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Biochemical and Biophysical Research Communications 347 (2006) 273–278
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Hyperphosphorylation at serine 199/202 of tau factor in the gerbil hippocampus after transient forebrain ischemia

Motohiro Morioka [a,*], Takayuki Kawano [a], Shigetoshi Yano [a], Yutaka Kai [a], Hiromasa Tsuiki [a], Yutaka Yoshinaga [a], Jun Matsumoto [a], Tatsumi Maeda [a], Jun-ichiro Hamada [c], Hideyuki Yamamoto [b], Kohji Fukunaga [d], Jun-ichi Kuratsu [a]

[a] Department of Neurosurgery, Kumamoto University School of Medicine, Japan
[b] Department of Pharmacology, Kumamoto University School of Medicine, Japan
[c] Department of Neurosurgery, Kanazawa University Graduate School of Medical Science, Japan
[d] Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Tohoku University, Japan

Received 13 June 2006
Available online 23 June 2006

Abstract

We examined the phosphorylation state of tau factor in hippocampal delayed neuronal death (DND) after transient forebrain ischemia. A transient phosphorylation increase at serine 199/202 but not serine 396 of tau factor after transient ischemia was clearly observed. Intraventricular injections of olomoucine and U-0126 (CDK5 and MAP kinase inhibitors, respectively) inhibited hyperphosphorylation. In contrast, wortmannin (PI3 kinase inhibitor) increased phosphorylation at serine 199/202 and corresponded with an increase in GSK3 phosphorylation. Our findings suggest that CDK5, MAP kinase, and GSK3 phosphorylate these sites after ischemia. We prepared recombinant normal human tau (N-Tau40) with TAT-HA protein and dephosphorylated-form human Tau-40 (D-tau40) in which 199/202 serines were changed to alanine by site-directed mutagenesis. Intraventricularly injected D-tau40 protected somewhat against DND while N-Tau40 did not. These data suggest that hyperphosphorylation at serine 199/202 of tau factor is induced by MAP kinase, CDK5, and GSK3, and contributes to ischemic neuronal injury.

Keywords: Ischemia; Hippocampus; Tau factor; Phosphorylation

Transient forebrain ischemia induced delayed neuronal death (DND) in the CA1 region of the hippocampus [1]. DND occurred slowly; morphologically these cells may appear normal as late as 2 days after ischemia. A better understanding of the intracellular processes in DND may make it possible to prevent neuronal degeneration after cerebral ischemic insult. Our studies have documented post-ischemic alterations in key kinases and phosphatases and in intracellular phosphorylation [2–6], and recently many authors reported alterations of phosphorylation activities. Although changes in the activity of these enzymes have been studied, the key substrates and their role in the death cascade remain unclear.

Tau factor is a microtubule-associated protein abundant in neuronal cells. It induces microtubule assembly and stabilizes microtubules by its phosphorylation states [7–12]. Hyperphosphorylation of tau factors may be a pathogenic process with a role in the initiation and progression of neurodegenerative diseases (tauopathy) including Alzheimer's disease (AD). Enzymes involved in tau phosphorylation are MAPK, glycogen synthase kinase 3 (GSK3), CDK5, CaM KII, cAMP-dependent protein kinase, calcineurin, and phosphatase 2A [3]. While more than 30 phosphorylation sites have been reported [13,14], knowledge regarding important sites and the mechanisms underlying their hyperphosphorylation remains insufficient.

* Corresponding author. Fax: +81 96 371 8064.
E-mail address: morioka@kaiju.medic.kumamoto-u.ac.jp (M. Morioka).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.06.096

The phosphorylation state of tau factor after cerebral ischemia has been studied by several authors. Within 2 h of ischemia (early period), tau was dephosphorylated *in vitro* and *in vivo* [15–17]. Wen et al. [18,19] reported site-specific hyperphosphorylation of tau after transient cerebral ischemia 24 h after ischemia and they suggested that ischemia induced AD-like tauopathy. Specific markers of AD, e.g., Alz-50-immunoreactive granules and amyloid precursor protein (APP), were found in ischemic brains [20,21] and ApoE4 is a genetic risk factor for both AD and stroke [22]. These observations suggest that AD and ischemic neuronal injury share a death pathway that includes hyperphosphorylation of tau (tauopathy). Here we examined changes in the phosphorylation state of tau factor in DND of gerbil hippocampus after transient forebrain ischemia.

