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Dysregulation of Tau Phosphorylation in Mouse Brain during Excitotoxic Damage

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Abstract

Glutamate receptor-mediated excitotoxicity is thought to contribute to the development of Alzheimer's disease (AD), but the underlying mechanism is unknown. In this study, we investigated the dynamic changes of tau phosphorylation and tau-related protein kinases and protein phosphatase 2A (PP2A) in the mouse brain during excitotoxicity induced by intraperitoneal injection of 20 mg/kg kainic acid (KA). We found that KA-induced excitotoxicity led to transient dephosphorylation of tau (within 6 hr post-injection), followed by sustained hyperphosphorylation of tau at multiple sites that are hyperphosphorylated in AD brain. The initial dephosphorylation of tau may result from activation of PP2A, and the sustained hyperphosphorylation may be due mainly to activation of cdk5 and down-regulation of PP2A during the later phase. Because abnormal hyperphosphorylation of tau plays a crucial role in neurodegeneration and in the formation of neurofibrillary tangles, our results suggest that glutamate receptor-mediated excitotoxicity might contribute to AD partially via promoting abnormal hyperphosphorylation of tau in AD brain.

Keywords

Alzheimer's disease; glutamate receptors; kainic acid; phosphorylation; protein kinases; protein phosphatase-2A; tau

INTRODUCTION

Alzheimer's disease (AD), one of the most devastating diseases of the aged, causes dementia and eventually death of affected individuals. In only less than 1% of cases, AD is caused by autosomal dominant mutations of presenilin-1, presenilin-2, or amyloid- β protein precursor. Most AD cases are believed to be caused by multiple etiological factors including genetic susceptibility (such as ApoE4 allele) and environmental and metabolic factors. Several of these etiological factors, such as multi-micro infarction, head trauma, and oxidative stress, lead to a common pathway of neuronal over-excitation involving the excitatory glutamate receptors. This type of excitotoxicity has been recognized as an important underlying mechanism in neurodegenerative disorders including AD [1,2]. However, how the glutamate receptor-mediated excitotoxicity contributes to brain pathology and clinical expression of AD is not understood.

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Abnormally hyperphosphorylated tau protein not only constitutes neurofibrillary tangles, which is one of the hallmark brain lesions of AD, but also plays a critical role in neurodegeneration [3–5]. Tau is a neuronal microtubule-associated phosphoprotein. The phosphorylation level of tau is regulated by tau protein kinases and phosphatases as well as other modifications, such as O-GlcNAcylation [6,7], of tau itself. A dysregulation of these enzymes and tau modifications is thought to lead to abnormal hyperphosphorylation in AD brain.

To investigate how excitotoxicity contributes to neurodegeneration of AD, we studied dysregulation of tau phosphorylation during excitotoxicity. The excitotoxic damage was induced by a single intraperitoneal injection of kainic acid (KA), which is a well-known excitatory and neurotoxic ligand that binds and activates the kainate receptor of the glutamate receptor family. We also studied alterations of tau kinases and phosphatases during the excitotoxic damage.

MATERIALS AND METHODS

Materials

The catalytic subunit of PKA was purchased from Sigma (St. Louis, MO). The largest isoform of recombinant human tau, tau₄₄₁, was expressed and purified from *E. coli*, as described previously [8]. Antibody Tau-5 that recognizes tau in a phosphorylation-independent manner was purchased from Chemicon International Inc. (Temecula, CA). Phosphorylation-dependent and site-specific tau antibodies (pT181, pS199, pS202, pT205, pT212, pS214, pT217, pT231, pS262, pS396, pS404, pS409, and pS422) and anti-phospho-GSK-3 α/β (Tyr279/Tyr216) were purchased from BioSource International (Camarillo, CA). Anti-ERK1/2, anti-phospho-ERK1/2(Thr202/Tyr204), anti-phospho-GSK-3 β (Ser9), anti-SAPK/JNK, and anti-phospho-SAPK/JNK (Thr183/Tyr185) were from Cell Signaling Technology, Inc. (Danvers, MA). Polyclonal antibodies against GSK-3 β and the catalytic subunit of PP2A (R123d) were raised in rabbits, as described previously [9,10]. Anti-CaMKII and anti-active-CaMKII (phosphorylated at Thr286) were purchased from Promega (Madison, WI). Anti-cdk5 and anti-p35/p25 were from SantaCruz Biotechnology, Inc. (SantaCruz, CA). Peroxidase-conjugated anti-mouse and anti-rabbit IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). ECL kit was from Amersham Pharmacia (Costa Mesa, CA). [γ -³²P]ATP was from ICN Biomedicals (Costa Mesa, CA).

