Valproate reduces tau phosphorylation via cyclin-dependent kinase 5 and glycogen synthase kinase 3 signaling pathways

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Abstract

Valproate (VPA) is a widely used anticonvulsant and mood-stabilizing drug. Recent studies have shown that VPA could reduce amyloid-β generation, and improve memory deficits in transgenic mouse models of Alzheimer's disease (AD). However, whether VPA affects tau phosphorylation and the underlying mechanism has not been established. Here, we showed that systemic treatment of APP and presenilin 1 double transgenic mice with VPA (50 mg/kg, once a day for 12 weeks), significantly reduced the levels of tau phosphorylation at the sites of Thr205, Ser396 and Thr231. Meanwhile, VPA treatment markedly reduced the activities of cyclin-dependent kinase 5 (CDK5) and glycogen synthase kinase 3β (GSK3β), two protein kinases involved in abnormal hyperphosphorylation of tau. In an okadaic acid-induced tau hyperphosphorylation SH-SY5Y cell model, the anti-tau-phosphorylation effect of VPA was further confirmed, accompanied by a marked decrease in the activities of CDK5 and GSK3β. Our present data suggest that the inhibitory effects of VPA on tau hyperphosphorylation might be mediated through both CDK5 and GSK3β signaling pathways.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by extracellular amyloid-β (Aβ) deposits in senile plaques, intracellular hyperphosphorylated tau aggregates in neurofibrillary tangles (NFTs) and selective cholinergic neuronal loss in the brain regions involved in learning and memory [15,22,27]. Aβ is generated from amyloid precursor protein (APP) after β- and γ-secretase cleavages [34]. Tau is a microtubule-associated protein and binds to microtubules in mature neurons, where it is involved in regulating the stability and dynamics of the microtubule network. Tau is a substrate for several protein kinases, and is phosphorylated at a large number of serine/threonine residues [16,29]. Tau phosphorylation is essential for the interaction of tau and microtubules. However, abnormally tau hyperphosphorylation, aggregated into paired helical filaments (PHFs), negatively regulates microtubule-binding and leads to destabilization of the microtubule network and neuronal death such as the case in AD [15,22,27].

The abnormal hyperphosphorylation of tau in AD is modulated by cyclin-dependent kinase 5 (CDK5) and glycogen synthase kinase 3β (GSK3β) [21,22,27]. Although there is no up-regulation of CDK5 and GSK3β detected in AD brain by biochemical analyses [31], the activation of both CDK5 and GSK3β is found in neuronal cultures exposed to Aβ peptides [2,40]. In APP and presenilin-1 (PS1) double transgenic mouse brain, both phosphorylated CDK5 and GSK3β are significantly increased compared with wild type mouse [9]. Importantly, inhibition of CDK5 and GSK3β activities results in a decrease in Aβ deposition and tau phosphorylation, and improves spatial learning and memory in AD transgenic mice [9]. Since CDK5 and GSK3 are the key kinases responsible for the phosphorylation of APP and tau [20,21,36], the candidate regulating these two kinases might be a potential strategy for prevention and treatment of AD.

Valproate (VPA) has been widely used as a highly effective anticonvulsant and mood-stabilizing drug for treating epilepsy and bipolar disorder. The involving mechanisms contain the inhibition of ion channels, enhancement of γ-aminobutyric acid...
transmission [7], regulation of GSK3 activity [46], activation of extracellular signal-regulated kinase [48], and protecting neurons through increasing neurogenesis and stimulating the release of neurotrophic factors from astrocytes [6]. Interestingly, recent studies have shown that VPA is a potential agent for prevention and treatment of AD [33]. VPA reduces Aβ generation and neurotic plaque formation through inhibiting GSK3β-mediated γ-secretase cleavage of APP, and improves memory deficits in AD transgenic model mice [33]. However, whether VPA affects tau phosphorylation and the underlying mechanism has not been established.

In the present study, VPA treatment of APP/PS1 transgenic mice was employed to evaluate the effect of VPA on tau phosphorylation. Our results indicate that VPA inhibited the activities of CDK5 and GSK3β, and consequently reduced tau phosphorylation in APP/PS1 mouse brain. Further, the anti-tau-phosphorylation effect of VPA and the underlying mechanism was confirmed with a tau hyperphosphorylated cell model, i.e. human neuroblastoma SH-SY5Y cells treated with okadaic acid (OA), a serine/threonine phosphatase inhibitor. The present data suggest that the neuroprotective effects of VPA on AD is not only related to the inhibition of Aβ generation, but also correlated to the reduction of tau phosphorylation through CDK5 and GSK3 signaling pathways.

