

# Alzheimer-like tau phosphorylation induced by wortmannin *in vivo* and its attenuation by melatonin<sup>1</sup>

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**KEY WORDS** Alzheimer disease; neurofibrillary tangles; tau proteins; glycogen synthase; wortmannin; melatonin; rats

## ABSTRACT

**AIM:** To investigate the *in vivo* induction of Alzheimer-like tau phosphorylation by wortmannin and the attenuation by melatonin. **METHODS:** Stereotaxic technique was used for administrating wortmannin or melatonin into rat lateral ventricle. The phosphorylation of tau was analyzed by immunocytochemistry and Western blot, and ultrastructural alteration in neuronal processes was detected by electron microscopy. **RESULTS:** Level of phosphorylated tau at paired helical filament-1 (PHF-1) epitope was elevated at 1 h, peaked at 6 h, and then decreased at 12 h and 24 h after injection of wortmannin  $10 \mu\text{mol} \cdot \text{L}^{-1}$ . The increased tau phosphorylation at particular epitope determined at 6 h was arrested by preinjection of melatonin  $10 \mu\text{mol} \cdot \text{L}^{-1}$  or  $100 \mu\text{mol} \cdot \text{L}^{-1}$  for 12 h. The wortmannin-induced hyperphosphorylation of tau was mainly detected in pyramidal neuron of hippocampus, and swelled axons and depredated myelin sheaths were also seen in the same region of the brain. **CONCLUSION:** Wortmannin induced *in vivo* Alzheimer-like hyperphosphorylation of tau at Ser396/404, and melatonin inhibited partially the pathological processes.

## INTRODUCTION

Abnormally phosphorylated microtubule-associated

protein tau (AD P-tau) is the major protein subunit of paired helical filaments (PHF) in brain of Alzheimer disease (AD) patients, and accumulation of PHF in affected neurons leads to the formation of neurofibrillary tangles (NFT) and neuropil threads<sup>1</sup>. Although the precise mechanism for tau hyperphosphorylation is not currently understood, it is widely recognized that an imbalanced regulation in phosphorylation (catalyzed by protein kinase) and dephosphorylation (catalyzed by protein phosphatase) system may play an important role in this pathological process.

*In vitro* studies have shown that various protein kinases, such as mitogen activated protein kinases (MAPK), protein kinase A (PKA), cyclin-dependent kinase (CDK), and glycogen synthase kinase-3 (GSK-3) phosphorylated tau at the same epitope were seen in Alzheimer disease brain<sup>2,7</sup>. Among them, GSK-3 is the most active enzyme in phosphorylating tau *in vitro* at Ser396/404, a pivotal amino acid in AD P-tau. Over-expression and activation of GSK-3 $\beta$  in transfected neuroblastoma cell line also induces hyperphosphorylation of tau. Furthermore, GSK-3 is co-localized with neurofibrillary tangles in AD affected neurons<sup>3,1</sup>. Taken together, it is suggested that GSK-3 is a crucial kinase involved in hyperphosphorylation of tau in AD brain.

GSK-3 has two isoforms, named  $\alpha$  and  $\beta$ , both of them are microtubule-binding proteins and enriched in brain<sup>1,1</sup>. GSK-3 is a downstream element of phosphatidylinositol-3 kinase (PI-3K), it is inhibited by protein kinase B (PKB) catalyzed phosphorylation at Ser-9 of GSK-3 $\beta$  and Ser-21 of GSK-3 $\alpha$ . PKB in activity is stimulated by PI-3K mediated phosphorylation<sup>5,1</sup>. Based on the above pathway, we speculated that wortmannin, a specific inhibitor of PI-3K, used as a specific stimulator for GSK-3<sup>6,1</sup>, might also phosphorylate tau *in vivo*.

Melatonin has been demonstrated to regulate the biological rhythm; it is also found recently that melatonin is effective in attenuating amyloid  $\beta$ -protein ( $A\beta$ ) toxicity<sup>7,8,1</sup> and Alzheimer-like tau phosphorylation in SY5Y neuroblastoma cells (manuscript in preparation).

