

## Prolonged Alzheimer-like Tau Hyperphosphorylation Induced by Simultaneous Inhibition of Phosphoinositol-3 Kinase and Protein Kinase C in N2a cells

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**Abstract** Co-injection of wortmannin (inhibitor of phosphatidylinositol-3 kinase, PI3K) and GF109203X (inhibitor of protein kinase C, PKC) into the rat brain was found to induce spatial memory deficiency and enhance tau hyperphosphorylation in the hippocampus of rat brain. To establish a cell model with durative Alzheimer-like tau hyperphosphorylation in this study, we treated N2a neuroblastoma cells with wortmannin and GF109203X separately and simultaneously, and measured the glycogen synthase kinase 3 (GSK-3) activity by  $\gamma$ - $^{32}$ P-labeling and the level of tau phosphorylation by Western blotting. It was found that the application of wortmannin alone only transiently increased the activity of GSK-3 (about 1 h) and the level of tau hyperphosphorylation at Ser<sup>396</sup>/Ser<sup>404</sup> and Ser<sup>199</sup>/Ser<sup>202</sup> sites (no longer than 3 h); however, a prolonged and intense activation of GSK-3 (over 12 h) and enhanced tau hyperphosphorylation (about 24 h) were observed when these two selective kinase inhibitors were applied together. We conclude that the simultaneous inhibition of PI3K and PKC can induce GSK-3 overactivation, and further strengthen and prolong the Alzheimer-like tau hyperphosphorylation in N2a cells, suggesting the establishment of a cell model with early pathological events of Alzheimer's disease.

**Key words** Alzheimer's disease (AD); tau; glycogen synthase kinase 3 (GSK-3); protein kinase C (PKC); phosphatidylinositol-3 kinase (PI3K); N2a cells

Alzheimer's disease (AD) is the most common neurodegenerative disease affecting the quality of life of an increasing number of people in the population. Neuro-pathological studies have demonstrated that neurofibrillary tangle (NFT) is one of the most prominent pathologic characteristics in a brain affected by AD, and the abnormal hyperphosphorylation of the microtubule-associated protein tau is the major component of the tangles. It has been reported that the abnormal hyperphosphorylation of tau may impair its microtubule-binding ability and thus lead to their aggregation into paired helical filaments (PHFs) [1,2]. As a proven experimental model for AD has yet to be established, there is still no specific and effective cure for the disease. Therefore, any breakthrough in establish-

ing an AD-like experimental model will be a significant contribution to the field.

It is well accepted that the imbalance between protein kinases and protein phosphatases plays a key role in tau hyperphosphorylation, and glycogen synthase kinase (GSK)-3 $\beta$  is one of the kinases commonly implicated in tau hyperphosphorylation [3,4]. As a downstream element of the phosphatidylinositol-3 kinase (PI3K)-mediated signaling pathway, GSK-3 is activated by the inhibition of PI3K/protein kinase B (PKB) and the phosphorylation level at Ser<sup>9</sup> of GSK-3 $\beta$  or Ser<sup>21</sup> of GSK-3 $\alpha$  will decrease [5]. Our recent study showed that the application of wortmannin (inhibitor of PI3K) led to only a transient (<3 h) activation of GSK-3 with a concurrent increase in phosphorylation of tau in N2a cells, which very much limits drug screening because the pathological change wears away too fast [6]. This result also suggests that a certain type of negative feedback regulation might exist in wortmannin-induced

Received: January 13, 2005 Accepted: March 11, 2005

This work was supported by grants from the National Natural Science Foundation of China (30430270, 30472030 and 30400068), Science and Technology Committee of China (G1999054007) and National Educational Committee of China (2001-171)

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DOI: 10.1111/j.1745-7270.2005.00050.x

GSK-3 activation. It has been reported that the inactivation of PKB may activate caspase-3 and lead to phosphorylation (inactivation) of GSK-3 $\beta$  at Ser<sup>9</sup> through up-regulation of the protein kinase C (PKC) [7]. We also showed that the activation of GSK-3 in the rat brain by the simultaneous brain injection of wortmannin and GF109203X (a highly selective inhibitor of PKC) led to enhanced tau hyperphosphorylation and spatial memory impairment in the rat [8]. This confirms the above hypothesis and it also suggests a useful animal model of Alzheimer-like cytoskeletal pathology. The evidence strongly suggests that by using the strategy of simultaneously inhibiting PI3K and PKC, a reliable cell model representing Alzheimer-like tau hyperphosphorylation may be established.