Kainic acid injection of mice

Male FVB mice (2.5–30 g body weight, 12 weeks old, obtained from Charles River Laboratories, Wilmington, MA) were housed individually in a 12-hour light/dark schedule with free access to food and water. A single dose of kainic acid (20 mg/kg body weight) was injected intraperitoneally [1]. The mice were then sacrificed 2.5, 6, 10, 24, 36, and 48 hours after injection, and the forebrains were immediately removed and homogenized in 9 volumes of buffer consisting of 50 mM Tris-HCl (pH 7.4), 8.5% sucrose, 10 mM β -mercaptoethanol, 2.0 mM EDTA, 2.0 mM benzamidine, and 2.0 μ g/ml each of aprotinin, leupeptin, and pepstatin. Part of the homogenates was centrifuged at 16,000 g at 4°C for 10 min to prepare the crude brain extracts. Animal use was in full compliance with the NIH guidelines and was approved by our institutional Animal Care and Use Committee.

Western blot analysis

Western blots of the mouse brain homogenates were carried out by using 10% SDS-PAGE and standard procedure, and developed by using enhanced chemiluminescence (ECL) kit (Pierce Biotechnology, Rockford, IL), as described previously [11]. The immunoreactivity of the blots was quantified by analyzing densitometry of the X-ray films using TINA 2.0 program.

PKA assays

Brain extracts were incubated in a PKA assay mixture (20 μ l) containing 50 mM Tris-HCl (pH 7.4), 0.1 mM EGTA, 4.0 μ M cyclosporine A, 0.2 μ M okadaic acid, 1.0 mM Na₃VO₄, 10 mM MgCl₂, 0.2 mM [γ -³²P]ATP (500 cpm/pmol), and 30 μ M malantide. Malantide is a specific peptide substrate for PKA assay [12]. After incubation at 30°C for 20 min, 10 μ l each of 10 mg/ml BSA and 40% trichloroacetic acid were added into the assay mixtures to terminate the reaction. The reaction mixture was left in wet ice for 30 min and then centrifuged at 2,300 g for 3 min. Aliquots of 10 μ l supernatants containing Malantide were spotted onto squares (1 $\times$ 1 cm) of P81 chromatography paper (Whatman International Ltd., Maidstone, UK). After washing with 75 mM phosphoric acid 5 times, the chromatography paper squares were dried and the radioactivities were counted in a scintillation counter to determine the ³²P-incorporation into Malantide.

PP2A assay

PP2A was first immunoprecipitated with R123d, as described previously [13]. Phosphatase activity was assayed in a reaction mixture (20 μ l) containing 50 mM Tris-HCl, pH 7.4, 1.0 mM MnCl₂, 10 mM β -mercaptoethanol, 0.2 mg/ml ³²P-tau, 1.0 mM NaCl, and immunoprecipitated PP2A, complex as described previously [13]. ³²P-tau was prepared by phosphorylation with PKA and cdk5. After incubation at 30°C for 20 min, the reaction was terminated, and the released ³²Pi was determined by Cerenkov counting after separation from ³²P-tau by ascending paper chromatography.