Materials and methods

All experiments were approved by the Animal Care and Use Committee of Kumamoto University. Male Mongolian Gerbils weighing 60–80 g were housed under constant environmental conditions (temperature 22 ± 2 °C, humidity 55 ± 5%, and 12/12 h light/dark cycle) in the Animal Research Center of Kumamoto University. They were acclimatized for 7 days and had free access to food and water before and after all procedures. Transient (5-min) global forebrain ischemia was induced as previously reported [2]. Briefly, anesthesia was induced with 4% halothane and maintained with 2–2.5% halothane, 30% O_2, and 70% N_2O via a facemask. The rectal temperature was monitored and maintained at 37–38 °C throughout the operation with a warming blanket. The bilateral common carotid arteries were surgically exposed and quickly occluded for 5 min with aneurysm clips. After their release, the animals were returned to their cages; they were sacrificed at 0, 6, and 12 h and at 1 and 2 days after ischemia induction. Their brains were removed immediately and the hippocampal CA1 and CA3 regions were resected under a microscope and stored at −80 °C until use.

All chemicals and recombinant proteins were delivered by right-side stereotactical intraventricular injection after anesthetization with pentobarbital (10 mg/kg, ip). As described elsewhere [2], following chemicals (2 μl) were injected through a burr hole at 2.0 mm posterior from bregma, 3.5 mm lateral and 2.5 mm depth from cortical surface. Wortmannin (100 μmol/L, Wako Pure Chemical Industries, Osaka, Japan), U0126 (500 μmol/L, Calbiochem-Novabiochem, San Diego, CA, USA), and Olomoucine (2.5 mmol/L, Calbiochem-Novabiochem, San Diego, CA, USA) were dissolved in phosphate-buffered saline with 2% dimethyl sulfoxide.

cDNA from human hTau-40 was used as normal tau (N-tau). In the dephosphorylated-form tau (D-tau), 199 and 202 serine was converted to alanine by site-directed mutagenesis (Quick Change™ site-directed mutagenesis kit; Stratagene, CA, USA). cDNA (N- and D-tau) was inserted into a TAT-HA plasmid [23] (pET28bTAT V1 plasmid; kindly provided by Prof. Dowdy, Howard Hughes Medical Institute, University of California). The inserted plasmid was transformed into *Escherichia coli*, BL21-S1, and the recombinant proteins were expressed and purified.

Protein extraction was as described elsewhere [2] using homogenizing buffer that included phosphatase inhibitors (1 mmol/L Na_3Vo_4, 30 mmol/L sodium pyrophosphate, and 50 mmol/L NaF). Equal protein amounts were loaded on each lane for SDS–PAGE and then the proteins were transferred for 1 h at 70 V to an Immobilon PVDF membrane (Millipore, Bedford, MA, USA). The primary antibodies were anti-general tau antibody (1:200, Sigma–Aldrich, St. Louis, MO, USA), anti-phosphorylated serine 199/202 tau antibody (1:200) [24], anti-phosphorylated serine 396 tau (1:200) [24], and anti-phosphorylated GSK3 (1:200, New England Laboratories). Western blots were performed with horseradish peroxidase-conjugated anti-rabbit antibody (1:5000, Vector, CA, USA) using enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech, Arlington Heights, IL, USA). The images were scanned and analyzed semiquantitatively using NIH image software.