2. Materials and methods

2.1. Animals and treatments

APP/PS1 (APPSwe/PS1ΔE9) double-transgenic mice were obtained from the Jackson Laboratory (West Grove, PA, USA). The mice were housed in a controlled environment (22–25 °C, 50% humidity), with standard diet and distilled water available ad libitum. The animal experiment protocols were approved by the animal ethics committee of China Medical University. APP/PS1 transgenic mice at the age of 20 weeks were randomly divided into vehicle control group and VPA treatment group (n = 6 each group). VPA group mice were intraperitoneally (i.p.) injected with VPA (50 mg/kg, Sigma; dissolved in saline) once a day for 12 weeks. Control mice were treated with the same dose of saline. At the age of 32 weeks, mice were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.) and sacrificed by decapitation. The brains were removed quickly and split into halves. The left hemisphere was frozen with liquid nitrogen and kept at −80 °C for Western blotting analyses. The right hemisphere was fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemical analysis.

2.2. Cell culture and treatments

Human neuroblastoma SH-SY5Y cells were cultured at 37 °C in humidified 5% CO2 in Dulbecco minimum essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 µg/ml streptomycin as described previously [47]. OA (Santa Cruz Biotechnology, dissolved in DMEM) was used to induce tau hyperphosphorylation [31,50,51]. The cells were treated with OA at 40 nM for 12 h prior to the treatment of VPA at 10 nM for 24 h.

2.3. Immunofluorescence and confocal analysis

For confocal immunofluorescence, culture cells were preincubated with normal donkey serum (1:20; Jackson Immunonostic Research Laboratory) for 1 h and then incubated overnight with a mixture of primary antibodies, mouse anti-tau-pThr231 (1:400; Invitrogen) and rabbit anti-phospho-CDK5 (Tyr15) (1:400; Abcam) or mouse anti-tau-pThr231 and rabbit anti-pS35/25 (1:200; Cell Signaling Technology). After several rinses with PBS, the cells were incubated for 2 h with a mixture of secondary antibodies, FITC-conjugated donkey anti-rabbit IgG (1:50; Jackson Immunoresearch Laboratory) and Texas Red-conjugated donkey anti-mouse IgG (1:50; Jackson Immunoresearch Laboratory). After rinsing, the cells were incubated with DAPI for 3 min, mounted with an anti-fading mounting medium and visualized using a confocal laser scanning microscope (SP2; Leica, Wetzlar, Germany). To assess nonspecific staining, control sections were incubated in each experiment with normal serum instead of primary antibodies followed by all subsequent incubations as described above.

2.4. Western blotting

Protein lysates preparation and Western blots were performed as described previously [45,47]. Briefly, brain tissues or culture cells were homogenized in ice-cold lysis buffer containing an inhibitor protease cocktail (1 mM PMSE, 10 µg/ml apro- tinin, 1 mM Na3VO4, 10 µg/ml leupeptin and 1 mM NaF). The homogenates were centrifuged at 12,000 rpm for 30 min at 4 °C. The supernatants were collected and total protein concentrations were measured using a standard Bradford method. Proteins (50 µg) separated by 10% SDS polyacrylamide gels were transferred onto PVDF membranes (Millipore; Temecula, CA, USA). The membranes were blocked in TBST solution (10 mM Tris/HCl pH 8.0, 150 mM NaCl, 0.1% Tween-20) supplemented with 5% non-fat milk for 1 h and then incubated with a primary antibody for 2 h at room temperature. Primary antibodies used included rabbit anti-GSK3β (1:1000; Cell Signaling Technology), rabbit anti-phospho-GSK3α (ser21)/β (ser9) (1:1000; Cell Signaling Technology), rabbit anti-phospho-GSK3β (1:1000; Cell Signaling Technology), rabbit antibody anti-phospho-GSK3β (1:1000; Cell Signaling Technology), mouse anti-CDK5 (1:5000; Abcam), rabbit anti-tau-pThr231 (1:500; Invitrogen), rabbit anti-tau-pThr205 (1:1000; Abcam), rabbit antibody anti-tau-pSer396 (1:1000; Abcam), rabbit anti-tau (1:400; Abcam), rabbit anti-CDK5 (1:5000; Abcam), rabbit anti-CDK5-pTyr15 (1:1000; Abcam), rabbit anti-p-CDK5 (1:500; Cell Signaling Technology), mouse anti-β-actin (1:5000; Santa Cruz Biotechnology) and mouse anti-GAPDH (1:10000; KC-5G, Kang Chen, 0811). The signals on membranes were revealed with an enhanced chemiluminescence kit (Pierce Biotechnology; Rockford, IL) and Chem Doc XRS with Quantity One software (Bio-Rad; Hercules, CA, USA). The quantifications of band intensity were performed using Image J (NIH; Bethesda, MD, USA).

