Prevention of isoproterenol-induced tau hyperphosphorylation by melatonin in the rat

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Abstract: Hyperphosphorylated microtubule-associated protein tau is the major protein component of neurofibrillary tangles in the brain of patients with Alzheimer’s disease (AD). Until now, there is no effective cure to arrest this hyperphosphorylation. The present study was designed to explore the in vivo preventive effect of melatonin on Alzheimer-like tau hyperphosphorylation. Isoproterenol, a β-receptor agonist, was used to induce tau hyperphosphorylation, and for preventive effect of melatonin, the rats were injected intraperitoneally with melatonin for 5 d before hippocampi infusion of isoproterenol. The level of tau phosphorylation was detected by Western blot and immunohistochemistry using sites specific antibodies (PHF-1 and Tau-1), and it was normalized by non-phosphorylation dependent total tau antibody (111e). The results by Western blot showed that the immunoreaction of tau at PHF-1 epitope was enhanced, and the reaction at Tau-1 epitope was weakened significantly at 48 h after injection of isoproterenol, suggesting hyperphosphorylation of tau at Ser 396/Ser 404 (PHF-1) and Ser199/Ser 202 (Tau-1) sites. Similar results were observed by immunohistochemistry staining, in which hyperphosphorylated tau was mainly detected in mossy fibers of hippocampal CA3 region. Pre-injection of rats with melatonin intraperitoneally arrested effectively the isoproterenol-induced tau hyperphosphorylation at both Tau-1 and PHF-1 sites, implying the preventive effect of melatonin in Alzheimer-like tau hyperphosphorylation.

Key words: Alzheimer’s disease; melatonin; isoproterenol; tau; hyperphosphorylation

褪黑素对异丙肾上腺素诱导大鼠 tau 蛋白过度磷酸化的预防作用

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摘 要: 异常过度磷酸化的微管相关蛋白 tau 是阿尔茨海默病(Alzheimer’s disease, AD)患者大脑中神经原纤维缠结的主要组成部分。迄今为止，尚无有效的措施阻止 tau 蛋白的过度磷酸化。为探讨褪黑素(melatonin, Mel)对 AD 患者 tau 蛋白过度磷酸化的预防作用，我们以 β 受体激动剂异丙肾上腺素(isoproterenol, IP)来复制 AD 患者 tau 蛋白过度磷酸化的动物模型。在大鼠双侧海马注射 IP 前，以褪黑素作为保护组药物，于腹腔连续注射 5 d。应用磷酸化位点特异性抗体(PHF-1 和 Tau-1)作免疫印迹和免疫组织化学检测 tau 蛋白的磷酸化水平，并用非磷酸化依赖的总 tau 蛋白抗体(111e)进行标准化。免疫印迹结果显示：在注射 IP 48 h 后，tau 蛋白在 PHF-1 位点的免疫反应显著增强，在 Tau-1 位点显著减弱，表明 tau 蛋白在 Ser396/Ser404 (PHF-1) 和 Ser199/Ser202 (Tau-1)位点有过度磷酸化。免疫组织化学染色结果显示，免疫印迹结果显示：在注射 IP 48 h 后，tau 蛋白在 PHF-1 位点的免疫反应显著增强，在 Tau-1 位点显著减弱，表明 tau 蛋白在 Ser396/Ser404 (PHF-1) 和 Ser199/Ser202 (Tau-1)位点有过度磷酸化。免疫组织化学染色结果显示，免疫印迹结果显示：在注射 IP 48 h 后，tau 蛋白在 PHF-1 位点的免疫反应显著增强，在 Tau-1 位点显著减弱，表明 tau 蛋白在 Ser396/Ser404 (PHF-1) 和 Ser199/Ser202 (Tau-1)位点有过度磷酸化。上述结果提示：褪黑素可预防大鼠脑组织中由异丙肾上腺素引起的 AD 患者 tau 蛋白的过度磷酸化。

关键词: 阿尔茨海默病; 褪黑素; 反式异丙肾上腺素; tau 蛋白; 异常磷酸化
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Alzheimer’s disease (AD) is a progressive neurodegenerative disease. The incidence of the disease increases sharply with rapid aging of the population, and it is now the fourth leading cause of death following cardiovascular disease, cancer and stroke in Western countries. AD is characterized clinically by the presence of progressive memory impairment and histopathologically by the formation of senile plaques and neurofibrillary tangles [1], the latter being positively correlated with the degree of dementia symptom in the patients [2]. Biochemically, neurofibrillary tangles are composed of bundles of paired helical filaments (PHF), the major protein subunit of PHF is the hyperphosphorylated microtubule-associated protein tau [3]. Hyperphosphorylated tau is not competent to promote microtubule assembly and has decreased affinity to bind to microtubules [4], which is believed to be the major cause for the disrupted neuronal cytoskeleton and neurofibrillary degeneration in AD patients [5]. To mimic an Alzheimer-like tau hyperphosphorylation, direct or indirect activators or inhibitors to specific kinases or phosphatases have been used. For instance, isoproterenol, a β-receptor agonist, is employed for the activation of protein kinase A (PKA) and induction of tau hyperphosphorylation [6,7].

