Hyperphosphorylation and Aggregation of Tau in Experimental Autoimmune Encephalomyelitis*

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Axonal damage is a major morphological correlate and cause of permanent neurological deficits in patients with multiple sclerosis (MS), a multifocal, inflammatory and demyelinating disease of the central nervous system. Hyperphosphorylation and pathological aggregation of microtubule-associated protein tau is a common feature of many neurodegenerative diseases with axonal degeneration including Alzheimer’s disease. We have therefore analyzed tau phosphorylation, solubility and distribution in the brainstem of rats with experimental autoimmune encephalomyelitis (EAE), an animal model of MS. Tau was hyperphosphorylated at several sites also phosphorylated in Alzheimer’s disease and became partially detergent-insoluble in EAE brains. Morphological examination demonstrated accumulation of amorphous deposits of abnormally phosphorylated tau in the cell body and axons of neurons within demyelinating plaques. Hyperphosphorylation of tau was accompanied by up-regulation of p25, an activator of cyclin-dependent kinase 5. Phosphorylation of tau, activation of cdk5, and axonal pathology were significantly reduced when diseased rats were treated with prednisolone, a standard therapy of acute relapses in MS. The underlying molecular mechanisms of transport impairment and axonal degeneration in MS are so far elusive. Axonal degeneration is also found in neurodegenerative diseases (i.e. Alzheimer’s disease, progressive supranuclear palsy, frontotemporal dementia linked to parkinsonism), which are characterized by pathological hyperphosphorylation and assembly of microtubule-associated protein tau into paired helical filaments (11, 12). The physiological function of tau is to bind to and stabilize microtubules in a phosphorylation-dependent way (11). In addition, tau is involved in regulation of anterograde axonal transport by influencing the attachment/detachment rate of molecular motors along microtubules (13). Pathological hyperphosphorylation of tau as seen in Alzheimer’s disease causes detachment of tau from microtubules that might lead to microtubule breakdown and disruption of axonal transport (loss of function). An imbalance of kinases and phosphatases has been proposed to contribute to the pathogenesis of diseases with paired helical filaments (14). A common hypothesis holds that tau hyperphosphorylation and subsequent detachment increases the pool of unbound tau beyond a critical concentration, thereby initiating its aggregation into paired helical filaments (gain of toxic function) (11, 12, 15, 16).

Multiple sclerosis (MS) is an inflammatory disease that leads to the destruction of myelin in the central nervous system and spheroids (6–9). Histopathological studies have shown that axonal damage in MS is associated with axonal accumulation of amyloid precursor protein (APP), which is transported in a kinesin-dependent fashion, indicating impairment of axonal transport (6). Because neurons are highly elongated cells, their function depends on efficient transport of proteins and organelles toward synapses. A disturbance in axonal transport would therefore cause energy depletion at synapses, eventually leading to complete transsection and degeneration of axons in MS. The underlying molecular mechanisms of transport impairment and axonal degeneration in MS are so far elusive.

Axonal degeneration is also found in neurodegenerative diseases (i.e. Alzheimer’s disease, progressive supranuclear palsy, frontotemporal dementia linked to parkinsonism), which are characterized by pathological hyperphosphorylation and assembly of microtubule-associated protein tau into paired helical filaments (11, 12). The physiological function of tau is to bind to and stabilize microtubules in a phosphorylation-dependent way (11). In addition, tau is involved in regulation of anterograde axonal transport by influencing the attachment/detachment rate of molecular motors along microtubules (13). Pathological hyperphosphorylation of tau as seen in Alzheimer’s disease causes detachment of tau from microtubules that might lead to microtubule breakdown and disruption of axonal transport (loss of function). An imbalance of kinases and phosphatases has been proposed to contribute to the pathogenesis of diseases with paired helical filaments (14). A common hypothesis holds that tau hyperphosphorylation and subsequent detachment increases the pool of unbound tau beyond a critical concentration, thereby initiating its aggregation into paired helical filaments (gain of toxic function) (11, 12, 15, 16).

