Activation of Glycogen Synthase Kinase 3 Induces Alzheimer-like Hyperphosphorylation of Cytoskeleton Protein and Cell Damage*

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Abstract Neurofibrillary tangles are the neuropathological hallmarks of Alzheimer disease (AD). Abnormally hyperphosphorylated tau and neurofilament (NF) are the components of neurofibrillary tangles. Hyperphosphorylation may be the result of an imbalanced regulation between protein kinases and protein phosphatases. Among the many kinases, glycogen synthase kinase-3(GSK-3) might be a key participator in neurodegeneration of AD. To investigate the role of GSK-3 on Alzheimer-like neurofibrillary degeneration, the wild type mouse neuroblastoma cell lines (N2awt) were treated with wortmannin (WT), an inhibitor of phosphatidylinositol 3-kinase (PI3K), and the effect of WT on cell metabolism, cell morphology, cell apoptosis, phosphorylation of NF and tau were detected, as well as the relationship between the alternations of these parameters and GSK-3 activity. It was found (1) that treatment of the cell with 1 μmol/L WT led to a transient (at 1h) activation of GSK-3 with a concurrent increase in phosphorylation of NF and tau. At 3h, the activity of GSK-3 was decreased and the hyperphosphorylation of NF was partially restored. (2) that WT decreased the cell metabolism detected by MTT assay in a dose dependent manner. (3) that treatment of the cell with 1 μmol/L WT for 1h or for 3h induced retraction of cell processes. (4) that no typical apoptotic damage was seen by transient stimulation of GSK-3 activity. It is suggested that transit overactivation of GSK-3 led to Alzheimer-like hyperphosphorylation of cytoskeleton protein and impairment in cell viability.

Key words Alzheimer disease, wortmannin, glycogen synthase kinase-3, neurofilament, phosphorylation

Alzheimer disease (AD) is the most common cause of dementia. Extracellular senile plagues (SP) composed of β-amyloid and intracellular neurofibrillary tangles (NFT) constituted of paired helical filaments (PHF) are neuropathological hallmarks of AD. Abnormally hyperphosphorylated tau is the major protein subunit of PHF. In addition to tau, it was also confirmed that abnormally hyperphosphorylated neurofilament (NF) was also an indispensable component of NFT[1, 2]. The destruction of cytoskeleton might be one of the most critical mechanisms in cell dysfunction and NFT formation seen in AD brain.

Generally the phosphorylated level of a protein is regulated by protein kinases and protein phosphatases. Among the many kinases, GSK-3 has been mostly implicated: GSK-3 is one of the most active enzymes in phosphorylating tau *in vitro*[3]. It is enriched in brain and active form of GSK-3 co-exists with neurofibrillary tangles[4]. Inhibition of GSK-3 arrests Aβ overproduction[5]. GSK-3 transgenic mice produce tangle-like structures and memory deficits[6, 7]. *In vivo*, activation of GSK-3 by brain injection of WT and GF-109203X led to hyperphosphorylation of tau and spatial memory impairment[8]. Therefore, GSK-3 might be served as a therapeutic target both in tau hyperphosphorylation and β-amyloid deposition.

In the present study, we have found that overactivation of GSK-3 in N2a cell led to AD-like hyperphosphorylation of tau and NF proteins, retraction of cell processes and a significant decrease in cell viability. No obvious cell apoptosis was observed during the activation of GSK-3 and hyperphosphorylation of the cytoskeleton proteins.

1 Materials and methods

1.1 Cell culture

Neuroblastoma N2awt cell was a generous gift from Dr. Xu (the Burnham Institute, La Jolla, California, USA). The cells were maintained in media containing 50% DMEM, 50% OPTI-MEM and 5% FBS (Gibico BRL, Gathersburg, MD, USA).

1.2 MTT assay

After cells were planted in 96-well plate for 24 h, they were treated with WT (Sigma, St. Louis, MO,

* This work was supported in part by grants from The National Natural Science Foundation of China (39925012, 30170221 and 30100057), The Science and Technology Committee of China (G1999054007) and The National Educational Committee of China (2001-171).