In the present study, we employed wortmannin in conjunction with GF109203X to treat N2a cells in a culture, and thus produced a cell model with sustained Alzheimer-like tau hyperphosphorylation (up to 24 h).

## Materials and Methods

### Measurement of cell viability and metabolism

N2a cells were propagated in Dulbecco's modified Eagle's medium (DMEM) and Opti-MEM I reduced-serum medium (1:1; *V/V*) with 5% fetal bovine serum (Gibco, NY, USA) at 37 °C in 5% CO<sub>2</sub> and 95% air. Then, the cells were grown and differentiated in 96-well culture plates at a density of  $1.5 \times 10^5$  cells in 100  $\mu$ l. The cells were exposed to various concentrations (1  $\mu$ M, 0.5  $\mu$ M and 0.25  $\mu$ M) of wortmannin (Sigma, St. Louis, MO, USA) and/or GF109203X (Calbiochem, San Diego, CA, USA) for 6 h at 37 °C. Then, 0.2% crystal violet or 1% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Promega, Madison, WI, USA) in phosphate-buffered saline (PBS) was added and the cells were incubated for 3 min or 4 h in the dark, and the absorbance at 570 nm was read (TECAN Austria, Salzburg, Austria). All assays were performed seven times.

### GSK-3 activity assay by $\gamma$ -<sup>32</sup>P-labeling

N2a cells were cultured in the absence or presence of 1  $\mu$ M wortmannin+1  $\mu$ M GF109203X, and harvested after 1 h, 3 h, 6 h and 12 h. The cells were lysed in 50 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES, pH 7.0), 10 mM  $\beta$ -mercaptoethanol (ME), 1 mM ethylenediaminetetraacetic acid (EDTA), 1.0 mM  $\beta$ -glycerol phosphate, 1.0 mM Na<sub>3</sub>VO<sub>4</sub>, 50 mM NaF, 1 mM phenyl methyl sulfonyl fluoride (PMSF), 2 mM benzamidine,

1  $\mu$ g/ml leupeptin, pepstatin A and aprotinin, each on ice, and centrifuged at 12,000 g for 5 min. The supernatants were used for the kinase assay and Western blotting. The protein concentration was determined by a bicinchoninic acid kit (BCA; Chemical Company Rockford, IL, USA) to ensure equal loading for the kinase assay and Western blotting. The GSK-3 activity was assayed by using phospho-GS (Sigma, St. Louis, MO, USA) as a kinase substrate. The samples were incubated with the substrates in a buffer containing 30 mM Tris (pH 7.4), 10 mM MgCl<sub>2</sub>, 10 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 2 mM sodium ethylene glycol-bis( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 10 mM  $\beta$ -ME and 0.2 mM [ $\gamma$ -<sup>32</sup>P]ATP (Beijing Yahui Biologic and Medicinal Engineering Company, Beijing, China) at 30 °C for 30 min. The reaction was stopped by adding 12.5  $\mu$ l of 300 mM *O*-phosphoric acid. Reaction mixture aliquots of 10  $\mu$ l were applied in duplicate onto phosphocellulose units. The filters were washed 3 times with 75 mM *O*-phosphoric acid. The radioactivity incorporated into the substrates was analyzed by liquid scintillation counting. The GSK-3 activity was expressed as cpm/pmol ATP/mg protein/min.