2.5. Statistical analysis

All values were expressed as mean ± standard deviation (SD). Statistical significance between control and VPA treatment groups was determined using Student’s t test. p < 0.05 was considered significant.

3. Results

3.1. VPA inhibits tau phosphorylation in the APP/PS1 mouse brain

We first examined whether VPA treatment could inhibit tau hyperphosphorylation in APP/PS1 mouse brain. Brain tissues from VPA-treated mice were subjected to Western blot analyses using antibodies against total tau, and different phosphorylation sites on tau, including Thr205, Ser396 and Thr231. As shown in Fig. 1, the steady-state levels of total tau showed no significant differences between VPA-treated mice and controls (Fig. 1). However, the expression levels of phospho-tau in the VPA-treated transgenic mouse brain were significantly reduced by 65% (Thr205, p < 0.01), 57% (Ser396, p < 0.01) and 54% (Thr231, p < 0.05), respectively, compared with controls (Fig. 1A and B). Collectively, these results suggest that VPA treatment inhibits tau hyperphosphorylation in APP/PS1 mouse brain.

3.2. VPA inhibits the activity of CDK5 and GSK3β in the APP/PS1 mouse brain

Both CDK5 and GSK3β are believed to be the most important kinases that regulate tau phosphorylation in AD brain [12,26,27]. To determine whether the inhibitory effect of VPA on tau phosphorylation was regulated by CDK5 and GSK3β, the levels of phospho-CDK5 and phospho-GSK3β were measured by Western blotting. As shown in Fig. 2, no significant differences were detected in the levels of total protein of CDK5 between the vehicle control and VPA-treated APP/PS1 mouse brain. However, a significant decrease in the levels of phospho-CDK5/CDK5 detected by a specific anti-phospho-CDK5 (Tyr15) antibody, was observed in the VPA-treated mice compared with vehicle controls. Quantitative analysis showed that the ratio of phospho-CDK5/CDK5 was decreased by 34% in the VPA-treated mouse brain, compared with the control (p < 0.05; Fig. 2A). Moreover, the expression level of phospho-GSK3β (Ser9) was increased in VPA-treated transgenic mouse brain, and the ratio of phospho-GSK3β/GSK3β was significantly increased by 39% (p < 0.05; Fig. 2B). However, the ratio of the phospho-GSK3α/GSK3α was not changed significantly in VPA-treated mouse brain, compared with vehicle controls (p > 0.05; Fig. 2B). These data suggest that VPA treatment significantly inhibits the activities of CDK5 and GSK3β in the APP/PS1 mouse brain.
Fig. 1. VPA inhibits tau phosphorylation in the APP/PS1 mouse brain. (A) The levels of phosphorylation of tau at the sites of Thr205, Ser396, and Thr231 in the cortex of vehicle control and VPA-treated APP/PS1 mice were examined by Western blot analysis. (B) The phosphorylation levels of tau at Thr205, Ser396 and Thr231 were decreased in VPA-treated mice, compared with the control. Densitometric values were normalized using GAPDH as an internal control. All values are mean ± SD (n = 6), *p < 0.05, **p < 0.01.

Fig. 2. Effect of VPA on the activities of CDK5 and GSK3 in the APP/PS1 mouse brain. Protein extracts from control and VPA-treated mouse brain were examined by Western blotting using antibodies against phospho-CDK5, CDK5 phospho-GSK3, GSK3 and GAPDH, respectively. (A) No significant differences of the total protein levels of CDK5 were detected between control and VPA-treated mice. However, treatment with VPA significantly reduced the ratio of phospho-CDK5/CDK5, compared with the control. (B) VPA did not alter the level of phospho-GSK3α/GSK3α, but the ratio of phospho-GSK3β/GSK3β was markedly increased in VPA-treated mouse brain, compared with the control. Densitometric values were normalized using GAPDH as internal control. All values are mean ± SD (n = 6), *p < 0.05.