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Received: December 22, 2003 Accepted: January 20, 2004

USA). WT was dissolved in dimethyl sulfoxide (DMSO) and aliquots were stored at -30℃. To determine the effective dose of WT, cells were cultured in the absence or in the presence of 0.1 μmol/L, 1 μmol/L or 10 μmol/L WT for 1 h. Then 5 g/L MTT was added 4 h later, the wells were rinsed with FBS-free media. Sequentially cells were incubated with DMSO for 30 min at 37℃ and A_{570} was measured.

1.3 GSK-3 activity assay by γ^{32}P-labeling

GSK-3 activity was assayed using phospho-GS (Sigma, St. Louis, MO, USA) as a kinase substrate. The samples were incubated with the substrates in buffer containing 30 mmol/L Tris pH 7.4, 10 mmol/L $MgCl_2$, 10 mmol/L NaF, 1 mmol/L Na_3VO_4, 2 mmol/L Na_4EGTA, 10 mmol/L β-ME, 0.2 mmol/L γ^{32}P-ATP (Beijing Yahui Biologic and Medicinal Engineering Co., Beijing, China) at 30℃ for 30 min. Reaction was stopped by adding 12.5 μl 300 mmol/L O-phosphoric acid. 10 μl incubation mixture was applied in duplicates to phosphor cellulose units. The filters were washed 3 times with 75 mmol/L O-phosphoric acid. And the radioactivity incorporated into the substrates was analyzed by liquid scintillation counting. GSK-3 activity was expressed as pmol phosphate incorporated/mg of protein/min at 30℃.

1.4 Western blot

The proteins were dissolved in sample buffer with 0.1 mol/L Tris, pH 6.8, 4% SDS, 20% glycerin, 0.2% bromophenolblue and 10% β-ME. Total protein concentration was determined by the BCA (Bicinchoninic Acid) assay, using BSA as standard. SDS-PAGE gel stained by Coomassie blue was used as inter-reference (Figure 1a lower lane). The proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Anti-GSK3β-Ser9-p (Cell Signaling Technology, Inc. Beverly, MA, USA) was used to detect the level of phosphorylated GSK3β at site Ser9 (inactivated form of GSK3β). The phosphorylation of tau was studied by monoclonal antibody PHF-1 (recognize phosphorylated tau Ser296/Ser404), tau-1 (recognize non-phosphorylated tau at Ser198/Ser202) and 111e (recognize phosphorylated and non-phosphorylated tau). Alkaline phosphatase-labeled goat anti-mouse IgG was used as second antibody and the blots were developed by 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT, Amersham Pharmacia Biotech, Piscataway, NJ, USA).

1.5 Immunocytochemistry

Cultured cells were incubated with primary antibody SMI31 (recognize phosphorylated NF) or SMI32 (recognize non-phosphorylated NF) (Sternberger Inc, Lutherville, MA, USA) in a humidified chamber for 12 h at 4℃, then with biotin labeled goat anti-mouse IgG (1∶200) for 45 min at 37℃, and with HRP labeled avidin (1∶200) for 30 min at 37℃. The slices were developed with diaminobenzidine (DAB, Sigma, St. Louis, MO, USA) system. The images were analyzed quantitatively by a Kodak Digital Science 1D software (Cold Spring Harbor Inc., Beijing, China). At least 200 cells were computed and analyzed in each experiment. To detect the actual phosphorylation state of NF, the increased ratio of SMI31 or SMI32 to each of the controls was calculated.

1.6 Transmission electron microscopy

The cells were washed with PBS and fixed for 1 h at 37℃ with 0.1 mol/L cacodylate, pH 7.0 containing 2.5% gluteraldehyde and 0.2% tannic acid. The EPON812 embedded sections were cut (LKBV ultramicrotome, Germany) and stained with uranyl acetate and lead citrate, viewed with an Opton EN/l0C transmission electron microscope (Opton, Germany).