MARK, microtubule-affinity regulating kinase; MAPK, mitogen-activated protein kinase; MOG, myelin-oligodendrocyte-glycoprotein; PP2A, protein phosphatase 2A; SP/TP motif, serin-proline/threonin-proline; CNP, 2’,3’-cyclic nucleotide 3’-phosphodiesterase.

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1 The abbreviations used are: MS, multiple sclerosis; APP, amyloid precursor protein; cdk5, cyclin-dependent kinase 5; EAE, experimental autoimmune encephalomyelitis; GS K-β, glycogen synthase kinase 3β;
In light of the conspicuous axonal abnormalities in MS, we wondered whether tau abnormalities contribute to neuronal dysfunction and degeneration in experimental autoimmune encephalomyelitis (EAE), an animal model of MS. Myelin-oligodendrocyte-glycoprotein (MOG)-induced EAE in rats resembles many characteristic features of MS including multifocal inflammation, demyelination, and axonal loss. Therefore, we characterized tau phosphorylation, solubility, and distribution in rats with acute brainstem EAE.

**EXPERIMENTAL PROCEDURES**

**Induction of EAE—**EAE was induced in female LEW.1N rats by intradermal injection of 50 μg MOG in saline emulsified (1:1) with complete Freund’s adjuvant (Sigma) containing 200 μg of Mycobacterium tuberculosis (strain H 37 RA; Difco Laboratories, Detroit, MI). Control rats were injected with complete Freund’s adjuvant alone. Rats were sacrificed 12–13 days after sensitization. The experiments were approved by the regional ethics board.

**Treatment of Animals—**Rats were treated intraperitoneally with prednisolone (20 mg/kg) starting on day 8 after sensitization as described (17). 8-Week-old C57Bl6 mice (n = 5) were fed with 0.2% (w/w) cuprizone (bis-cyclohexanone oxalidihydrazone) (Sigma) in ground breeder chow for 5 weeks. Subsequently, brains were snap-frozen in liquid nitrogen for further biochemical analysis (see below). Brains from age-matched animals (n = 5) maintained on a normal diet served as controls.

**Western Blotting—**Brainstems of LEW.1N rats were dissected, snap-frozen in liquid nitrogen, and weighed. Tissue was homogenized in ice-cold lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 20 mM NaF, 1 mM Na3VO4, 2 mM EGTA, 0.5% Triton X-100, and 0.1% SDS) and protease inhibitor mixture (Complete, Roche Diagnostics). The homogenates were centrifuged and the protein content in the supernatant was determined. The supernatants were subjected to immunoblotting analysis. As a phosphorylation-independent monoclonal antibody Tau-5 (BD Pharmingen) was used. Monoclonal antibodies directed against phosphorylated tau epitopes were AT-8, AT-10, AT-180 (Innogenetics, Gent, Belgium), 12E8 (P. Seubert, Elan Pharmaceuticals, South San Francisco, CA), TG-3 and PHF-1 (kindly provided by P. Davies, New York). The rabbit polyclonal anti-phospho-MAPK, rabbit polyclonal anti-phospho-independent-MAPK, monoclonal anti-phospho-GSK-3β, and polyclonal phosphorylation-independent GSK-3β were from New England Biolabs (Beverly, MA). Rabbit anti-p35 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Immunoblots were measured using Scion Image software. Statistical differences were determined with Student’s t-test.

**Isolation of Insoluble Tau—**Brainstems were homogenized in lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 20 mM NaF, 1 mM Na3VO4, 2 mM EGTA, 0.5% Triton X-100, and 0.1% SDS) and protease inhibitor mixture and centrifuged twice at 10,000 × g for 35 min. The resulting supernatant was removed and recentrifuged at 100,000 × g for 30 min. The resulting pellet was re-extracted with 70% formic acid to recover the insoluble material. For isolation of crude paired helical filaments (18) brainstems were homogenized in a buffer containing 10 mM Tris-HCl, 0.8 mM NaCl, 1 mM EGTA, 10% sucrose and protease inhibitors, and centrifuged at 27,000 × g for 20 min. The pellet was washed once. Both supernatants were combined, adjusted to 1% Sarkosyl, and incubated for 1 h at 37 °C, followed by centrifugation at 100,000 × g for 35 min. The resulting pellet containing crude insoluble tau was resuspended in 8 M urea for 30 min. The resulting insoluble pellet was collected by centrifugation and redissolved in 8 M urea.

