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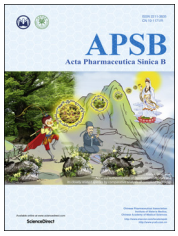

ORIGINAL ARTICLE

Potassium 2-(1-hydroxypentyl)-benzoate improves depressive-like behaviors in rat model

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KEY WORDS

PHPB;
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Abstract Potassium 2-(1-hydroxypentyl)-benzoate (PHPB) is a novel drug candidate for acute ischemic stroke. PHPB has been also shown to be beneficial for some neurodegenerative diseases. In this study, we demonstrated that PHPB improved depressive-like behaviors induced by chronic unpredictable mild stress (CUMS) in rats. Male SD rats were subjected to the stress for five weeks. PHPB (30 and 100 mg/kg) or fluoxetine (FLX 10 mg/kg, as positive control) was administered orally from the third week in CUMS procedure. The behavioral tests were applied and then the biochemical studies were carried out. PHPB or FLX treatment rescued the behavioral deficiency in CUMS-exposed rats. Meanwhile, PHPB normalized the enhanced level of serum corticosterone, improved hippocampal and serum BDNF levels, as well as p-CREB level in hippocampus. In addition, PHPB could reverse the reduced level of extracellular 5-HT and its metabolite 5-HIAA in prefrontal cortex (PFC) of depressed rats. In summary, our results showed that PHPB improved depression-like behaviors in CUMS-exposed rats. The mechanisms might relate to the reverse of neurotrophic disturbance in the brain, reducing excessive HPA axis response and facilitating the release of 5-HT.

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1. Introduction

In the present study, we investigated the anti-depression effects and the mechanisms of potassium 2-(1-hydroxypentyl)-benzoate (PHPB, Fig. 1), which is a novel drug candidate for treatment of acute cerebral ischemic stroke. PHPB, pro-drug of 3-*n*-butylphthalide (D,L-NBP), was synthesized based on a primary naphtha component from seeds of *Apium graveolens* Linn[1]. Previous studies showed that PHPB has neuroprotective effects in multiple animal models and cell-based studies. For example, it could reduce infarct volume in the cerebral ischemic animal model[2] and improve the neurobehavioral deficits, rescued cerebral hypoperfused animals from learning and memory deficits[3], decreased the hyperphosphorylation of tau protein in APP/PS1 transgenic Alzheimer's disease (AD) mouse model[4] and it could also attenuate neuronal apoptosis and neuronal inflammation *in vitro* and *in vivo*[5,6]. Currently PHPB is in phase II/III clinical trial, it might be a promising novel drug for ischemic stroke and might be also for neurodegenerative diseases.

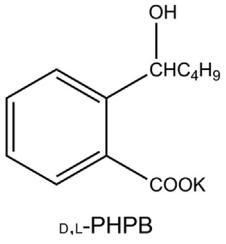

Figure 1 Chemical structure of PHPB. $C_{12}H_{15}O_3K$, FW: 246.4.

Depression is a well-known psychiatric disorder with high morbidity or mortality, which is one of the most severe health issues at the present time. Depressive symptoms are numerous, including long lasting depressed mood, anxiety, pessimism and suicidal tendencies[7]. During last several decades, some antidepressants were applied in clinic, such as tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs) and monoamine oxidase inhibitors (MAOIs). They exert effects by increasing the levels of monoamines, 5-hydroxytryptamine (5-HT) and/or noradrenaline (NE). However, most of these drugs have undesirable side effects[8,9]. Therefore, to explore novel antidepressant agents with high efficiency and low toxicity is necessary.

In addition, plenty of preclinical or clinical studies have indicated that the pathophysiology of depression is associated with cerebral injury and hippocampal volume decrease, which may be induced by lasting or severe stress events, subsequently results in hyperactivation of hypothalamic-pituitary-adrenal (HPA) axis[10,11]. It has been also suggested that brain-derived neurotrophic factor (BDNF) and hippocampal neurogenesis play an important role in the pathophysiology of depression, or at least in the mechanism of antidepressant action[12,13]. BDNF levels are reduced in the postmortem brains of depressed patients as well as in the animal models of depression as reported[14,15]. Thus, increasing BDNF level and improving hippocampal neurogenesis may be a potential antidepressant strategy[16].