1.7 TUNEL labeling

Cell samples were fixed with a freshly prepared paraformaldehyde solution (4% in PBS, pH 7.4) at room temperature for 1 h. The slides were rinsed with phosphate-buffered saline (PBS) and incubated in permeabilisation solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 min on ice (4℃). TUNEL reaction mixture was added into the samples and the slides were incubated for 60 min at 37℃ and analyzed by light microscopy (TUNEL kit was from Boehringer Mannheim GmbH, Germany).

1.8 Statistical analysis

Data were expressed as $\bar{x} \pm s$ and analyzed using SPSS 10.0 statistical software (SPSS Inc., Chicago, Illinois, USA). The One-Way ANOVA procedure followed by LSD's post hoc tests was used to determine the different means among groups ($P < 0.05$).

2 Results

2.1 WT induces transit activation of GSK-3 and phosphorylation of GSK-3 at Ser9

To determine the effect of WT on GSK-3 activity and the level of Ser-9-phosphorylated GSK3, cells were treated with 1 μmol/L WT for 1 h, 3 h, or 6 h, respectively. It was found that at 1 h after the treatment, GSK-3 activity increased significantly ($P < 0.01$) to the control level with a concurrent decrease in expression of inactivated form of GSK-3 (i.e. Ser-9-phosphorylated GSK-3). The activity of GSK-3 was obviously lower than that of control at 3h, it was restored to the control level at 6h after the treatment, and the expression of phosphorylated GSK-3 was restored to the normal control level at these two time points (Figure 1). This data suggests a transit activation of GSK-3 induced by 1 μmol/L WT.

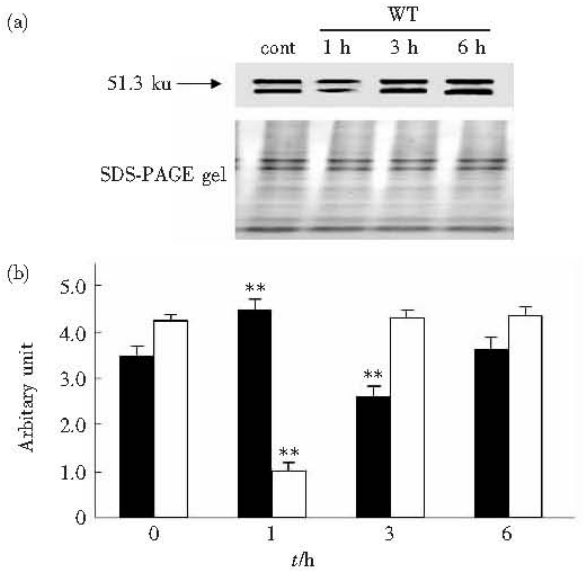

Fig. 1　Effect of WT on GSK-3 activity and expression of Ser9-phosphorylated GSK-3

(a) Expression of Ser9-phosphorylated GSK-3 induced by 1 μmol/L WT for 1 h, 3 h and 6 h detected by Western Blot. (b) Activity of GSK-3 measured by ^{32}P-labeling and quantitative analysis of Ser9-phosphorylated GSK-3. With increased GSK-3 activity observed at 1 h, the level of inactivated Ser9-phosphorylated GSK-3 decreased significantly. SDS-PAGE gel stained by Coomassie blue according to BCA assay of protein concentration (20 g/L) was used as interference. Data are ($\bar{x} \pm s$) of eight observations. $^{**}P < 0.01$ vs control. ■: GSK-3 activity; □: Ser9-GSK-3β.