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Whether melatonin also attenuates tau phosphorylation *in vivo* is not known. In the present study, we tested our working hypothesis and tried to answer the above raised questions.

## MATERIALS AND METHODS

**Drugs and reagents** Wortmannin was from Sigma. Monoclonal antibody PHF-1, which specifically recognizes tau phosphorylated at Ser396/404, was a generous gift from Dr Davis (Albert Einstein College of Medicine, USA).

**Animals** Wistar rats ( $\delta$ , 275 g  $\pm$  25 g, Grade II, Certificate No TJLA2000 - 3) were supplied by Center of Laboratory Animal, Tongji Medical College, Huazhong University of Science and Technology.

**Administration of wortmannin or melatonin into rat brain** Wortmannin ( $10 \mu\text{mol} \cdot \text{L}^{-1}$ )  $5 \mu\text{L}$  was administered into rat lateral ventricle. To study the effect of melatonin on wortmannin-induced hyperphosphorylation of tau, equal volume of melatonin with concentration of  $1 \mu\text{mol} \cdot \text{L}^{-1}$ ,  $10 \mu\text{mol} \cdot \text{L}^{-1}$ , and  $100 \mu\text{mol} \cdot \text{L}^{-1}$  were injected at 12 h before administration of wortmannin. At each condition, equal volume of artificial cerebrospinal fluid (aCSF) was injected as control. The drugs were delivered to the place of 1.2 mm posterior and 2.2 mm lateral to bregma, and 3.5 mm in depth to skull and the position was determined by stereotaxic apparatus<sup>(9)</sup>.

### Immunocytochemistry and Western blotting

For immunocytochemistry, the brain was fixed in Zamboni's solution for 12 h after *in situ* perfusion with the same solution<sup>(10)</sup>, then the blocks including hippocampus were paraffin-embedded, and the sections were cut into 6  $\mu\text{m}$  in thickness. After deparaffinization and rehydration, the sections were incubated with PHF-1 (1:500) for 12 h at 4  $^{\circ}\text{C}$ , incubated with anti-mouse IgG-HRP for 1 h at 37  $^{\circ}\text{C}$ , then developed with diaminobenzidine (DAB) system<sup>(3)</sup>. Western blotting was carried out according to the procedure described previously<sup>(3)</sup>. The level of tau phosphorylation was analyzed by using Kodak Digital Science 1D software.

**Electron microscopy** Brain was fixed in 4% paraformaldehyde-0.05% glutaraldehyde for 12 h after *in situ* perfusion, and the ultrastructure was detected according to the method previously described<sup>(11)</sup>.

**Statistical analysis** Results were expressed as  $\bar{x} \pm s$  and analyzed by ANOVA followed by Student-Newman-Keul's test.

## RESULTS

**Wortmannin-induced Alzheimer-like phosphorylation of tau *in vivo*** Compared with vehicle control, the level of tau bound to PHF-1 was increased to 1.6, 3.6, 4.6, 3.0, and 1.3 folds at 1 h, 3 h, 6 h, 12 h, and 24 h after administration of wortmannin, respectively ( $P < 0.01$ , Tab 1, Fig 1). The increased binding of tau to the same antibody was arrested to 65% and 34% respectively, by pre-injection of melatonin  $10 \mu\text{mol} \cdot \text{L}^{-1}$  or  $100 \mu\text{mol} \cdot \text{L}^{-1}$  ( $P < 0.01$ ), although the reaction of tau to the antibody on these conditions was still higher than that of vehicle control ( $P < 0.01$ ). No significant difference was observed by pre-injection of  $1 \mu\text{mol} \cdot \text{L}^{-1}$  of melatonin ( $P > 0.05$ , Tab 2, Fig 2). The data suggested that hyperphosphorylation of tau at Ser-396/404 was induced *in vivo* by injection of wortmannin, and the abnormal modification of tau at this epitope was inhibited partially by pre-injection of melatonin. Immunocytochemistry data demonstrated that positive reaction of PHF-1 to neuronal cell bodies and dendrites was only detected in hippocampal CA1 region of wortmannin treated rats, but not in aCSF injected control (Fig 3), which further confirmed that wortmannin could induce *in vivo* hyperphosphorylation of tau.