### Western blotting

The proteins were dissolved in a sample buffer with 0.1 M Tris (pH 6.8), 4% sodium dodecyl sulfate (SDS), 20% glycerin, 0.2% bromophenol blue and 10%  $\beta$ -ME, and separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE). They were then transferred to polyvinylidene difluoride (PVDF) membranes. The phosphorylation of tau was monitored using the monoclonal antibody PHF-1 (recognizes phosphorylated tau Ser<sup>396</sup>/Ser<sup>404</sup>), tau-1 (recognizes non-phosphorylated tau at Ser<sup>199</sup>/Ser<sup>202</sup>) and the polyclonal antibody R134d (recognizes total tau). An enhanced chemiluminescent (ECL) substrate kit (Pierce, Rockford, IL, USA) was used to visualize the phosphorylation, which was exposed to Fuji film (Fuji Photo Film Company Limited, Tokyo, Japan). PHF-1, tau-1 and R134d were gifts from Dr. Peter DAVIES (Albert Einstein College of Medicine, Bronx, NY, USA), Dr. Lester BINDER (Northwestern University, Chicago, IL, USA) and Dr. Inge GRUNDKE-IQBAL (NYS Institute for Basic Research, Staten Island, NY, USA), respectively. The protein bands were quantitatively analyzed by using Kodak Digital Science 1D software (Eastman Kodak Company, New Haven, CT, USA), and the number of protein bands was expressed as a relative optical density.

### Statistical analysis

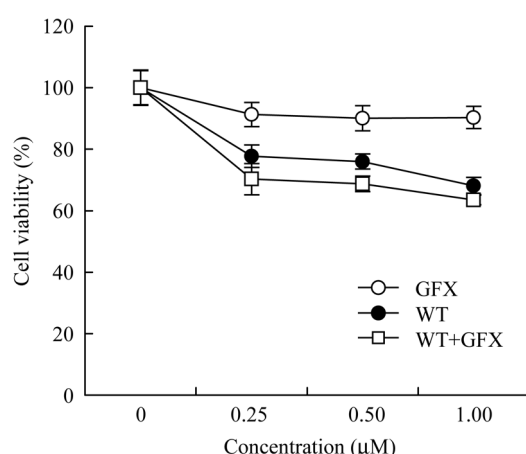
All experiments were repeated at least three times, with

similar findings. The results were expressed as mean $\pm$ SD and analyzed with SPSS 10.0 statistical software (SPSS Inc., Chicago, Illinois, USA). The one-way analysis of variance (ANOVA) procedure, followed by least significant differences (LSDs) *post hoc* tests, was used to determine the different means among groups ( $P<0.05$ ).

## Results

### Effect of wortmannin and GF109203X on cell viability

To study the optimum concentrations of wortmannin and GF109203X in N2a cells, we cultured cells in various concentrations (1  $\mu$ M, 0.5  $\mu$ M and 0.25  $\mu$ M) of wortmannin and GF109203X for 6 h, respectively. The effects of wortmannin and GF109203X on the viability of the cells were investigated by MTT assay. We observed that the relative cell viability decreased to 77.7%, 76.0% and 68.1% of the control level ( $P<0.01$ ) following treatment of the cells with wortmannin at concentrations of 0.25  $\mu$ M, 0.5  $\mu$ M and 1  $\mu$ M, respectively. A similar inhibition of cell viability (70.3%, 68.7% and 65.5%,  $P<0.01$ ) was observed when wortmannin and GF109203X were used in combination, but GF109203X used alone at concentrations of 0.25  $\mu$ M, 0.5  $\mu$ M and 1  $\mu$ M did not change cell viability significantly (**Fig. 1**). Therefore, we chose wortmannin and/or GF109203X at the optimum