2.2　WT induces hyperphosphorylation of NF and tau proteins

The phosphorylated level of NF was detected by immunocytochemistry (Figure 2). Phosphorylated NF recognized by SMI31 was mainly located in cell processes (Figure 2a) and non-phosphorylated NF was detected both in cell processes and in cytoplasm (Figure 2b). After treatment with WT for 1 h or 3 h, the immunoreaction of phosphorylated (SMI31) (Figure 2 c, e) and non-phosphorylated (SMI32) NF was significantly enhanced in cytoplasm (Figure 2d, f). The immunoreaction of SMI31 reached to the peak at 1 h (Figure 2c) and of SMI32 at 3 h (Figure 2f). At 6 h, the immunoreaction for both SMI31 and SMI32 tends to restore to the control level (not shown). To detect the actual phosphorylation state of NF, the increased ratio of SMI31 or SMI32 to each of the controls was calculated. It was shown that the increased ratio of SMI31 at 1 h (176.9%) and 3 h (125.6%) was higher than that of SMI32 at 1 h (10.4%) and 3 h (38.6%) after WT treatment. It suggested that NF was hyperphosphorylation at 1 h and 3 h after WT treatment.

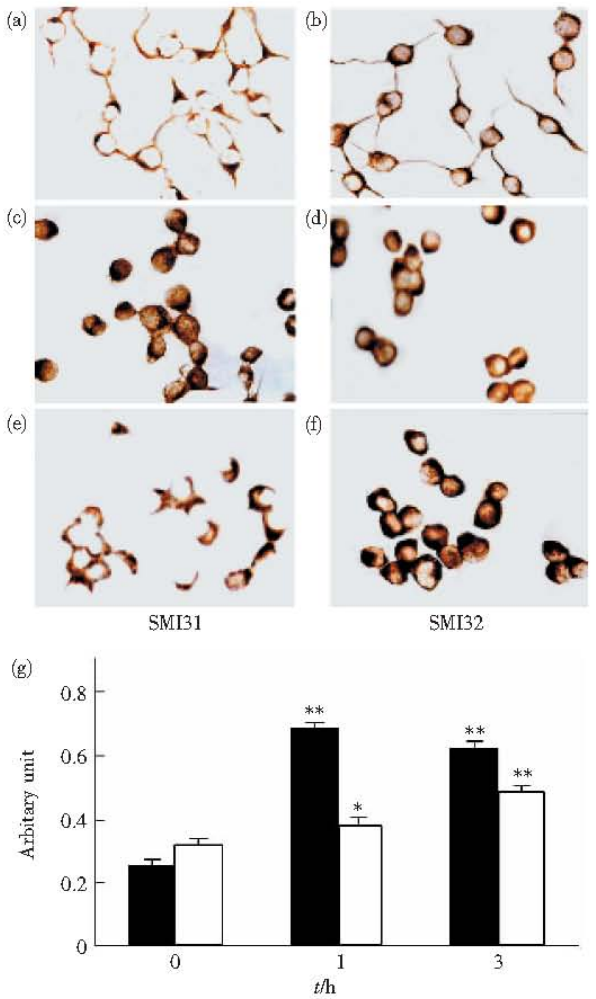

Fig. 2　Effect of WT on NF phosphorylation by immunocytochemistry

(a) NF is distributed evenly in cell body and cell processes in normal condition (a, b). CA led to accumulation of both phosphorylated and unphosphorylated NF in the cytoplasma, especially at hillock for 1 h (c, d) and 3h (e, f) (Magnification ×400). (g) Quantitative analysis of WT-induced neurofilament phosphorylation determined by SMI31 and SMI32. The level of both phosphorylated and non-phosphorylated NF was increased, but the increased rate was higher in phosphorylated form (see text). ■: SMI31; □: SMI32.

The phosphorylated level of tau was detected by Western blot. After the treatment of WT for 1h, the immunoreaction of Tau-1 decreased significantly compared with the control. No obvious change was observed for PHF-1. It suggested that treatment of WT led to hyperphosphorylation of tau at Ser198/Ser199/Ser202 via activation of GSK-3 in N2a cells (Figure 3).

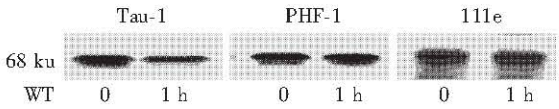

Fig. 3 Effect of WT on tau and NF phosphorylation
After treatment of WT for 1 h, the immunoreaction of Tau-1 decreased significantly compared with the control. No significant change was observed for PHF-1 and 111e.