**Immunohistochemistry—**Immunohistochemistry was performed on adjacent serial sections of paraffin-embedded brainstem sections using standard streptavidin-biotin-peroxidase methods or immunofluorescent dyes. Primary antibodies were used against the following targets: APP, monoclonal antibody 22C11 (Chemicon); phosphorylated tau (AT-8, AT-100, PHF-1, TG-3); and pS396/p404 regulatory subunit of cdk5. Bielchowsky silver, Gallyas silver, hematoxylin-eosin, and luxol fast blue stainings were done using standard methods.

**Results**

**Hyperphosphorylation of Tau in Rats with EAE—**To study the mechanisms of axonal pathology in MS, we used LEW.1N rats with MOG-induced hyperacute EAE as an experimental model (19). As in acute MS, active demyelination is accompa-
Activation of MAPK and p25/cdk5 in EAE—To determine the molecular mechanisms for increased tau phosphorylation, the activity of several known tau-directed protein kinases, including MAPK, GSK-3β, cdk5, and MARK were studied (Fig. 2, A and B). Immunoblot analysis using an anti-phospho MAPK (Erk1/2) antibody, which recognizes only the activated form of MAPK, showed a ~2.5-fold increase in MAPK activity in EAE brain lysates compared with that in adjuvant alone immunized or naive rats. The overall expression levels of MAPK were not changed. To assess the activity of cdk5, we determined the levels of cdk5 activators, p35 and its proteolytic fragment p25. The proteolytic conversion of p35 to p25 has been implicated in aberrant cdk5 activity leading to tau hyperphosphorylation, cytoskeleton disruption, and neuronal death (22–25). There was a striking increase in the p25/p35 ratio in EAE brainstems, suggesting conversion of p35 to p25 in EAE. The activity of the tau-directed kinase GSK-3β, which phosphorylates Ser-262, was not altered in EAE.

In addition to kinase activation, inactivation of phosphatases can result in hyperphosphorylation of tau (26, 27). We therefore determined the activity of the major tau-directed phosphatase, PP2A, in duplicate samples by a colorimetric assay using a phosphopeptide substrate in the PP2A-specific reaction buffer (27, 28). The peptide was dephosphorylated to the same extent by homogenates obtained from rats with EAE compared with controls, indicating that the activity of the tau-directed phosphatase, PP2A, is not changed (Fig. 2C).

Hyperphosphorylated Tau and p25/p35 in Degenerating Neurons of Rats with EAE—Previous neuropathological studies have demonstrated axonal dilatations and spheroids in rats with MOG-induced EAE as well as in human MS brains (6–9). It has been shown recently that damaged axons and spheroids stain strongly positive with an antibody against APP (6). Whereas physiological levels of axonal APP are not detected by this method, APP accumulation is found in damaged axons possibly because of failure of axonal transport (6). We detected prominent APP staining in dilated axons and spheroids on paraffin-embedded brainstems of LEW.1N rats with EAE (Fig. 3A). To analyze whether pathologically hyperphosphorylated tau was localized in neurons with axonal injury, we performed immunohistochemical stainings with PHF-1, AT-8, and 12E8 antibodies. All three antibodies prominently stained axons in EAE brains, particularly dilated and irregularly shaped axons with MOG-induced EAE as well as in human MS brains (6–9). We next evaluated the immunoreactivity with TG-3, an antibody that recognizes phosphorylated tau (pT231) with abnormal conformation-dependent antibody MC-1 was used (data not shown). In contrast, only weak immunoreactivity of AT-8, PHF-1, and 12E8 was observed in adjuvant-immunized control rats. We next evaluated the immunoreactivity with TG-3, an antibody that recognizes phosphorylated tau (pT231) with abnormal conformation and detects early stages of paired helical filaments (29, 30). TG-3 displayed extensive staining of abnormal axons and spheroids in rats with EAE, indicating a pathological conformation shift of tau (Fig. 3). Similar results were observed when the conformation-dependent antibody MC-1 was used (data not shown). In contrast, an altered compartmentalization of tau with accumulation of amorphous and granular tau deposits in the soma of neurons within demyelinating plaques was detected by PHF-1 and Bielschowsky stainings (Fig. 3B).