As the mechanisms for tau hyperphosphorylation and the formation of neurofibrillar tangles in AD are still not understood, there is no effective treatment to inhibit this pathological process. It is observed that production of melatonin, a pineal gland secreted hormone, declines progressively with aging [8,9], and the level of melatonin in the cerebrospinal fluid of AD patients is lower than that in normal age-matched population [10,11]. These data suggest an intrinsic link between the deficits of melatonin and development of AD. Recently, we have also found that simultaneous injection of melatonin with isoproterenol into the hippocampi [7], or with wortmannin into the brain ventricle [12] or with calyculin A into cell culture [13] attenuates hyperphosphorylation of cytoskeletal proteins. As the application of brain infusion is limited, and the preventive effect of melatonin is not known, we have investigated in the present study whether periphery pre-administration of melatonin prevents isoproterenol-induced tau hyperphosphorylation by pre-injection intraperitoneally of melatonin for 5 d after hippocampal infusion of isoproterenol.

1 MATERIALS AND METHODS

1.1 Animals

Wistar rats [male, (250±20) g, n=18] were supplied by the Center of Animal Laboratory, Tongji Medical College, Huazhong University of Science and Technology. The rats were raised in room temperature (25 °C) with light to dark cycle of 12 h/12 h.

1.2 Drugs and reagents

Isoproterenol (IP) and melatonin (Mel), purchased from Sigma (St Louis, MO USA) were diluted in 1% dimethyl sulfoxide (DMSO) before hippocampal injection for instant application. Monoclonal antibodies Tau-1 (reacts with Ser199/Ser202 non-phosphorylated tau) and PHF-1 (reacts with Ser396/Ser404 phosphorylated tau), and polyclonal antibody 111e (reacts with total tau) were kind gifts from Drs. Binder (Northwestern University, Chicago, Illinois, USA), Davis (Albert Einstein College of Medicine, Bronx, NY, USA) and Iqbal (NYS Institute for Basic Research, Staten Island, NY, USA), respectively. Secondary antibodies for Western blot were from Amersham Pharmacia Biotech (Little Chalfort, Buckinghamshire, England). Detection kit (Histostain-SP) for immunohistochemistry was from Zymed Laboratory (South San Francisco, CA, USA).

1.3 Animal grouping and stereotoxic stereotaxic injection

Eighteen rats were divided into 3 groups and saline or drugs were injected into hippocampi bilaterally (2 µL each) as follows: normal saline (NS) group, IP group (20 mmol/L), and Mel plus IP group (pre-injection of 10 mg/kg Mel intraperitoneally for 5 d before bilateral hippocampi bilateral infusion of IP). The concentration of IP and Mel was determined according to our previous study [6,7]. The rats were deeply anesthetized intraperitoneally with 60 g/L chloral hydrate. The injection was set at a position 4.2 mm posterior to the bregma and 2 mm lateral from the midline and 3 mm in depth from the skull surface to reach the hippocampus [14]. The microsyringe needle of injection was left in the hippocampi for 5 min after the injection.

1.4 Western blot

Rat hippocampi were quickly dissected out with cold homogenizing buffer containing (mmol/L) Tris–HCl 50 , pH 7.0, mercaptoethanol 10, EDTA 1.0, phenylmethylsulfonyl fluoride 0.1, and 2.0 µg/ml each of aprotinin, leupeptin, pepstatin for 3 min at 4 °C, and were stored at −70 °C. The phosphorylation of tau in the above samples was analyzed by Western blots using 10% SDS-PAGE as described originally by Laemmli [15]. The separated protein bands were transferred onto the nitrocellulose membrane and probed with specific anti-tau antibodies as described. Phosphorylation-independent antibody (R111e) was used to detect total tau. Two phosphorylation-dependent antibodies (Tau-1 and PHF-1) were employed to monitor the phosphorylation of tau at the specific sites. The blots were developed with
peroxidase-conjugated secondary antibody and visualized by enhanced chemiluminescence (ECL) system.