2.3 WT induces cell retraction and decreased cell viability

Cells were cultured in the absence or in presence of 0.1 μmol/L, 1 μmol/L or 10 μmol/L of WT for 1 h. And cell morphology was observed by phase contrast microscopy. It was found that in the control group the border of the cells was clear with plenty of processes, and treatment of the cells with 0.1 μmol/L or 1 μmol/L of WT induced retraction of cell processes (Figure 4). When the concentration of WT was increased to 10 μmol/L, the cells were almost all detached from the plate (not shown).

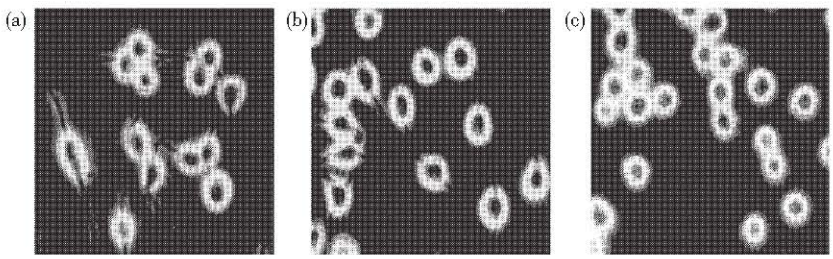

Fig. 4 Effect of WT on cell morphology
Compared with control (a), treatment of the cells with 0.1 μmol/L (b) or 1 μmol/L (c) for 1 h of WT induced retraction of cell processes. (Magnification ×400)

MTT method was used to analyze the influence of WT on cell viability. Statistical data displayed that the value of A_{570} decreased notably with increased concentration of WT (Figure 5).

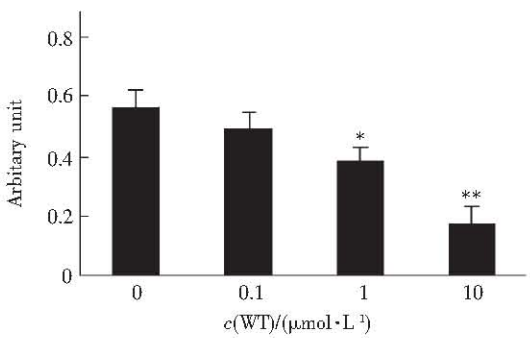

Fig. 5 Effect of WT on cell viability
A dose-dependent MTT reduction was observed at 1 h in the presence of WT. Data are $(\bar{x} \pm s)$ of eight observations. $*P<0.05$, $**P<0.01$ vs control.

2.4 No typical apoptosis was observed by treatment of WT

N2awt were treated with 1 μmol/L WT for 1h and cell apoptosis was detected by TUNEL and electromicroscopy. Although nuclear staining was seen in WT-treated sample, no notable difference was detected when compared with control group. By electromicroscopy, it was seen that the nucleus membrane was intact and the nucleolus was clear in control. After WT treatment for 1 h, the nucleolus vanished, and diffused accumulation of chromatin around the nuclear membrane was seen (Figure 6).

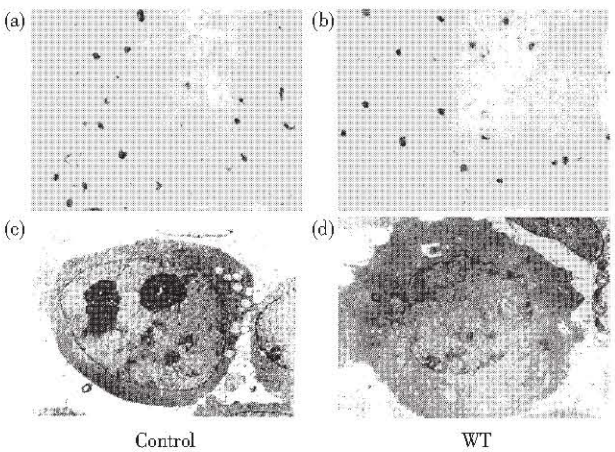

Fig. 6 Effect of WT on cell apoptosis detected by TUNEL (a, b) and electromicroscopy (c, d)
Compared with control (a, c), no typical apoptosis was detected after 1 μmol/L WT treatment for 1 h (b, d). (Magnification for (a) and (b) is ×100 and for (c) and (d) is ×6000)