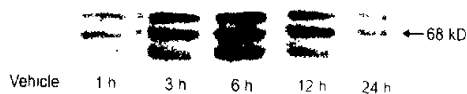


Fig 1. Immunoblot of PHF-1 tau after injection of wortmannin  $10 \mu\text{mol} \cdot \text{L}^{-1}$  determined at time points shown under each lane.

Tab 2. Attenuation of wortmannin ( $10 \mu\text{mol/L}$ )-induced tau phosphorylation at 6 h by different concentrations of melatonin.  $n = 6$ .  $\bar{x} \pm s$ .  $^*P < 0.01$  vs vehicle control.  $^{\#}P > 0.05$ ,  $^{\dagger}P < 0.01$  vs wortmannin.

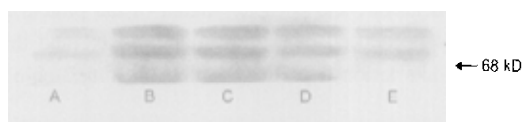
Treatment	$10^{-1} \times$ Phospho-tau
Vehicle control	$8.64 \pm 0.14$
Wortmannin	$34 \pm 5^*$
Wortmannin + melatonin $1 \mu\text{mol/L}$	$32 \pm 3^{\dagger}$
Wortmannin + melatonin $10 \mu\text{mol/L}$	$22 \pm 3^{\#}$
Wortmannin + melatonin $100 \mu\text{mol/L}$	$11.7 \pm 1.7^{\dagger}$

Data were expressed as sum intensity of PHF-1 positive tau by Western blot.

**Wortmannin-induced axonal lesion in rat brain** The caliber of axons was significantly enlarged at 6 h after injection of wortmannin when compared with

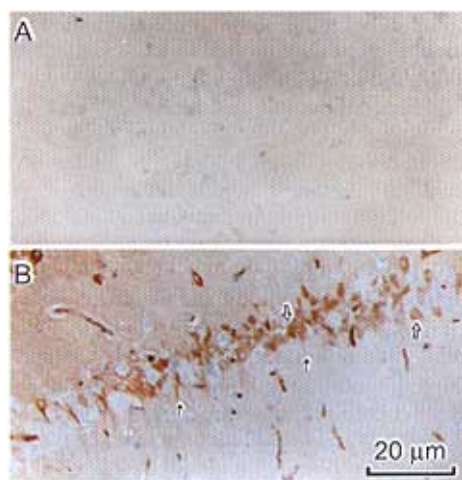
**Tab 1. Kinetics of tau phosphorylation induced by wortmannin  $10 \mu\text{mol} \cdot \text{L}^{-1}$ .  $n = 6$ .  $\bar{x} \pm s$ .  $^*P < 0.01$  vs vehicle control.**

Treatment	$10^{-1} \times$ Sum intensity of PHF-1 positive tau				
	1 h	3 h	6h	12 h	24 h
Vehicle control	$8.02 \pm 0.23$	$7.8 \pm 0.3$	$7.98 \pm 0.14$	$7.97 \pm 0.09$	$7.84 \pm 0.21$
Wortmannin	$12.6 \pm 2.7^*$	$28 \pm 5^*$	$37 \pm 5^*$	$24 \pm 6^*$	$10.5 \pm 1.7^*$