Previous studies have shown that the TG-3, AT-8, and PHF-1 epitopes can be generated in vivo by cdk5 (31, 32). Because we found a conversion of p35 to p25, indicating an activation of
from treated and untreated groups were sacrificed. As expected, the severity of the disease, measured by the clinical score of each animal, and the number of inflammatory infiltrates were significantly reduced in rats that had been treated with prednisolone. Previous work in a rat model of autoimmune optic neuritis has provided evidence that prednisolone treatment may induce apoptosis in retinal ganglia cells (17).

We therefore evaluated the extent of axonal damage by immunohistochemistry for APP and the tau-specific phosphoepitopes PHF-1 and TG-3 shows injured axons and spheroids in cross-sections of brainstems from rats with EAE. B, amorphous protein aggregations in the soma of neurons in demyelinating plaques, stained by Bielschowsky or with PHF-1 antibody. C, axonal swellings reveal prominent staining with an antibody directed against the regulatory subunits p25/p35 of cdk5 and phosphorylated tau (PHF1). D, no co-localization of pMAPK and PHF-1, indicating that up-regulation of pMAPK is most likely caused by inflammatory cells. Scale bars: 10 μm in A, and 25 μm in B.

cdk5 toward tau phosphorylation in EAE brains, we analyzed the localization of the regulatory subunits of cdk5 in rats with EAE. Immunostaining with a C-terminal p35 antibody recognizing both p35 and p25 revealed intense staining of axons with abnormal profiles and hyperphosphorylated tau (Fig. 3C). In contrast, we did not detect any significant co-localization of pMAPK and PHF-1 (Fig. 3D). Taken together, these results indicate that cdk5, but not MAPK is involved in hyperphosphorylation of tau in EAE.

**Tau Insolubility and Aggregation, but No Formation of Paired Helical Filaments in Rats with EAE**—The accumulation of hyperphosphorylated tau in dilated axons and spheroids as well as its pathological conformation, which is known to precede formation of paired helical filaments, is suggestive of axonal tau aggregations. Because paired helical filaments are highly insoluble, we analyzed the insolubility of tau in 0.1% SDS and 0.5% Triton X-100. EAE brainstems were extracted in lysis buffer, and the detergent-soluble and -insoluble fractions were subjected to immunoblotting analysis. Whereas tau was barely detectable in the detergent-insoluble fraction of control brains we found significant amounts of insoluble tau in EAE brainstems (Fig. 4). The overall levels of tau did not differ between EAE and controls. These data show that in addition to hyperphosphorylation, tau also partially forms detergent-insoluble aggregates in EAE. An established biochemical method for isolation of paired helical filaments is to take advantage of their insolubility in 1% Sarkosyl (18, 33). In contrast to its insolubility in 0.1% SDS and 0.5% Triton X-100, tau from diseased animals was soluble in 1% Sarkosyl. Taken together, these findings demonstrate aggregation of tau without the formation of paired helical filaments.