3 Discussion

GSK-3 is a juncture of three signal transduction cascades, i.e. the Wnt, the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3-kinase (PI3K)[9]. Activation of PI3K/PKB pathway commences when insulin or growth factors bind their respective receptors[10, 11]. This facilitates the phosphorylation of PKB. Subsequently, PKB can phosphorylate Ser9 of GSK-3β or Ser21 of GSK-3α, and thus inhibits the enzyme activity[12]. Therefore, regulation of PI3K/PKB pathway and the subsequent

activation/inhibition of GSK-3 is one mechanism by which the activity of GSK-3 is regulated. In the present study, we chose WT to inhibit PI3K and found that after treatment of 1 μmol/L WT for 1 h, the activity of GSK-3 was significantly increased accompanied by a decrease in the level of Ser9-phosphorylated GSK-3. This testifies that in N2awt PI3K/PKB pathway participates in the regulation of GSK-3 activity. At 3h after the treatment, GSK-3 activity decreased to an even lower level than that of the control, suggesting certain type of negative feedback regulation or antagonistic mechanisms in the cell line. It was reported that inactivation of PKB in SY5Y cell line might activate caspase-3, which led to GSK-3 phosphorylation and inactivation through activating protein kinase C (PKC)[13]. Additionally, dephosphorylation of GSK-3αTyr^{279} or GSK-3βTyr^{216}, the required phosphorylation sites for the activity of GSK-3, may also participate in this regulating process[14]. Additionally, tau phosphorylation by GSK-3 is also regulated by its prephosphorylation by some non-proline-directed protein kinases, such as protein kinases A[15].

GSK-3, by virtue of its role as a major tau kinase, is a leading candidate for initiating pathologic tau hyperphosphorylation[16~18]. GSK-3 forms a complex with tau in the microtubule fraction from the bovine brain[19] and is co-localized with phosphorylated tau during development[20]. A number of studies suggest that hyperphosphorylation of tau by GSK-3 accelerates neurodegeneration and induces fibrillary tau-immunoreactive inclusions *in vivo* and *in vitro*[21]. In the present study, we have found that treatment of N2a cell with WT for 1h induces tau hyperphosphorylation at Ser198/Ser199/Ser202 via activation of GSK-3. Although only a transit overactivation of GSK-3 by WT, the cell damage both morphologically and metabolically was already very significant. This implies that extremely subtle imbalanced phosphorylation of tau protein may govern the neuropathological changes taking place in AD brain.

In addition to tau, NF also hyperphosphorylated and accumulated in AD brain. NF acts as modulators of caliber of large myelinated axons as well as axonal transport[1]. NF is required for the axonal regeneration. It was reported that hyperphosphorylation of NF led to dissociation of microtubules from NF and destruction of cell structure[22, 23], although the physiologic level of NF phosphorylation facilitates their incorporation into a stationary network and in slowing transport[24]. After hyperphosphorylation, NF loses its configuring function[25]. Besides, hyperphosphorylated NF resists to proteolysis contributes to the NF accumulation in the nucleus[26]. In the present study, we have observed that treatment of the cell with WT enhances phosphorylation of NF, this is in agreement with the abnormality of NF observed in AD, including perikaryal accumulation, dystrophic neurite formation and down regulation of NF expression.