Fig. 3. Effect of VPA on tau phosphorylation in OA-treated SH-SY5Y cells. (A) The expression levels of total tau and phosphorylated tau at the sites of Thr205, Ser396, and Thr231 were examined by Western blot analysis. (B) Quantification results showed that the levels of phosphorylated tau at Thr205, Thr396 and Thr231 were significantly decreased in VPA-treated cells, compared with the control. Vehicle control was treated with OA only. Densitometric values were normalized using GAPDH as internal control. Data are presented as mean ± SD (n = 6), *p < 0.05, **p < 0.01.
3.3. VPA inhibits tau phosphorylation in OA-treated SH-SY5Y cells

To investigate the effect of VPA on tau phosphorylation in vitro, SH-SY5Y cells were treated with OA to induce tau phosphorylation and the effect of VPA was observed by co-treatment on cells with VPA for 24 h. Western blot was performed to assess the levels of tau phosphorylation in culture cells (Fig. 3A). Quantitative analysis showed that the levels of total tau were not changed significantly between control and VPA-treated cells. However, VPA treatment significantly reduced the levels of tau phosphorylation by 40% at Thr205 (p < 0.05), 27% at Ser396 (p < 0.01) and 16% at Thr231 (p < 0.05), respectively (Fig. 3A and B). Furthermore, immunofluorescence labeling and confocal analysis showed that VPA reduced the fluorescence intensity of phospho-tau (Thr231; Figs. 4A and 5A). These results suggest that VPA inhibits hyperphosphorylation of tau in OA-treated SH-SY5Y cells.

3.4. VPA inhibits the activities of CDK5 and GSK3β in culture cells

It has been reported that calpain-induced cleavage of p35 to p25 could induce aberrant CDK5 activation [28]. The activated CDK5 is responsible for abnormal tau hyperphosphorylation [2]. Therefore, the expression levels of p35 and p25 were examined in SH-SY5Y cells treated with OA and VPA. Confocal analysis showed that VPA reduced the fluorescence intensity of p35/p25 (the antibody recognized both p35 and p25, see immunoblotting results in Fig. 4B), accompanied by a reduction of phospho-tau (Thr231) (Fig. 4A). Immunoblotting data showed that VPA treatment did not change the expression levels of p35 in OA-treated cells, while the ratio of p25/p35 was reduced significantly by 28%, compared with the control (p < 0.05; Fig. 4B).

The immunofluorescence intensity of phospho-CDK5 was reduced in VPA-treated cells, revealed by confocal analysis (Fig. 5A). Western blotting results showed that VPA treatment significantly decreased the ratio of phospho-CDK5/CDK5 by 38%, compared with the control (p<0.01; Fig. 5B). Furthermore, the ratio of phospho-GSK3β/GSK3β was significantly increased by 48% in VPA-treated cells, compared with OA-treated control cells (p<0.01; Fig. 5C).

Collectively, these data suggest that VPA could inhibit the activities of CDK5 and GSK3β in OA-treated SH-SY5Y cells.

4. Discussion

In AD brain, hyperphosphorylated tau aggregating in NFTs is one of the major pathological features, and contributes to neuronal degeneration [1,3,5,12]. Therefore, attenuation of tau phosphorylation is a novel therapeutic strategy for AD. Tau441 was phosphorylated by GSK3β at Ser199, Thr205, Thr212, Thr231, Ser396 and Ser404, and by CDK5 at Ser199, Ser202, Thr205, Thr212, Thr217, Thr231, Ser396 and Ser404 [14,25]. Thus, GSK3β and CDK5 largely phosphorylate the same serine and threonine residues of tau. Interestingly, VPA possibly protects neurons through its effects on the phosphorylation of tau and β-catenin in HIV-1-associated dementia (HAD) [10]. On the basis of recent findings that VPA inhibits Aβ production in vitro [33,39], and reduces neuritic plaque formation and improves memory deficits in AD transgenic mice [33], the present study further evaluated the effect of VPA on tau phosphorylation in vitro and in vivo. Our data showed that VPA treatment significantly reduced the levels of tau phosphorylation at Thr205, Ser396, and Thr231 in the brain of APP/PS1 transgenic mouse and OA-treated neuroblastoma SH-SY5Y cells. Many kinases have been believed to play important role in the phosphorylation of tau, including GSK3β, CDK5 and PKA [14].
Fig. 5. VPA inhibits the activities of CDK5 and GSK3β in OA-treated SH-SY5Y cells. (A) Immunofluorescence labeling and confocal analysis showed that VPA reduced the fluorescence intensity of phospho-tau (green) and phospho-CDK5 (red) in OA-treated SH-SY5Y cells. Scale bar = 10 μm. (B) Immunoblotting results showed that treatment with VPA decreased the ratio of phospho-CDK5/CDK5 compared with the control, suggesting that VPA could inhibit the activity of CDK5 in OA-treated SH-SY5Y cells. (C) The levels of phosphorylated GSK3β and total GSK3β were examined by Western blotting. Treatment with VPA markedly increased the ratio of phospho-GSK3β/GSK3β, compared with the control, suggesting that VPA could inhibit the activity of GSK3β in OA-treated SH-SY5Y cells. Densitometric values were normalized using GAPDH as an internal control. All values are mean ± SD (n=6), **p < 0.01.