**Prednisolone Treatment Reduces Kinase Activation and Tau Phosphorylation in Rats with EAE**—High-dosage prednisolone treatment is the standard therapy regime in acute relapses of MS (1, 2). We therefore tested whether the treatment with prednisolone could inhibit the pathological cascade leading to hyperphosphorylation and aggregation of tau in EAE rats. Rats were treated with 20 mg/kg prednisolone intraperitoneally from day 8 post-immunization to day 12. At day 12 animals from treated and untreated groups were sacrificed. As expected, the severity of the disease, measured by the clinical score of each animal, and the number of inflammatory infiltrates were significantly reduced in rats that had been treated with prednisolone. Previous work in a rat model of autoimmune optic neuritis has provided evidence that prednisolone treatment may induce apoptosis in retinal ganglia cells (17).

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perphosphorylation of tau is part of a specific pathway triggered by an inflammatory attack in the central nervous system of rats with EAE.

**DISCUSSION**

Here we show that 1) tau derived out of EAE brains is abnormally hyperphosphorylated at sites that define tau pathology in Alzheimer’s disease; 2) that hyperphosphorylation is most likely because of an activation of cdk5 rather than to decreased phosphatase activity; 3) that pathologically hyperphosphorylated tau is localized in injured neurons of EAE brains; and 4) that tau becomes partially insoluble and undergoes a conformational shift that is thought to precede paired helical filament formation, whereas aggregation into paired helical filaments is not observed.

These findings raise the question of how tau could mediate the axonal damage that occurs in active MS lesions. The fact that the binding of tau is regulated by phosphorylation within the repeat region (e.g., Ser-262) has led to the hypothesis that tau hyperphosphorylation leads to a release of tau from microtubules, followed by microtubule breakdown and transport decay (loss of function). In addition, hyperphosphorylated tau could be neurotoxic by itself or in its aggregated form (gain of toxic function). Consistent with the loss of function hypothesis we found significant tau phosphorylation at Ser-262, one site within the repeat domain that strongly inhibits microtubule binding and causes detachment of tau from microtubules when phosphorylated. It is therefore possible that hyperphosphorylation of tau during acute inflammation disengages tau from microtubules, causing their destabilization and impairment of axonal flow. Furthermore, we observed significant abnormal phosphorylation of SP/TP motifs in the flanking regions of tau such as PHF-1 (pS396/pS404), AT-8 (pS202/pT205), AT-180 (pT231/pS235), and AT-100 (pT212/pS214) epitopes.

To identify downstream kinases involved in the sequen- tial toxic hyperphosphorylation of tau at SP/TP motifs we performed immunoblotting with several activity dependent antibodies against active MAPK, inactive OSK-3β, and upstream activators of cdk5 because all of these have been described to phosphorylate tau in vivo and in vitro. In contrast to GSK-3β, which was found to be down-regulated we detected an increase in MAPK activity as well as an increase in the ratio of cdk5 activators p25/p35. Considering that MAPK is also expressed in astrocytes and microglial cells it is possible that the detected activated MAPK could originate from glial cells rather than neurons (36). Indeed, we did not detect significant co-localization of pMAPK and PHI-1 in EAE. In contrast, cdk5 and its activators p25/p35 are primarily expressed in neurons (see also Fig. 3C) and have been localized and purified from brain microtubules (37, 38). In addition, transgenic mice overexpressing p25/cdk5 display increased tau phosphorylation at SP/TP motifs in the flanking region as well as tau aggregation and neurodegeneration (23–25). Cdk5 is recruited to the neuronal membrane by its interaction with membrane-anchored p35 (39). Aberrant activation of cdk5 occurs when the myristoylated domain of p35 is cleaved to p25, leading to release of p25-bound cdk5 into the cytoplasmic compartment. Several lines of evidence indicate that only p25 but not p35-bound cdk5 phosphorylates tau in vitro, thereby inducing pathological alterations in neurons (22–25, 40, 41).
It is interesting to note that conversion of p35 to p25 is regulated by the calcium-dependent cysteine protease calpain as activation of calpain together with impaired calcium homeostasis has been observed in EAE and MS brains (42, 43). It is therefore tempting to speculate that increased calcium influx might trigger a cascade of pathological events that lead to calpain activation, followed by conversion of p35 to p25 and increased and pathological tau phosphorylation at epitopes such as PHF-1, AT-8, and AT-100.