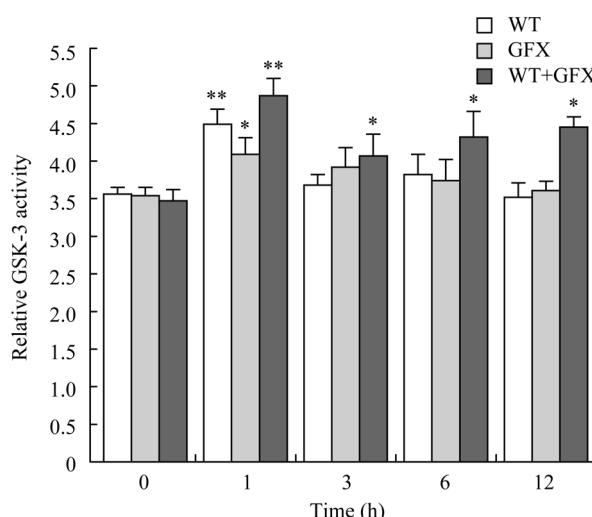
**Fig. 1** Effects of wortmannin (WT) and GF109203X (GFX) on the cell viability

Administration of WT alone ( $P<0.01$ ) or WT plus GFX ( $P<0.01$ ), but not GFX alone, significantly decreased cell viability. Data is presented as mean $\pm$ SD of seven independent observations.

concentration of 1  $\mu$ M for the following experiments.

### Effect of wortmannin and GF109203X on GSK-3 activity

The GSK-3 activity was measured by  $\gamma$ - $^{32}$ P-labeling protein kinase assay. We observed that the activity of GSK-3 was increased transiently at 1 h by treatment of the cells with 1  $\mu$ M wortmannin alone ( $P<0.01$ ), and it was restored to the normal control level at 3 h. Treatment of the cells with GF109203X alone also elevated the GSK-3 activity at 1 h ( $P<0.05$ ), and it was also restored at 3 h. In contrast, a sustained activation of GSK-3 to 137% ( $P<0.01$ ), 115% ( $P<0.05$ ), 122% ( $P<0.05$ ) and 125% ( $P<0.05$ ) of the control level at 1 h, 3 h, 6 h and 12 h, respectively, was achieved by simultaneous administration of wortmannin and GF109203X to the system (**Fig. 2**).