The gerbils were anesthetized with pentobarbital perfused through the ascending aorta with cold 0.1 M phosphate buffer (pH 7.4); fixation was with 4% paraformaldehyde in 0.1 M phosphate buffer. All perfusion solutions contained phosphatase inhibitors (1 mmol/L Na_3Vo_4, 30 mmol/L sodium pyrophosphate, and 50 mmol/L NaF). The brains were removed, post-fixed overnight in the same fixative solution containing phosphatase inhibitors, and cut with a vibratome. For immunohistochemical staining we used the free-floating method [2]. The primary antibodies were anti-general tau antibody (1:2000, Sigma–Aldrich), anti-phosphorylated serine 199/202 tau antibody (1:1000), and anti-phosphorylated serine 396 tau (1:1000). We used the Vectastain ABC kit; the chromogen was 3,3′-diaminobenzidine.

Western blots were scanned and analyzed semiquantitatively using NIH image software. All values were expressed as the mean ± SD. Overall statistical significance for differences among groups was tested by one-way analysis of variance followed by multiple comparisons between groups or between control- and experimental groups using Dunnett's multiple comparison test. Differences of $p < 0.05$ were considered significant.

Results and discussion

Tau phosphorylation after transient ischemia

Neuronal somata of some neurons in the CA1 region of control animals manifested phosphorylated tau at 199/202 serine (199/202P-tau) (Fig. 1A). After 5-min transient ischemia, 199/202 serine was dephosphorylated immediately; at 12 and 24 h post-ischemia, there was an increase of phosphorylation in all neurons. In contrast, the distribution of 396 serine phosphorylation (396P-tau) exhibited no obvious change (Fig. 1B). Western blot analysis confirmed these findings (Fig. 1C and D). All immunostained bands of 199/202P-tau, ranging from 65 to 45 kDa, corresponded to the time course of our immunohistochemical findings. The pattern of 396P-tau gel morbidity differed from P199/202-tau and showed no hyperphosphorylation.

To examine whether hyperphosphorylation of 199/202 serine is specific for ischemic neuronal cell death, we investigated the dynamics of P199/202-tau under other ischemic conditions and in other regions. In the current gerbil model, we detected no neuronal death in the CA3 region, and there was no hyperphosphorylation of P199/202-tau (data not shown). In animals with ischemic tolerance, produced by the induction of 2-min ischemia 24 h before 5-min ischemia induction, we did not observe hyperphosphorylation of P199/202-tau in the CA1 region (Fig. 1F).

Others reported that tau factor was dephosphorylated within 2 h after the induction of central nervous system ischemia [15–19]. Wen et al. [19] documented site-specific hyperphosphorylation of tau in the peri-infarct area at 24 h after 1-h transient MCA occlusion; the hyperphosphorylated sites were 202-, 214-, 402-serines and 231 threonine. The phospho-tau epitopes co-localized with the apoptotic marker TUNEL. Our findings of tau factor hyperphosphorylation in the later stage after ischemia induction coincide with those of Wen et al., suggesting

