. The cell extracts obtained from cultures treated for 1 and 16 h with AICAR expressed increased specific PP2A activities that were elevated nearly 3-fold over the control (Fig. 5C). The concomitant elevations of both PP2A and GSK activities probably could explain the stable Tau phosphorylation.

Influence of PP2A on Akt and GSK-3β Phosphorylation—The observation that the phosphorylation of GSK-3β at Ser-9 was...
reduced during AICAR treatment needed to be explained. To test a possible cross-talk between PP2A and GSK-3β, we treated neurons with okadaic acid, which is an inhibitor of the PP2A. The inhibition of PP2A increased the phosphorylation of GSK-3β at Ser-9. This effect depended on the concentration of the inhibitor. This was tested in a range described to be suitable for a specific PP2A inhibition (20–100 nM) (Fig. 6). Using the same extracts, we investigated the phosphorylation of Akt at Ser-473. Unlike the case of the GSK-3β, here we could not detect any changes in the phosphorylation of this kinase (Fig. 6).

FIGURE 4. Changes in Tau phosphorylation at the epitopes. Tau-1 (Ser-199/202), AT8 (Ser-202/Thr-205), PHF1 (Ser-396/Ser-404), and 12E8 (Ser-262) during treatments with LY294002 (15 μM) (A), insulin (200 nm boost) (B), and AICAR (1 mM) for 0.5, 1, 6 and 16 h; n = 3, for each treatment and time point (C). The means ± S.D. of band volumes (OD × area) were calculated and depicted in percent (mean value of controls set to 100%). Protein load is 1 μg/lane. Exception, the 16-h values after LY294002 and AICAR treatment were adjusted to balance the specific loss in total protein contents detected after 16 h (Fig. 2). Note that phosphorylation at GSK-3β/PP2A-dependent sites (Tau-1, AT8, and PHF1) were largely stable during the first 6 h. AICAR and LY294002 treatment induced significant dephosphorylation after 16 h (p values, <0.05). The 12E8 epitope was significantly dephosphorylated during treatments with AICAR (p values for 1 h, <0.001; 6 h, <0.05; 16 h, <0.005) and LY294002 treatments (p values for 0.5 h, <0.001; 1 h, <0.01; 6 h, <0.05; 16 h, <0.005). Treatments with insulin significantly increased phosphorylation at Ser-262 during the first 6 h (p values for 0.5 h, <0.005; 1 h, <0.01; 6 h, <0.05).
Inhibition of mTor Raptor with Rapamycin—To prove that the effects elucidated by AICAR resulted from an inhibition of mTor raptor, we inhibited mTor raptor with rapamycin. We determined Akt, GSK phosphorylation, and PP2A activity after an incubation with 20 nM rapamycin for 1 h.

Effects on the Phosphorylation of Proteins and PP2A Activity—The Akt phosphorylation at Ser-473 was significantly elevated (Fig. 7A), whereas the GSK phosphorylation at Ser-9 was significantly reduced (Fig. 7B). Concentrations of total protein were not affected (Fig. 7, A and B). The PP2A activity was significantly increased (>3-fold over control) (Fig. 7C).

LY294002 (15 μM) on the specific activity of PP2A. Protein extracts from neuronal cell cultures were analyzed and treated for 1 and 16 h with the inhibitor or with solvent, respectively. Note that LY294002 strongly increased the specific PP2A activity (>4-fold), even after prolonged treatments (p values for 1 h, <0.0005; 16 h, <0.0005). B, effect of insulin boosts (200 nM) on the specific activity of PP2A. Protein extracts from neuronal cell cultures were analyzed, treated for 1 and 6 h with insulin, or with the solvent serving as control. Note that insulin significantly decreased the specific PP2A-activity (<0.5-fold) measured at these points, (p values for 1 h, <0.05; 6 h, <0.05). C, influence of AICAR treatments (1 mM) on the specific activity of PP2A. Protein extracts from neuronal cell cultures were analyzed, treated for 1 and 16 h with the AMPK activator, or with the solvent PBS. Note that AICAR induced strong increases in the specific PP2A activity (<3-fold) at the two points (p values for 1 h, <0.001; 16 h, <0.005).

Inhibition of mTor Raptor with Rapamycin—To prove that the effects elucidated by AICAR resulted from an inhibition of mTor raptor, we inhibited mTor raptor with rapamycin. We determined Akt, GSK phosphorylation, and PP2A activity after an incubation with 20 nM rapamycin for 1 h.