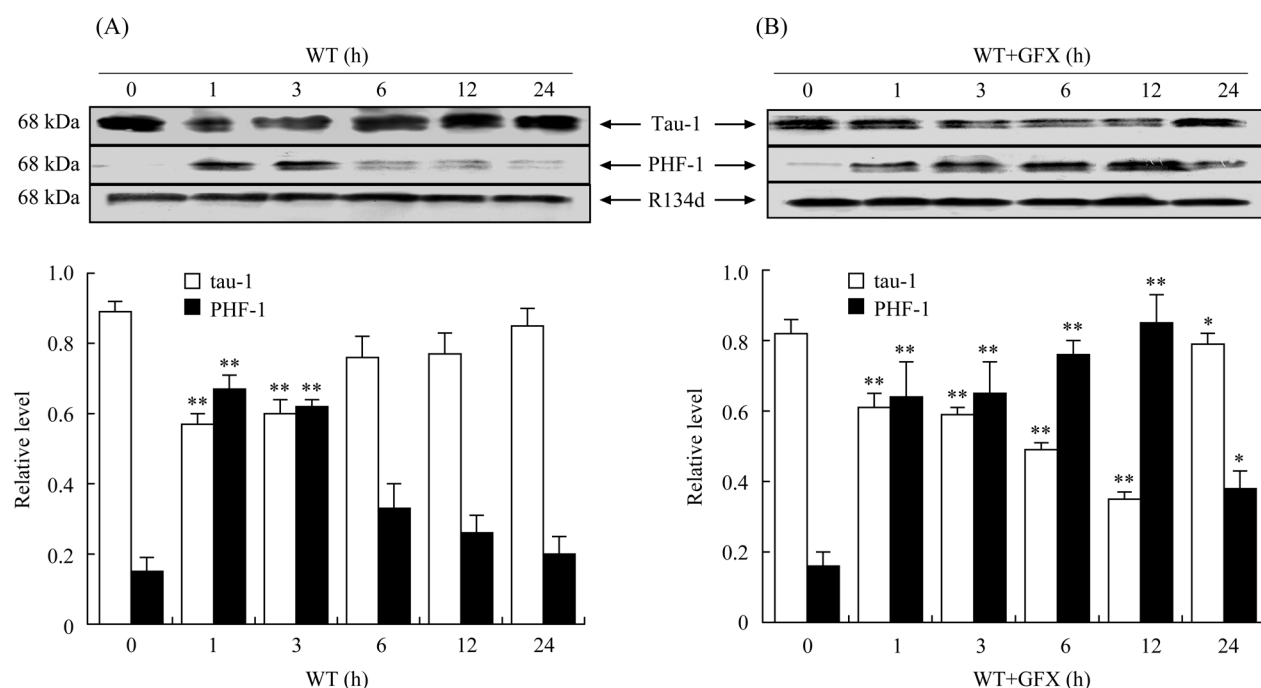


**Fig. 2** Effects of wortmannin (WT) and GF109203X (GFX) on GSK-3 activity

GSK-3 activity was increased temporarily by WT alone at 1 h compared to the control, and the activity was then restored at 3 h. GFX alone also elevated GSK-3 activity at 1 h. WT and GFX applied together led to a sustained higher GSK-3 activity at 1 h, 3 h, 6 h and 12 h, respectively. \*\* $P<0.01$  vs. control; \* $P<0.05$  vs. control.

### Effect of wortmannin and GF109203X on tau phosphorylation

As GSK-3 phosphorylates Ser<sup>396</sup>/Ser<sup>404</sup> (PHF-1 sites) and Ser<sup>199</sup>/Ser<sup>202</sup> (tau-1 sites) efficiently and it does not



**Fig. 3** Effects of wortmannin (WT) and WT plus GF109203X (GFX) on tau phosphorylation

(A) WT treatment alone only induced tau hyperphosphorylation at 1 h and 3 h. (B) WT plus GFX induced a sustained tau hyperphosphorylation for 24 h. The phosphorylation level of tau shown under each blot was normalized by the total level of tau probed by R134d. \*\* $P < 0.01$  vs. control; \* $P < 0.05$  vs. control.

phosphorylate Ser<sup>262</sup>/Ser<sup>356</sup>, we measured the phosphorylation level of tau by using PHF-1 and tau-1. We observed that the immunoreaction of tau-1 (binds to the unphosphorylated epitope of tau) decreased significantly at 1 h and 3 h ( $P < 0.01$ ), and it was reversed to the normal level at 6 h after treatment of the cells with wortmannin (1  $\mu$ M). The immunoreaction of PHF-1 (binds to the phosphorylated epitope of tau) was opposite to that of tau-1. It is suggested that the treatment of the cells with wortmannin alone only leads to transient hyperphosphorylation (for no more than 3 h) of tau at Ser<sup>199</sup>/Ser<sup>202</sup> (tau-1 epitope) and Ser<sup>396</sup>/Ser<sup>404</sup> (PHF-1 epitope) [Fig. 3(A)]. When the cells were treated with wortmannin (1  $\mu$ M) and GF109203X (1  $\mu$ M) simultaneously, the hyperphosphorylation of tau at the same epitopes was prolonged to 24 h, although the peak phosphorylation was seen at 12 h [Fig. 3(B)]. No obvious change in the immunoreactivity of tau was detected for R134d (binds to total tau) (Fig. 3).

## Discussion

In the AD brain, neurofibrillary tangles are found in the neuronal cell bodies and apical dendrites in the form of

aggregated PHFs, which are assembled from hyperphosphorylated tau [9]. GSK-3 $\beta$ , one of the most active enzymes in phosphorylating tau *in vitro*, has been directly linked to several of the key pathological mechanisms of AD [10]. GSK-3 $\beta$  activity is regulated by the PI3K pathway, which is initiated by insulin; phosphorylation and thus inactivation of GSK-3 $\beta$  takes place through the intermediate mediation of PKB. A previous study of SY5Y cells has shown that wortmannin treatment can induce the transient activation of GSK-3 $\beta$  and tau hyperphosphorylation; however, these effects were reversed by the simultaneous fragmentation (activation) of PKC [7]. Thus, blocking this negative effect of PKC activation may prevent the rapid inactivation of GSK-3 $\beta$  during administration of wortmannin and thus produce a model with sustained tau hyperphosphorylation. We have tested and confirmed this hypothesis in the rat brain system [8]. To establish a cell model with prolonged tau hyperphosphorylation in the present study, we used wortmannin to activate GSK-3 $\beta$ , and GF109203X to inhibit PKC in N2a cells at the same time. We found that the sustained activation of GSK-3 $\beta$  and durative Alzheimer-like tau hyperphosphorylation (up to 24 h) was achieved by this method. The alterations in N2a cells induced by