RESULTS

Changes of tau phosphorylation during excitotoxic damage induced by KA administration

To study how neuronal excitotoxicity affects tau phosphorylation, we determined alterations of tau phosphorylation at various time points after intraperitoneal injection of KA, which is the commonly used method to induce glutamate receptor-mediated neuronal excitotoxicity and seizures in rodents [1]. Alterations of tau phosphorylation at individual phosphorylation sites were measured by Western blots by using a battery of phosphorylation-dependent and site-specific tau antibodies. We found that KA administration induced marked two-phase changes of tau phosphorylation at all phosphorylation sites examined. During the first phase (within 6 hr post-injection), tau phosphorylation was markedly decreased at all the sites studied, whereas the total tau level in the brain was not markedly affected (Fig. 1). A slightly faster gel mobility of tau was also observed with phosphorylation-independent tau antibody during this phase (Fig. 1A, bottom blot), which is consistent with previous observations that the less phosphorylated tau moves faster than the more phosphorylated tau in SDS-PAGE [14,15]. After 6 hr post-injection of KA (phase 2), tau was quickly rephosphorylated, and after 10 hr post-injection, the phosphorylation level became much higher than that of the vehicle-injected control mice at all the phosphorylation sites examined except at Thr181, Ser214, and Ser404. The phosphorylation level of tau at phase 2 reached 3-to 4-fold higher than that of control mice at Ser199, Ser202, Thr205, Ser396, and Ser422, and more than 5-fold higher at Thr212, Thr217, and Ser262 (Fig. 1B). Based on the patterns of dynamic changes, KA-induced alterations of tau phosphorylation could be divided into two groups. At the group 1 sites (Fig. 1B, upper panel), tau was first dephosphorylated at phase 1 and then became hyperphosphorylated during the entire phase 2 till 48 hr post-injection, the longest time point we studied. Tau at the group 2 sites (Fig. 1B, lower panel) was quickly hyperphosphorylated at the early hours of phase 2 (6–10 hr post-injection), and then it declined. Taken together, these results indicate that KA-induced excitotoxicity causes a rapid, short-term hypophosphorylation of tau, followed by a sustained, long-term hyperphosphorylation of tau.

Changes of tau kinases in the mouse brain during excitotoxic damage

To study the mechanism by which KA administration causes the two-phase alterations of tau phosphorylation, we investigated the levels and activation of glycogen synthase kinase-3 β (GSK-3 β) and cyclin-dependent protein kinase 5 (cdk5), two protein kinases most implicated in tau phosphorylation, during excitotoxic damage. Because GSK-3 β activity is negatively regulated by phosphorylation at Ser9 (major regulator) and positively regulated by phosphorylation at Tyr216 [16,17], we determined GSK-3 β activation by measuring the phosphorylation of GSK-3 β at these two sites. Cdk5 activation was monitored by measuring the cleavage of its activator, p35, into a more active form, p25 [18]. We found that although KA treatment did not change the levels of GSK-3 β or cdk5 in mouse brains, it altered the activation of these two kinases in a time-dependent manner (Fig. 2A). During the first phase (within 6 hr) after KA injection, both GSK-3 β and cdk5 were activated, as evidenced by a decrease in Ser9 phosphorylation of GSK-3 β and an increase in p25 generated from p35 (Fig. 2B). However, GSK-3 β activation started to reverse after 6 hr post-injection. On the other hand, cdk5 was activated in a time-dependent manner and reached a highly activated state after 10 hr post-injection (Fig. 1B).

Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) is another tau kinase [19,20]. Binding of Ca²⁺/calmodulin to CaMKII induces phosphorylation of the kinase at Thr286 in the autoinhibitory domain and results in activation of the kinase to 1,000-fold [21–23]. Thus, we determined CaMKII activity by measuring phosphorylation of CaMKII at Thr286. We found that during KA-induced excitotoxicity, phosphorylation of CaMKII at Thr286 was markedly decreased, although the total level of CaMKII was unchanged (Fig. 2C, D). These results suggest that CaMKII activity was down-regulated during excitotoxic damage.

cAMP-dependent protein kinase (PKA) is also involved in regulation of tau phosphorylation [24–26]. We, thus, determined its activity in mouse brains after KA injection and found that the profile of its time-dependent changes was similar to that of CaMKII activation, except it was inhibited to a much smaller extent than CaMKII (Fig. 2D).