**Fig 2. Effect of melatonin on wortmannin ( $10 \mu\text{mol} \cdot \text{L}^{-1}$ )-induced tau phosphorylation. A: Vehicle control, B: wortmannin  $10 \mu\text{mol} \cdot \text{L}^{-1}$ , C: wortmannin + melatonin  $1 \mu\text{mol} \cdot \text{L}^{-1}$ , D: wortmannin + melatonin  $10 \mu\text{mol} \cdot \text{L}^{-1}$ , E: wortmannin + melatonin  $100 \mu\text{mol} \cdot \text{L}^{-1}$ .**

stages with immunocytochemical and silver impregnation techniques<sup>[12,13]</sup>, ie, no tangle, beginning stage of tangles, classic tangles, "early" ghost tangles, and "late" ghost tangles. The beginning stage of tangles is characterized by fibrillary inclusions in neuronal soma and axon, which is positively stained by anti-phosphorylated

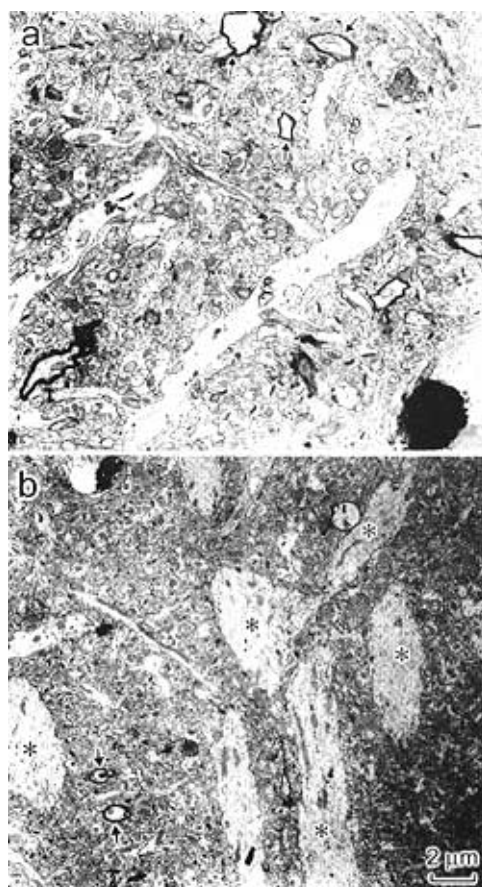


**Fig 3. Immunocytochemical staining of PHF-1 in rat hippocampus CA1 region detected at 6 h after injection of wortmannin  $10 \mu\text{mol} \cdot \text{L}^{-1}$ .  $\rightarrow$ : the spiny dendrites and axon.  $\square$ : neuronal soma.  $\times 200$ .**

aCSF injected control (Fig 4), suggesting a swelling lesion in axon was induced by administration of wortmannin.

## DISCUSSION

In Alzheimer disease, neurofibrillary tangles were first emerged in entorhinal cortex, then in limbic system, and finally, all neocortex<sup>[12]</sup>. A progressive pattern of neurofibrillary tangle development is divided into five



**Fig 4. Electron microscopy shown ultrastructural change induced by injection of wortmannin  $10 \mu\text{mol} \cdot \text{L}^{-1}$  detected at 6 h. a: vehicle control. b: wortmannin  $10 \mu\text{mol} \cdot \text{L}^{-1}$ .  $\blacktriangledown$ : normal axon.  $*$ : abnormal axon.  $\times 4000$ .**

tau antibodies. At this stage, the neuronal processes, such as spiny dendrites and axon are remained intact. The classic tangles and "early" ghost tangles react stronger with anti-phosphorylated tau than the beginning stage of tangles, and the spiny dendrites are disappearing and only neuronal soma is seen. The "late" tangles have no reaction with anti-phosphorylated tau, but react with anti-ubiquitin. In all stages, the distal segments of the dendrites become dilated and partial myelin sheaths and oligodendrocytes become losing. In the present study, we demonstrated that wortmannin induced tau hyperphosphorylation at Ser396/404, and phosphorylated tau was distributed both in axon and cytoplasm of pyramidal neuron in hippocampus. We also observed that the caliber of neuronal axon was dilated after treatment with wortmannin. According to the above classification, we believe that the topographic change of brain induced by wortmannin is similar to the beginning stage of tangles as seen in Alzheimer disease.