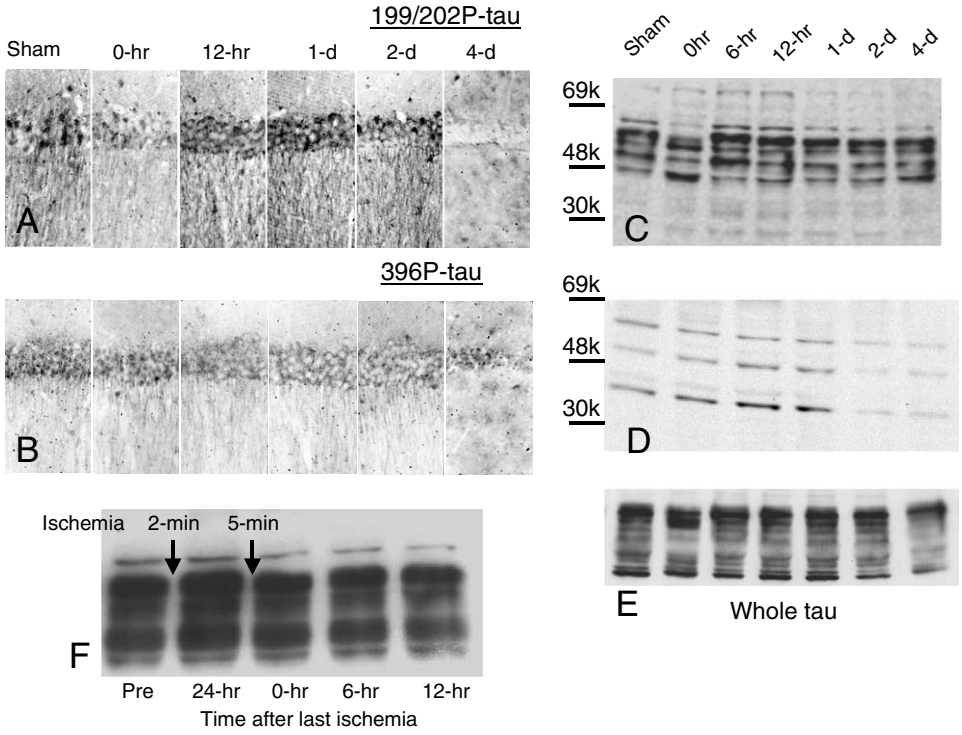

Fig. 1. Immunohistochemistry and Western blotting of hippocampal CA1 region after 5-min ischemia using anti-phospho199/202 serine (A,C) and anti-phosho396 serine (B,D) antibodies. Although 199/202 serine was hyperphosphorylated 6–12 h after ischemia, neither phospho-396 serine nor the whole tau protein amount (E) showed obvious changes until 1 day after ischemia (E). In gerbils rendered ischemia-tolerant by 2-min ischemia induction, hyperphosphorylation of 199/202 serine was not found even after 5-min lethal ischemia (F).

that hyperphosphorylation of specific sites of tau factor occurs in the later stages after ischemia. The CA1 region of gerbils rendered ischemia-tolerant did not show hyperphosphorylation, nor did other regions showing no cell death in the hippocampus subjected to 5-min lethal ischemia. Our data suggest that hyperphosphorylation of 199/202 serine of tau is a finding specific for ischemic cell death.

What kind of kinase contributes to hyperphosphorylation of P199/202-tau?

To identify the kinases that contribute to hyperphosphorylation of P199/202-tau, we investigated the effects of kinase inhibitors injected intraventricularly (Fig. 2A and B). At 12 h post-ischemia, 199/202P-tau intensity increased to $175.5 \pm 14.7\%$ (mean $\pm$ SD, $p < 0.05$ compared to the control) Olomoucine, a CDK5 inhibitor, showed the strongest inhibition of P199/202-tau hyperphosphorylation at 12 h post-ischemia ($140.1 \pm 17.4\%$ of the control; $p < 0.05$ compared to ischemic animals not treated with olomoucine). U-0126, a MAP-kinase inhibitor, inhibited phosphorylation but not to a statistically significant degree ($157.2 \pm 11.9\%$ of the control). CDK5 phosphorylates tau factor at several epitopes relevant in AD [13,26–30]. CDK5 and its activator p35 are highly expressed in neurons [31,32] and the post-ischemic activation of CDK5 has been reported [18,33,34]. Ser202, Thr205, and Ser396 of tau are the phosphorylated sites of CDK5 and their hyperphosphorylation contributes to AD pathology [25,27]. Our data suggest that CDK5 is a key kinase for hyperphosphorylation of tau after transient ischemia and that it plays a major role in hippocampal DND.

Interestingly, wortmannin, a PI-3 kinase inhibitor, produced a statistically significant increase in P199/202-tau phosphorylation ($244.0 \pm 15.6\%$ of the control; $p < 0.05$ compared to ischemic animals not treated with wortmannin). Wortmannin is an inhibitor of PI3-K whose downstream target is Akt, which is activated by phosphorylation by PI3-K [35,36]. Activated Akt phosphorylates and inhibits GSK3α/β [37,38]. The inhibition of PI-3 kinase induces a decrease in Akt activity that results in decreased GSK-3 phosphorylation (Fig. 2C). The decrease in GSK-3 phosphorylation results in the activation of GSK-3. Based on our findings we suggest that after ischemia, Akt, activated by PI3-K, inhibits GSK3 by phosphorylation. Wortmannin inhibited Akt activation and GSK3 phosphorylated tau factor. In our study, MAPK inhibitor somewhat inhibited hyperphosphorylation of tau at 199/202. Only olmoucine partially inhibited DND ($46.9 \pm 22.8\%$ inhibition, $p < 0.05$) 7days after ischemia. We postulate that CDK5 is a major kinase for 199/202 serine hyperphosphorylation of tau and that MAPK and GSK contribute to post-ischemic hyperphosphorylation.