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Effects on Tau Phosphorylation at the GSK-3β-independent Site Ser-262—To verify the assumption that a coordinated activity of PP2A and GSK-3β prevented net changes in Tau phosphorylation at corresponding sites, we looked for a phosphorylation site that was independent either from PP2A or GSK-3β. We investigated several sites with phosphospecific antibodies (AT100, AT180, AT270, and 12E8). From the sites tested, only the phosphorylation at Ser-262, recognized by 12E8, fully met these requirements. 12E8-positive Tau strongly increased after treatments with okadaic acid but remained unchanged after an incubation with lithium chloride (Fig. 1), indicating that the phosphorylation at Ser-262 is regulated by PP2A but not by GSK-3β. We probed the protein samples with 12E8. We found that both AICAR and LY294002 treatments induced a decline in the phosphorylation (−16 h), whereas insulin evoked a transient increase (−6 h) in the phosphorylation of this site (Fig. 4, A–C). It is important to emphasize that the phosphorylation patterns are congruent with the activity of the PP2A under such conditions (see above).

DISCUSSION

As already mentioned, several studies analyzing the effect of insulin on Tau phosphorylation showed conflicting results. The application of insulin to SH-SY5Y neuroblastoma cells (15, 17) and primary cortical neuron cultures of rat (16) increased the phosphorylation of Tau at GSK3-β-dependent sites, whereas under comparable conditions the phosphorylation of Tau was reduced in NT2 cells, associated with the inhibition of GSK-3β (14). Also the results coming from in vivo studies with mice are not unequivocal. Both hyperinsulinemia (18) and streptozotocin-induced insulin deficiency (19) increased the phosphorylation of Tau protein in brains.

Our results differ from those cited above. The data show that long term interferences in the insulin/PI3K signaling cascade do not necessarily alter phosphorylation of Tau at GSK-3β/PP2A-dependent sites. We analyzed the phosphorylation sites for which the specific involvement of GSK-3β and PP2A is well documented (23–28), and the data from our experiments performed with lithium chloride and okadaic acid confirm these findings. Our data indicate that the activities of the kinase and phosphatase are tightly coupled and that interferences in the signaling lead to coordinated changes in the activity of the GSK-3β and PP2A in the same direction. Both enzymes were simultaneously activated during an inhibition of PI3K with LY294002 and repressed during a stimulation of PI3K with insulin. We conclude that the induced changes in activities of both enzymes cancelled each other so that the Tau phosphorylation at GSK/PP2A-dependent sites was largely stable.

On the other hand, the investigation of Tau at Ser-262 (epitope for 12E8) showed that the phosphorylation is indeed affected in cases when only one of the enzymes, in this case the PP2A, is involved in the regulation. It was evident that treatments induced changes in the phosphorylation which matched the actual PP2A activity. The activity of the participating kinase, presumably protein kinase A (29), was not determined in this study. However, the postulated coordinated regulation of PP2A and GSK-3β implies an efficient signal transduction between the activity regulating PI3K/insulin and mTor pathways. This is discussed below and illustrated in Fig. 8.

Interference in the PI3K/Insulin Pathway and Effects on Tau Phosphorylation—GSK-3β and PP2A were simultaneously activated during an inhibition of PI3K with LY294002 and repressed during a stimulation of PI3K with insulin. Under such conditions the activity of Akt kinase seems to be of particular importance. This kinase not only phosphorylates and inhibits
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GSK-3β but also seems to influence the activity of PP2A. It is described that Akt phosphorylates TSC2 and decreases GTPase-activating function of the TSC1-TSC2 complex (30). As a consequence GTP charging of the Ras homologue enriched in brain (Rheb) is enhanced (31), providing the major mechanism for the acute activation of the mTOR complex. The regulatory mechanism is not fully understood. However, the phosphorylation of mTor at Ser-2448 seems to be essential for its activation (32). We observed reduced phosphorylation of mTor at Ser-2448 when Akt was inhibited (PI3K inhibition) and increased phosphorylation when Akt was activated (insulin boost). There is evidence that mTor regulates the activity of PP2A and the type 2A-related phosphatase STT4 in yeast. Several studies have shown that the inhibition of the kinase with rapamycin activates the PP2A (33–35). Moreover, it has been shown that mTor restrains the activity of PP2A when the kinase is activated via the insulin pathway (34). Our observations that the activity of PP2A changes according to the concentration of putative active mTor and that the selective inhibition of mTor raptor with rapamycin-activated PP2A indicate that this type of regulation also occurs in neurons. Questions remain about the mechanism by which mTor controls the PP2A activity. The regulation may involve the direct phosphorylation of PP2A by mTor (33). Alternatively, the kinase may phosphorylate α4, a mammalian homologue of yeast Tap42, which then forms a complex with the catalytic subunit of PP2A (36, 37) and regulates the phosphatase activity (38).