It has generally been assumed that filamentous tau aggregations as they are described in Alzheimer’s disease, frontotemporal dystrophies, and other tauopathies might be direct mediators of neuronal toxicity because the clinical progression of Alzheimer’s disease correlates with distribution and amount of tau aggregates (44, 45). Tau is a highly soluble protein because its sequence consists mostly of hydrophilic residues. The exact molecular mechanisms of its abnormal aggregation into paired or straight helical filaments are not completely understood. In _in vitro_ studies propose extrinsic (polyanions, oxidative environment) as well as intrinsic factors (increased tau concentration, tau mutations that promote β-structure) as possible reasons for aggregation (46). Hyperphosphorylation has also been assumed to cause pathological aggregation of tau.

The antibody TG-3 recognizes a conformation-dependent epitope that has been reported to precede paired helical filament formation (29). Because we observed a marked TG-3 and MC-1 staining of injured axons, we tested tau solubility and aggregation in the EAE model. We could identify one fraction of tau that had become insoluble in 0.5% Triton X-100, 0.1% SDS, indicating the formation of tau aggregates. Amorphous aggregates in the soma of neurons and axonal spheroids that are stained by PHF-1 and Bielschowsky silver impregnation could be the morphological correlate of the 0.5% Triton X-100, 0.1% SDS-insoluble tau fraction. A widely accepted biochemical method to isolate paired helical filaments of Alzheimer brains is based on their insolubility in 1% Sarkosyl. However, no tau reactivity could be detected in the Sarkosyl-insoluble fraction of EAE brainstem lysates. In line with the absence of Sarkosyl-insoluble tau aggregates, no paired helical filaments were observed by Gallyas or Bielschowsky staining. Insolubility and therefore aggregation of tau in the absence of paired helical filaments raises the question of the relevance of these findings for axon degeneration in EAE. It is poorly understood whether hyperphosphorylated tau is toxic by itself or oligomeric tau aggregations or stable fibrils are required to cause cellular dysfunction. Evidence is now accumulating that formation of paired helical filaments is not necessary for tau toxicity. Phosphorylation of tau in a temporally ordered series can already cause neurodegeneration in the absence of aggregation (47). Furthermore, it has been shown that misfolded and oligomerized proteins can cause cellular dysfunction before the deposition of stable fibrils (48). Small protein assemblies that can activate tau can in MS and EAE lesions correlate with the extent of inflammation, suggesting that the primary insult is an inflammatory attack (6–9). However, the causal relationship of inflammation, demyelination, and axonal degeneration has been difficult to determine. To address this question we used a genetic model of axonal damage, which is triggered by oligodendrogial dysfunction instead of inflammation. CNP-deficient mice display axonal loss in the absence of demyelination or inflammation. Although the axonal degeneration observed in this model displays similar ultrastructural features such as APP positive axonal swellings and spheroids, hyperphosphorylation of tau is only found in EAE, but not in CNP-deficient mice. In addition, cuprizone-induced demyelination did not lead to tau pathology in axons. These data indicate that demyelination _per se_ is not sufficient to induce changes in tau phosphorylation. Furthermore, one can speculate that inflammation as seen in EAE triggers a specific pathway of axonal damage, which is distinct from the one caused by oligodendrogial dysfunction or demyelination. This raises the question to which extent axonal damage can be reversed by reducing the inflammatory load and how steroid treatment that is the standard therapy for acute relapsing MS influences axonal damage. Our findings suggest that a prednisolone pulse treatment during the active phase of inflammation does not only reduce the amount of inflammatory infiltrates, but also the extent of axonal damage.

In summary our data provide evidence that axonal damage in EAE is associated with tau hyperphosphorylation and aggregation. These pathological tau alterations can be partially prevented by early prednisolone treatment. These findings are of particular relevance because the amount of axonal damage is a major determinant of persistent neurological deficits in MS patients. Our results might open new perspectives for understanding molecular pathology and treatment of multiple sclerosis.

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