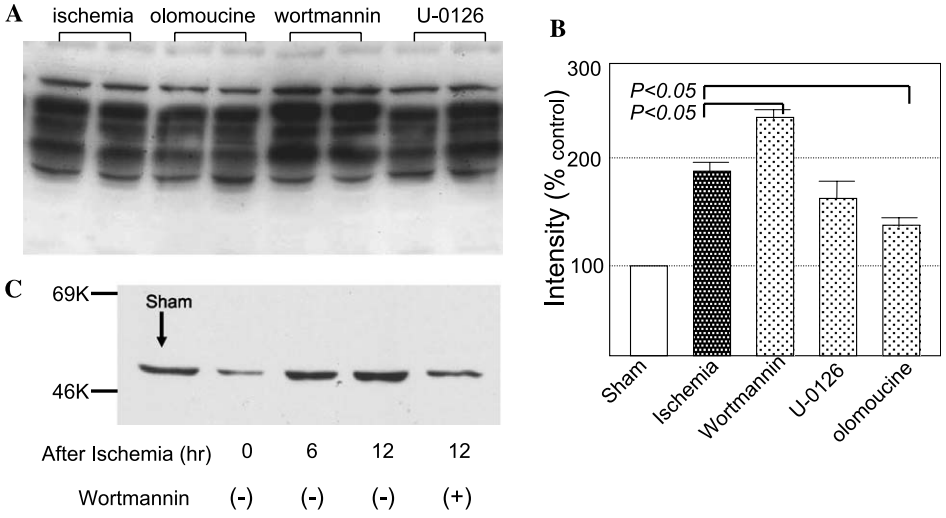

Fig. 2. Effect of various kinase inhibitors on 199/202-serine phosphorylation (A,B), and the phosphorylation state of GSK-3 kinase after ischemia (C). The inhibitors of CDK5 ($p < 0.05$) and MAPK (olomoucine and U-0126, respectively) decreased hyperphosphorylation, while PI-3 kinase inhibitor (wortmannin) increased phosphorylation ($p < 0.05$). The phosphorylation of GSK-3 increased after ischemia; wortmannin decreased phosphorylation.

Not only kinase, phosphatases may contribute to the hyperphosphorylation of tau factor, including Ser/Thr phosphatase protein 1, 2A, 2B (calcineurin), and 2C [13]. Elsewhere we have reported that the activity of calcineurin was, while that of phosphatase 2A was not, dramatically decreased in the rodent hippocampus after transient forebrain ischemia [3,4]. A decrease in calcineurin activity may play an important role in the mechanism(s) underlying DND.

The effect of recombinant human tau on ischemic neuronal cell death

We prepared recombinant human tau-40 conjugated to TAT-HA protein. In the preparation of control normal tau (N-tau40), 199/202 serine was phosphorylated in *E. coli* (Fig. 3A). Western blot analysis, using anti-199/202P tau antibody, showed faint immunoreactivity against D-tau40 (Fig. 3B). At 24 h after intraventricular injection, we