We also observed that although treatment of the cells with WT led to significant decreased cell viability with cytoskeleton (tau and NF) hyperphosphorylation, but no typical apoptosis was observed. This result is in consistent with the observation received in SY5Y cells that tau phosphorylation seemed to be anti-apoptosis[27]. In another experiment, we also found that treatment of the cells with CA led to an NF hyperphosphorylation and aggregation with minimal apoptosis[28]. Although some people believe that progressive cell loss in specific neuronal populations often associated with typical cytoskeletal protein aggregations is a pathological hallmark of neurodegenerative disorders, the nature, time course and molecular causes of cell death and their relation to cytoskeletal pathologies are still unresolved. Based on recent data in human brain, as well as in animal and cell culture models, a picture is beginning to emerge suggesting that, in addition to apoptosis, other forms of programmed cell death may participate in neurodegeneration[29]. Better understanding of the molecular players will further elucidate the mechanisms of cell death in these disorders and their relations to cytoskeletal abnormalities. Therefore, although many *in vivo* and *in vitro* data are in favor of apoptosis involvement in neurodegenerative processes, there is considerable evidence supporting that very complex events may contribute to neuronal death with possible repair mechanisms, the elucidation of which may prove useful for future prevention and therapy of neurodegenerative disorders.

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GSK-3 在阿尔茨海默病样细胞骨架蛋白过度磷酸化中的作用*

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摘要 神经原纤维缠结是阿尔茨海默病（Alzheimer disease，AD）的特征性病理改变. 蛋白激酶和蛋白磷酸酯酶失衡可导致骨架蛋白的异常过度磷酸化，而异常过度磷酸化的 tau 和神经丝（neurofilament，NF）是神经原纤维缠结的组成部分. 在众多激酶中，糖原合酶激酶-3（glycogen synthase kinase-3，GSK-3）可能是 AD 神经退行性变起重要作用. 为深入探讨 GSK-3 在 AD 样神经退行性变中的作用，以磷酯酰肌醇三磷酸激酶（phosphatidylinositol 3-kinase，PI3K）的特异性抑制剂渥曼青霉素（wortmannin，WT）处理野生型鼠成神经瘤细胞株（wild type mouse neuroblastoma cell lines，N2a wt），系统观察 WT 处理 N2a wt 不同时间点（1 h、3 h、6 h）细胞代谢率、细胞形态、细胞骨架蛋白 tau 和 NF 的磷酸化状态改变以及细胞的命运，并分析了 GSK-3 活性与上述参数改变之间的相关性. 结果发现：1 μmol/L WT 处理细胞 1 h，GSK-3 活性与未经 WT 处理的对照组相比明显增高，并伴有 Ser9 磷酸化的 GSK-3 水平的降低；NF 磷酸化程度增强，tau 在 Ser198/Ser199/Ser202 位点的磷酸化增强. 1 μmol/L WT 处理细胞 3 h，GSK-3 活性与对照组和处理 1 h 组相比明显下降，NF 磷酸化程度较 1 h 降低，但仍高于正常水平. 1 μmol/L WT 处理细胞 6 h，细胞形态、GSK-3 活性、Ser9 磷酸化形式的 GSK-3β 的表达、NF 磷酸化程度与对照组相比均无明显改变. WT 呈剂量依赖性降低细胞代谢率. 1 μmol/L WT 处理细胞 1 h 和 3 h 导致细胞变圆，突起变短甚至消失. 1 μmol/L WT 处理细胞 1 h，用 TUNEL 法和电子显微镜技术未观察到细胞凋亡. 研究结果提示：在 N2a 细胞中过度激活 GSK-3 可导致神经细丝和 tau 蛋白的 AD 样过度磷酸化，从而引起神经细胞的 AD 样退行性变.

关键词 阿尔茨海默病，渥曼青霉素，糖原合酶激酶-3，神经细丝，磷酸化
学科分类号 R745.7，Q513

* 国家自然科学基金（39925012、30170221 和 30100057），国家科技部基金（G999054007）和国家教育部基金（2001-171）资助项目.
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收稿日期：2003-12-22，接受日期：2004-01-20