this method resemble what has been observed in the AD brain, not only in terms of tau hyperphosphorylation and GSK-3 $\beta$  overactivation, but also in terms of the decreased activity of PKC [11].

The PI3K signaling pathway plays a critical role in mediating survival signals in a wide range of neuronal cells, and it can be regulated by insulin, estrogen or neurotrophins to maintain normal cell functions. It has been reported that the physiological or pathological decline of insulin and estrogen in the human body may contribute to a higher incidence of aging-related diseases, including AD [12]. In addition, the PKC activity and level are significantly lower in patients with AD than in age-matched normal individuals, and the loss of inositol 1,4,5-triphosphate receptors and PKC in the entorhinal cortex and hippocampus correlates with AD-related neurofibrillary changes [13,14]. Our recent study further shows that the injection of wortmannin and GF109203X into the left ventricle of the rat brain leads to spatial memory impairment, with tangle-like neurons seen in the CA3 and CA4 regions of the rat brain [8]. These results imply that the decline of PI3K and PKC may promote the formation of neurofibrillary tangles in AD, probably through long-term GSK-3 $\beta$  overactivation.

Besides the involvement of GSK-3 $\beta$  in tau phosphorylation that may contribute to the formation of neurofibrillary tangles, GSK-3 $\beta$  has been linked to all of the primary abnormalities associated with AD. These include interactions between GSK-3 $\beta$  and components of the plaque-producing amyloid system, and interactions of GSK-3 $\beta$  with presenilin and other AD-associated proteins [15]. Our most recent results in another study also showed that the overactivation of GSK-3 $\beta$  led to the dysfunction of axonal transportation in N2a cells, which leads to a particular interest in establishing such an AD cell model (data not shown).

Additionally, it has been reported that the activation of PKC (which leads to inactivation of GSK-3 $\beta$ ) promotes  $\alpha$ -secretase-mediated proteolysis of the  $\beta$ -amyloid precursor protein (APP), which precludes the formation of  $\beta$ -amyloid [16,17]. Therefore, modulating the activity of PKC is the desired objective not only in establishing an experimental model, but also in developing effective drugs.

In the present study, we only observed strong PHF-1 staining in the plasma region of the cells, but we did not detect any tangle-like structure by immunofluorescence staining of the cells. Furthermore, after treatment with wortmannin and GF109203X for 24 h, the morphologic manifestation for most of the cells recovered from swelling, and the borders of many cells became clear again

although they were much shorter and there were fewer such occurrences than in the control group. The aspect of each cell also seemed irregular (data not shown). This suggests that there may be mechanisms protecting cells from being disturbed and even destroyed by the administration of drugs, which may include the corresponding oppositional function of the protein phosphatases, such as PP-2A and PP-1 [18]. Further study is needed to explore these detailed mechanisms although the cell model established in this study is sound for representing Alzheimer-like tau hyperphosphorylation.

In conclusion, we have found in the present study that the simultaneous down-regulation of PI3K and PKC can lead to a more sustained activation of GSK-3 and thus a prolonged Alzheimer-like hyperphosphorylation of tau in N2a cells.

## Acknowledgements

The authors thank Drs. Khalid IQBAL, Inge GRUNDKE-IQBAL and Chengxin GONG from NYS Institute for Basic Research (Staten Island, NY, USA) for the discussion and the supply of reagents.

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Edited by  
**Nai-He JING**