We observed dephosphorylation of Tau at GSK-3β/PP2A-dependent sites after prolonged inhibition (16 h) of PI3K (mTor), which was accompanied by a loss of total GSK-3β in the protein extracts. It is known that mTor together with P70 S6 kinase regulate initiation and elongation stages of translation. This is established for example by the phosphorylation of E4-binding proteins and eEF2 kinase (39). We think that this translational effect unbalanced the system. The specific activity of the PP2A was still at a high level, whereas the GSK protein, although activated, was reduced in quantity.

Interference in the mTor Pathway and Effects on Tau Phosphorylation—In the second part of the experiment, in which we inhibited mTor with AICAR and rapamycin, the phosphorylation of Tau remained unchanged at GSK-3β/PP2A-regulated sites until the concentration of total GSK-3β decreased (probably caused by translational effects described above).

The observation that GSK-3β and PP2A were both simultaneously activated under such conditions may deliver the explanation for the largely stable phosphorylation of Tau (<16 h) at corresponding sites. AICAR stimulates the AMPK (40–42). The AMPK phosphorylates TSC2 at sites that increase GTPase activity of the TSC1/2 complex (43), thereby decreasing fractional Rheb-GTP charging, providing the major mechanism for the acute inhibition of mTOR signaling by ATP depletion. AICAR treatment reduced the phosphorylation of mTOR at Ser-2448, indicating that mTor was less active under such conditions. This in return may cause the concomitant increase in PP2A activity by the mechanism described above. In addition, reduced mTor/P70 S6 kinase signaling results in less activated P70 S6 kinase and may thereby cause the observed increase in Akt phosphorylation at Ser-473. Such a regulation of Akt by mTor via P70 S6 kinase is well documented in other cells (44–46). It is based on a P70 S6 kinase-mediated phosphorylation of insulin receptor substrate 1, which regulates the proteasome-mediated degradation of the substrate (47, 48). In contrast to Akt at Ser-473, the phosphorylation of GSK-3β at Ser-9 was reduced. A similar effect of AICAR on GSK phosphorylation has been described by King et al. (49). However, this was surprising insofar as we expected an Akt-mediated increase in phosphorylation at this site. We found that PP2A dephosphorylates GSK at Ser-9, whereas Akt phosphorylation at Ser-473 was not affected by the phosphatase. From the observation that AICAR induced a significant increase in PP2A activity, we conclude that PP2A acted antagonistically to Akt in GSK-3β phosphorylation at Ser-9, resulting in a net decrease in phosphorylation. This could represent an additional mechanism for the regulation of Tau phosphorylation at PP2A/GSK-3β-dependent sites. PP2A has been reported to dephosphorylate GSK-3β at Ser-9 in muscle cells and recently also in human neuroblastoma cells (50, 51). However, there is also good indication that phosphorylated Ser-473 of Akt is a substrate for PP2A in cells. This discrepancy may be explained by the observation that the specific composition of PP2A varies in different cell types and during development, which potentially influences its substrate specificity (52–56).

The results obtained with AICAR were verified by treatment of the neurons with rapamycin. Complexed with the immunophilin FKBP12, rapamycin inhibits the activity of mTor complex 1 (raptor) (57, 58). Also under such a condition, Akt phosphorylation at Ser-473 was increased, whereas phosphorylation of GSK-3β at Ser-9 was reduced, concomitant with a marked increase in the activity of PP2A. The stimulations of PP2A by rapamycin and AICAR were in a similar range. These results indicate that AICAR-induced changes in the GSK-3β, Akt phosphorylation and PP2A activity are primarily caused by an inhibition of the mTor raptor kinase.

Taken together, a coordinated regulation of PP2A and GSK-3β seems to ensure balanced Tau phosphorylation at corresponding sites. This may help in preventing severe changes in Tau phosphorylation under conditions when neurons undergo transient fluctuations either in insulin or nutrient supply. The results do not support the idea that diabetes type II-related disturbances in insulin/PI3K signaling pathway directly lead to hyperphosphorylation of Tau at GSK-3β-dependent sites. It was shown by Sengupta et al. (59) that only a sequential interference in the activities of multiple kinases induced Alzheimer-like hyperphosphorylation of Tau. Also secondary effects, like diabetes-associated hypothermia, may promote hyperphosphorylation (60). Finally, according to the cascade hypothesis, β-amyloid is an essential factor for the formation of tangles. β-Amyloid competes with insulin as a substrate for the insulin-degrading enzyme. Diabetes type II-associated hyperinsulinemia might therefore reduce β-amyloid degradation and promote plaque formation with downstream consequences for the neuronal cytoskeleton.
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