The kinetic change of tau phosphorylation induced by wortmannin was further investigated by quantitative Western blot. We found that the phosphorylation of tau at Ser396/404 was started to increase at 1 h, reached to peak at 6 h after injection. Then it was decreased at 12 h, remained this low level until 24 h. The data indicated that single dose injection of wortmannin could only induce transit phosphorylation of tau at Ser396/404 *in vivo*. It may also explain why we could only see the beginning stage of the tangles as discussed above.

Two possibilities may be relevant to the decreased phosphorylation of tau after 6 h of wortmannin treatment: 1) the catabolism of wortmannin leads to a decreased concentration of the drug and 2) activating in inhibitory mechanism against wortmannin-induced GSK-3 activation. It was reported in SY5Y cell line that wortmannin could inhibit PKB via inhibition of PI-3K, which might activate caspase-3 in apoptosis system. Activation of caspase-3 could activate novel protein kinase C $\delta$  (nPKC $\delta$ ) and thus phosphorylated GSK-3 at Ser9 or Ser21, and led to inactivation of GSK-3<sup>[14]</sup>. Therefore, to keep a chronic and consistent environment for tau hyperphosphorylation as seen in Alzheimer disease brain, one should pay attention to this negative feedback in regulation of GSK-3 activity.

It was also shown in the present study that melatonin could attenuate phosphorylation of tau at Ser396/404 induced by wortmannin *in vivo*. It was reported that melatonin could attenuate A $\beta$  toxicity via its eliminating

oxygen free radicals induced by A $\beta$ <sup>[7]</sup>. Until most recently, the procession of activating GSK-3 via wortmannin and phosphorylation of tau by GSK-3 has not been found to be relevant to oxidant stress yet. Melatonin can rapidly cross through the blood-brain barrier after systemic administration and reach neuronal compartment<sup>[7,8]</sup>. Therefore, melatonin may have potential therapeutic value in Alzheimer disease.

In conclusion, the phosphorylation of tau protein at Ser396/404 is induced by wortmannin *in vivo*, and this Alzheimer-like lesion is partially inhibited by melatonin.

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### 渥曼青霉素在体诱导 tau 蛋白阿尔采末样磷酸化以及褪黑激素的拮抗作用

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**关键词** 阿尔采末病; 神经原纤维缠结; tau 蛋白; 糖原合酶; 渥曼青霉素; 褪黑激素; 大鼠

**目的:** 研究渥曼青霉素能否在体诱导 tau 蛋白阿尔采末样磷酸化以及褪黑激素的对这种效应的拮抗作用。 **方法:** 采用立体定位技术向大鼠侧脑室注射渥曼青霉素和褪黑激素, 免疫组织化学和免疫印迹技术观察磷酸化 tau 蛋白的分布及含量, 电子显微镜观察轴突结构。 **结果:** 注射渥曼青霉素 ( $10 \mu\text{mol}\cdot\text{L}^{-1}$ ) 6 h 后, 海马 CA1 锥体神经元 tau 蛋白的 Ser-396/404 位点被磷酸化, 神经元轴突膨胀, 部分髓鞘脱落。免疫印迹结果表明, 渥曼青霉素注射后 1 h 到 6 h, tau 蛋白的 Ser-396/404 位点磷酸化逐渐增加, 6 h 到 24 h 逐渐减少, 6 h 为 tau 蛋白的 Ser-396/404 磷酸化的峰值。提前 12 h 注射褪黑激素 ( $10 \mu\text{mol}\cdot\text{L}^{-1}$  和  $100 \mu\text{mol}\cdot\text{L}^{-1}$ ), 可部分对抗渥曼青霉素 ( $10 \mu\text{mol}\cdot\text{L}^{-1}$ ) 注射 6 h 后诱导的 tau 蛋白 Ser-396/404 磷酸化。 **结论:** 在体注射渥曼青霉素可诱导大鼠 tau 蛋白阿尔采末样磷酸化, 褪黑激素可部分拮抗这一损伤效应。

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