KA administration has been shown to activate MAPK (mitogen-activated protein kinases) pathways [27,28], and these kinases also phosphorylate tau [29–31]. We, therefore, studied the activity of ERK1/2 and JNK, the two most relevant components of the MAPK pathways, during excitotoxic damage by Western blots developed with antibodies specific to the active, phosphorylated ERK1/2 or JNK. We found that both of the kinases, especially JNK, were activated at the earlier phase of excitotoxicity, and the activation peaked at 10 hr post-injection of KA (Fig. 2E, 2F).

Changes of tau phosphatase in the mouse brain during excitotoxic damage

Protein phosphatase 2A (PP2A) is the major protein phosphatase that regulates tau phosphorylation *in vivo* and accounts for ~70% of the total tau phosphatase activity in the mammalian brain [32]. Therefore, we determined PP2A activity against phosphorylated tau as a substrate. We found that the PP2A activity was first activated (at 2.5–6 hours) and then decreased during excitotoxic damage induced by KA administration (Fig. 3). These two-phase changes matched the times of the two-phase changes of tau phosphorylation (compare Fig. 3 with Fig. 1).

DISCUSSION

Glutamate receptor-mediated excitotoxicity has been implied in the pathogenesis of AD, although the underlying mechanism is not well understood. In this study, by using an *in vivo* model of neuronal excitotoxicity induced by KA, we observed that neuronal excitotoxicity

induced marked two-phase changes of tau phosphorylation. In the early phase (within 6 hr after KA injection), tau underwent dephosphorylation at all phosphorylation sites examined. After this initial phase, tau protein became highly phosphorylated, and tau hyperphosphorylation appeared to last for a long time at the majority of the phosphorylation sites studied. Because abnormal hyperphosphorylation of tau has been shown to be crucial to neurodegeneration and formation of neurofibrillary tangles in AD [3,4], our observations suggest that chronic neuronal excitotoxicity might contribute to AD via promoting abnormal hyperphosphorylation of tau.

The longest isoform of human tau contains 80 serine or threonine residues. Nearly half of these residues have been reported to be modified by phosphate groups in tau isolated from AD brain [33,34]. The phosphorylation sites we examined in this study are all among these sites that are hyperphosphorylated in AD. Phosphorylation of tau at these sites has been shown to reduce or diminish the biological activity of tau to stimulate microtubule assembly, to convert tau into a toxic molecule, and to promote its self-polymerization into neurofibrillary tangles [8,20,35–41]. Tau dephosphorylation at these sites in the early phase of KA-induced neuronal excitotoxicity might represent an initial protective response of neurons to KA.

Tau phosphorylation is regulated by several protein kinases in the brain. The kinases that have been most implicated in tau phosphorylation include GSK-3 β , cdk5, PKA, CaMKII, and stress-activated protein kinases. We, therefore, studied the dynamic alterations of these kinases, hoping to reveal the major tau kinases responsible for the two-phase changes of tau phosphorylation during excitotoxicity. We found that both GSK-3 β and cdk5 were activated during the first phase, and then GSK-3 β returned to its basal activity, whereas cdk5 was further activated during the second phase. These results suggest that GSK-3 β might not contribute to the observed changes of tau phosphorylation during excitotoxicity, whereas the marked activation of cdk5 during 6–10 hours post-injection could contribute to the rapid phosphorylation of tau during the same time period. Because the activation of GSK-3 β was assessed only by determining the phosphorylation at Ser9 and Tyr216 of the molecule, which are not the full determinants of the kinase activity [42], the role of GSK-3 β cannot be absolutely excluded. Both PKA and CaMKII activities were down-regulated during the first phase of excitotoxicity, which might contribute partially to the decreased tau phosphorylation at the relevant phosphorylation sites at this phase. However, the activities of these two kinases, especially of CaMKII, were still lower during the second phase in the KA-injected brains as compared to these in normal control brains. Thus, the marked hyperphosphorylation of tau at this latter phase could not be explained by the alteration of these two kinases. Activity of MAPK (ERK) and JNK were first activated and peaked 10 hours after KA injection, and then declined dramatically. However, the changes of the kinase activities did not correlate to the two-phase changes of tau phosphorylation. Because the actual kinase activities toward tau were not determined directly, the above analyses can only suggest possible involvement of the major tau kinases in the alteration of tau phosphorylation during KA-induced cytotoxicity.