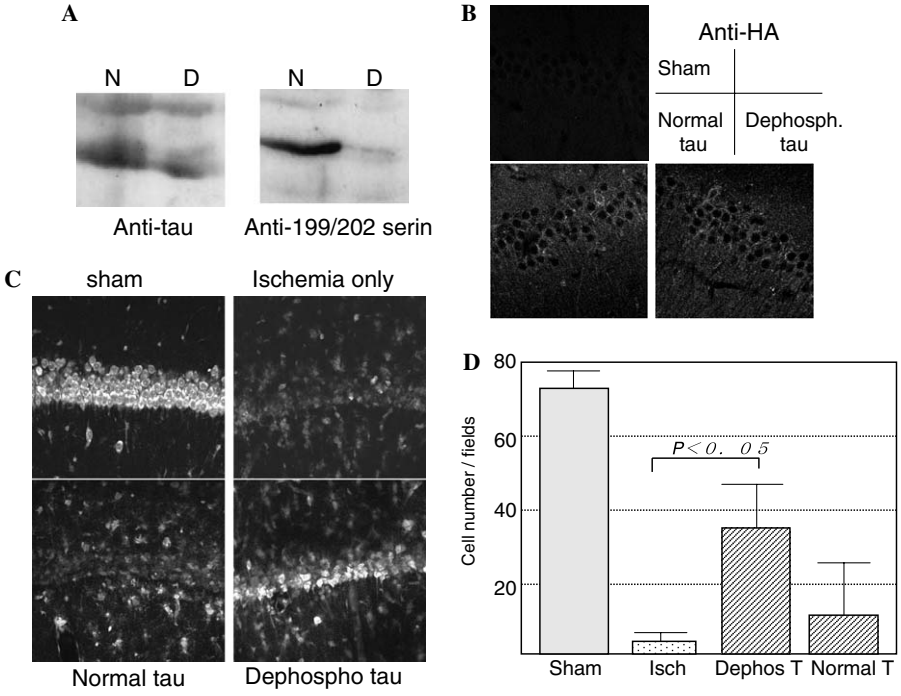

Fig. 3. Effect of TAT-dephosphorylated form tau. Western blot analysis of recombinant human tau proteins (N, normal tau; D, dephosphorylated form tau) showed that dephosphorylated form tau did not react with anti-phosphorylated 199/202 serine tau (A). Immunohistochemistry using anti-HA antibody showed that both TAT-recombinant proteins that had been injected into the lateral ventricle were detected in the cytosol of CA1 pyramidal neurons (B). Dephosphorylated tau protected against delayed neuronal death ($p < 0.05$; C, propidium-iodide staining and D).

detected anti-HA antibody immunoreactivity in neuronal somata in the CA1 region. We induced 5-min ischemia 12 h after ventricular injection and 7 days later, we investigated the inhibitory effect of recombinant tau protein against DND. D199/202 tau did, while N-tau did not, inhibit neuronal cell death (viable neurons; $49.4 \pm 24.0\%$ vs. $8.4 \pm 4.3\%$, $p < 0.05$) (Fig. 3C and D). Thus, the injection of D-tau40 into the lateral ventricle of our gerbils inhibited DND although N-tau40 did not. Western blots showed that TAT-N-tau was phosphorylated at 199/202 serine. We postulate that TAT-N-tau was phosphorylated in *E. coli* during the preparation of this protein and that this may explain the failure to protect against DND. TAT-D-tau also failed to protect completely from DND. While we examined only two phosphorylation sites of tau, 199/202 and 396 serines, at least 30 sites (from Thr39 to Ser422) have been found to exhibit various patterns of hyperphosphorylation in diseases such as AD [13]. In our study we focused on two serine sites that are known to play a role in AD and although these sites are considered important phosphorylation sites for DND, there may be others. Our findings document that hyperphosphorylation of 199/202 serine plays an important role in ischemic neuronal cell death. Although Ser396 was not hyperphosphorylated in our model, Noble et al. [25] reported Ser396 hyperphosphorylation in tangle formation. It is possible that there are differences in the pathology of AD and ischemic neuronal death.

Our findings suggest a common pathway of neuronal cell death in ischemia and degenerative diseases such as AD, although the sites of phosphorylation of tau factor differ somewhat. Studies are underway in our laboratory to elucidate the role of hyperphosphorylated tau in human neurodegenerative diseases.

Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Sports, Science and Culture of Japan.

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