Several studies have shown that tau hyperphosphorylation is associated with the activities of the CDK5 and GSK3β kinases in AD brain [12,17,18,27,42]. Thus, we further analyzed the mechanisms underlying the inhibitory effect of VPA on tau phosphorylation by examining the activities of CDK5 and GSK3β kinases in our animal and cell models.

CDK5 is believed to be an important kinase that regulates both APP and tau phosphorylation in the brain [24,26]. A recent study has revealed that inhibition of CDK5 activity with all-trans retinoic acid could attenuate Aβ deposition and tau phosphorylation in APP/PS1 transgenic mice [9]. In copper-exposed APP/PS1/tau triple transgenic mice, increased tau phosphorylation was closely correlated with aberrant CDK5/p25 activation [23]. The activation of c-Abl tyrosine kinase by Aβ promotes tau phosphorylation through Tyr15 phosphorylation-mediated CDK5 activation [5]. Infections with CDK5 inhibitory peptide (CIP) suppressed the aberrant tau phosphorylation and protected against Aβ1-42-induced apoptosis in primary rat cortical neurons [52]. In the present study, the activity of CDK5 has been examined in our in vivo and in vitro AD models. Our results showed that VPA treatment significantly reduced the levels of phospho-CDK5, but did not affect the total protein levels of CDK5, in APP/PS1 mouse brain and in OA-treated SH-SY5Y cells. Furthermore, we examined the effect of VPA on the expression levels of p35 and p25 in OA-treated SH-SY5Y cells. Immunofluorescent analysis showed that VPA reduced the fluorescence intensity of p35/p25, detected by an antibody recognized both p35 and p25. Western blotting results showed that VPA treatment did not change the expression levels of p35, but the ratio of p25/p35 was significantly reduced. It is known that CDK5 is activated by its activator, p35, or its proteolytic cleavage product, p25 [24,30,43]. The abnormally hyperphosphorylated tau found in p25-overexpressing transgenic mouse brain suggests that the deregulation of CDK5 activity by association with p25 leads to tau hyperphosphorylation in neurodegenerative diseases such as AD [24,50]. In the present study, we found that VPA significantly reduced the activity of CDK5 and the ratio of p25/p35, suggesting...
that the anti-tau-phosphorylation effect of VPA is likely through the inhibition of the CDK5/p25 pathway, which has not been reported yet.

GSK3β is another major kinase associated to tau hyperphosphorylation and Aβ generation [12,13,27,44]. GSK3β activity is regulated by its phosphorylation state. Phosphorylation of GSK3β on Tyr216 increases its activity [19], and phosphorylation at Ser9 inhibits kinase activity [8,38]. Recent studies have shown that GSK3β inhibitors might be beneficial for AD therapy. Treatment with NP12, a novel GSK3β inhibitor, significantly reduces tau phosphorylation and rescues memory deficits in a transgenic mouse model of AD [35]. 17β-Estradiol could attenuate tau hyperphosphorylation at multiple related sites, including Ser396/404, Thr231, Thr205 by increasing the level of GSK3β at Ser9 [37]. Lithium, a well-known GSK3β inhibitor, could inhibit tau hyperphosphorylation in living neurons [32,41], and reduce Aβ plaque burden in the AD transgenic mouse brain [39,41]. VPA, similar to lithium, is a mood stabilizer and GSK3β inhibitor, could reduce GSK3β-mediated γ-secretase cleavage of APP and Aβ production in vitro and in vivo [33]. However, to our knowledge, whether VPA has the effect on anti-tau-phosphorylation has never been investigated in APP/PS1 transgenic mouse brain. In the present study, we showed that administration of VPA significantly reduced the activity of GSK3β kinase, accompanied by decreased levels of tau phosphorylation in the transgenic mice and OA-treated SH-SYSY cells. These data suggest that inhibition of GSK3β activity might be involved in the inhibitory effect of VPA on tau phosphorylation.

In summary, the present study indicates that VPA has inhibitory effects on tau phosphorylation by a mechanism believed to inhibit both CDK5 and GSK3β signaling pathways. Together with previous findings that administration of VPA resulted in reduced Aβ plaque burden and improvement of memory deficits in AD transgenic mice [33], our data provide insights for the use of VPA, a widely used anticonvulsant and mood-stabilizing drug, as an efficient approach to the prevention and treatment of AD.

Competing interests

The authors declare that they have no competing interests.

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