In addition to protein kinases, tau phosphorylation is also regulated by PP2A [13,14,43–46]. A down-regulation of PP-2A in the brain may partially underlie the abnormal hyperphosphorylation of tau in AD [13,47–50]. Here, we observed that PP2A activity was increased during the first phase of excitotoxicity, when tau was dephosphorylated. These results suggest that the PP-2A activation might contribute to tau dephosphorylation at the early phase of KA-induced excitotoxicity. Ten hours after KA injection, PP-2A activity returned to its normal level and continued to decline after 26 hr post-injection. Therefore, the overall hyperphosphorylation of tau at the group 1 sites during this phase appears to result from the dramatic activation of cdk5 and the decline in PP2A activity.

Binding of KA to the kainate receptor of the glutamate receptor family opens sodium and potassium channels and induces alteration of multiple signaling pathways. Alterations of

several protein kinases, upon KA treatments, have been reported, but the results were inconsistent, probably due to the various models used and at the various time points examined [51–57]. In the present study, we investigated several tau-related kinases and found that all of them were dynamically regulated in a time-dependent manner, and each kinase had its own dynamics of alteration. The molecular basis underlying these dynamic changes of protein kinases and PP2A is complicated and requires further investigation.

In conclusion, by using a KA-injected mouse model of excitotoxicity, we found dynamic two-phase changes of tau phosphorylation – initial dephosphorylation followed by sustained hyperphosphorylation – during excitotoxicity. The initial dephosphorylation of tau might result from activation of PP2A, and the sustained hyperphosphorylation might be due mainly to activation of cdk5 and inhibition of PP2A during the second phase. These studies suggest that chronic excitotoxicity might contribute to AD via promoting abnormal hyperphosphorylation of tau.

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Fig. 1.

Changes of tau phosphorylation during KA-induced excitotoxic damage. (A) Homogenates of forebrains of mice sacrificed at the indicated time points after injection with KA were analyzed by Western blots developed with phosphorylation-dependent and site-specific tau antibodies, as indicated at the right side of the blots. Tau-5 is a phosphorylation-independent tau antibody for determining total tau level. (B) The blots shown in panel A were quantified densitometrically, and the relative phosphorylation (i.e., immunoreactivity with the individual phosphorylation-dependent tau antibody), where those of control samples were defined as 100, are shown. These data had been normalized with the total tau level, as determined by Tau-5 blot. All data are the means of 2–4 determinations.

Fig. 2.

Changes of tau kinases during KA-induced excitotoxic damage. (A, C, E) Brain homogenates of mice sacrificed at the indicated time points after KA injection were analyzed by Western blots developed with antibodies against the total or phosphorylated kinases, as indicated at the right side of the blots. (B, D, F) Relative levels of phosphorylated kinases or p25 at various time points after KA injection. Level of each phosphorylated kinase was quantified after being normalized with the level of the corresponding kinase. The PKA graph is its kinase activity determined by using *in vitro* kinase assay of immunoprecipitated PKA. All data are the means of 2–4 determinations.

Fig. 3. Alteration of PP2A activity induced by KA administration. PP2A was immunoprecipitated from the crude brain extract of mice after KA injection and then assayed for its phosphatase activity by using phosphorylated tau as a substrate. Data are presented as mean $\pm$ SD ($n = 4$).