1.5 Immunohistochemistry
Rats were deeply anesthetized and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde solution. Their brains were dissected out from the skull and post-fixed in the paraformaldehyde solution for about 6 h. Sections (30 µm) were sliced through the area of the hippocampal formation using Vibratome (LANCER, S100, TPI, Germany). Free-floating sections were blocked with 0.3% H2O2 in absolute methanol for 10 min and non-specific sites were blocked with sheep serum for 30 min at room temperature. Sections were then incubated for 48 h with primary antibodies, and developed with biotinylated horse-radish peroxidase (HRP) and diaminobenzidine (DAB) system.

2 RESULTS

2.1 Effect of melatonin pre-treatment on IP-induced tau hyperphosphorylation determined by Western blot
The immunoreaction of phosphorylated tau was stronger at PHF-1 (Fig. 1) epitope, and weaker at Tau-1 (Fig. 2) epitope in IP-injected rats than that of control rats, suggesting tau hyperphosphorylation at Ser396/Ser404 (PHF-1 epitope) and Ser199/Ser202 (Tau-1 epitope) induced by IP. Pretreatment of melatonin significantly attenuated IP-induced tau hyperphosphorylation at PHF-1 and Tau-1 epitopes (Fig. 1 and Fig. 2). The level of phosphorylated tau at above sites was normalized by total level of tau, which was not altered by IP or melatonin treatment (Fig. 3).

![Fig. 1. Effects of IP and Mel pretreatment on tau phosphorylation at PHF-1 epitope.](image1)

![Fig. 2. Effects of IP and Mel pretreatment on tau phosphorylation at Tau-1 epitope.](image2)

![Fig. 3. Effect of IP and Mel pretreatment on level of total tau probed by R111e.](image3)

2.2 Effect of melatonin pre-treatment on IP-induced tau hyperphosphorylation determined by immunohistochemistry
The immunostaining of tau was much stronger for PHF-1 and much weaker for Tau-1 in CA3 and CA4 regions of hippocampi in IP-rats than in control rats. Conversely, the

![Fig. 4.](image4)
staining was much weaker for PHF-1 and much stronger for tau-1 at the same regions of hippocampi in melatonin pretreated rats (Fig.4). The altered staining was mainly located in the mossy fibers in CA3 region of the hippocampi (Fig.4, panels G, H, I and P, Q, R). These results further confirmed the hyperphosphorylation of tau at Ser396/Ser404 and Ser199/Ser202 sites, and the attenuation of melatonin on IP-induced tau hyperphosphorylation.

3 DISCUSSION

Alzheimer’s disease (AD) is the most prevalent dementia affecting the quality of life to a fast growing number of senile populations. One of the major pathological hallmarks found in AD brain is the presence of numerous neurofibrillary tangles mainly composed of hyperphosphorylated tau. Although the precise mechanism for tau hyperphosphorylation...
in AD brain is not known, it is widely accepted that hyperphosphorylation of cytoskeletal proteins found in AD brain is due to an imbalanced protein phosphorylation and dephosphorylation system. Studies have shown that a variety of neuronal protein kinases are capable of phosphorylating tau both in vivo and in vitro [16-19]. Among them, protein kinase A (PKA) is one of the most implicated [20]. On the basis of these findings, we have established successfully an Alzheimer-like tau hyperphosphorylation animal model by hippocampal infusion of isoproterenol, an indirect PKA activator [6-7]. By using this model, we have investigated the preventive effect of melatonin on tau hyperphosphorylation, and found that intraperitoneal injection of melatonin for 5 d can efficiently protect tau from isoproterenol-induced hyperphosphorylation at both PHF-1 (Ser396/Ser404) and tau-1 (Ser199/Ser202) epitopes.

The mechanism for the function of melatonin in preventing isoproterenol-induced tau hyperphosphorylation is not currently understood. It is reported that melatonin participates in regulating comprehensive biological functions, such as biological circadian rhythm, sexual maturity, breeding, euro-endocrine and immunological reinforcement such as biological circadian rhythm, sexual maturity, and antioxidant [23,24]. Melatonin can enhance neuronal activities related to learning and memory [25] and reduce b-amyloid (Ab) burdens both in vitro and in vivo [26,27], and the neuroprotective properties of melatonin against Ab-mediated toxicity does not require binding of melatonin to the membrane receptor but is likely the result of the antioxidant and anti-amyloidogenic features of the agent [28]. According to all these data, we speculate that the preventive effect of melatonin on isoproterenol-induced tau hyperphosphorylation may also involve its antioxidant properties or even direct regulation to of the kinase activity. Further study is required to confirm this speculation.

As melatonin is ready to pass the blood brain barrier and has little side-effect, it may become a potential approach to interfere Alzheimer-like neurodegeneration.

REFERENCES