According to our previous studies, we proposed that PHPB might be also used for treatment of depression. It might become a novel candidate of antidepressant. However, the pharmacological effects and the mechanisms of PHPB in treatment of depression remain to be elucidated. In the current study, we used chronic unpredictable mild stress (CUMS), the classic animal model of depression, to observe the roles of PHPB and investigate the mechanisms related to major pathological changes during depression. We investigated the action of PHPB on imbalanced neuroendocrine and neuronal transmitters, such as the activity of HPA axis, levels of BDNF and 5-HT production and metabolism in brain.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats weighing 180–200 g (Vital River Laboratory Animal Technology Company, Beijing, China) were used at the beginning of the experiment. The animals were housed under a 12-h light/12-h dark cycle (lights on at 7:30 a.m., lights off at 7:30 p.m.) at room temperature 24 ± 1 °C and humidity 40%–60% with free access to food and water for one week except when animals are subjected to stressors during the CUMS procedure. Animal use procedures were in accordance with the National Institutes of Health Guide for the Care and were approved by the Animal Care Committee of the Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, China.

2.2. Drugs and reagents

PHPB was offered by the Department of Synthetic Pharmaceutical Chemistry of the Institute of Materia Medica (Beijing, China) with purity of 99.1%, Fluoxetine (FLX) was purchased from Sigma–Aldrich (St Louis, MO, USA). Both PHPB and FLX were dissolved in distilled water and administrated intragastrically to the rats.

2.3. CUMS procedure and drug treatments

Except for the control group (non-stressed group), the animals exposed to CUMS were subject to a total of 12 mild and unpredictable stressors (2 per day) in a variable sequence for five consecutive weeks. The exact stressors and sequence used are shown in Table 1, adapted from previous studies[17] with minor modifications. Animals were randomly allocated to one of the following seven groups: (1) vehicle (distilled water), (2) PHPB (100 mg/kg, i.g.), (3) FLX (10 mg/kg, i.g.), (4) CUMS+vehicle1 (distilled water), (5) CUMS+PHPB (30 mg/kg), (6) CUMS+PHPB (100 mg/kg), (7) CUMS+FLX (10 mg/kg). Drugs were administered by intragastric administration (i.g.) between 8:00 and 9:00 once a day. The control rats were left undisturbed in a separate room and were handled daily.

Table 1 Chronic unpredictable stress procedure.

Day	Stressor
1	Water deprivation 24 h; exposure to noise 3 h
2	Food deprivation 24 h; soiled cage 24 h
3	Paired housing 12 h; overnight illumination
4	Tail pinch 1 min; hot stress in oven at 45 °C 6 min
5	Shaker stress 45 min (170 rpm.); cage tilt 45° 12 h
6	Forced swimming at 10 °C for 6 min
7	Reversal of the light/dark cycle (light off 12 h and light on overnight)

2.4. Behavioral assessment

2.4.1. Open field test (OFT)

The open field apparatus is an arena (125 cm in diameter with 40 cm boundary walls), the floor of which is divided into 25 equal squares (25 cm × 25 cm) by white line. Rats were individually placed in the center of the arena, and their locomotor activity including the number of crossing, rearing and grooming (cleaning the face or licking/scratching the various part of the body) were recorded for 5 min.

2.4.2. Sucrose preference test (SPT)

The sucrose preference test was performed as described previously[18] with minor modifications. Briefly, prior to testing, rats were trained to adapt to the sucrose solution (1%, *w/v*): two bottles of sucrose solution were placed in each cage for 24 h, and then one bottle of sucrose solution was replaced with distilled water for 24 h. After adaptation, rats were deprived of water and food for 20 h. Then 1-h baseline test was performed, in which rats could select to drink from two preweighed bottles, one with 1% (*w/v*) sucrose solution and the other with water. After 1 h, the weights of consumed sucrose solution and water were recorded and the sucrose preference was calculated by determining the percentage of total fluid consumption accounted by ingestion of sucrose solution (Eq. (1)):

$$\text{Sucrose preference } (\%) = (\text{Intake}_{\text{sucrose solution}} / \text{Intake}_{\text{sucrose intake+water}}) \times 100 \quad (1)$$

Fluid consumption was recorded by weighing the bottles.

2.4.3. Forced swimming test (FST)

The FST followed the method described by Porsolt's report[19] with minor modifications. The rats were placed in a plexiglas cylinder (18 cm internal diameter, 50 cm height) filled with 23–25 °C water. All rats were forced to swim for 6 min, and the total duration of immobility was recorded during the last 4 min of the test. The definition of immobile status was that the mouse was floating in the water without any movement; only small motions are required to maintain its head above the water.

2.5. Biochemical analysis

2.5.1. Blood sampling

The blood samples were collected after the last behavioral assessments by decapitation of rats. All the blood sampling was performed in the light stage of the subjects, which was strictly between 8:30 and 11:30 a.m. and the blood sampling was conducted on Day 38, which was the first day after the behavioral test, when all the 5-week CUMS was finished. Blood samples were collected and allowed to coagulate at room temperature for 1 h followed by 1 h at 4 °C, subsequently centrifuge at a speed of 2000 rpm for 10 min (3–30 K, Sigma, MO, USA). Serum was separated and stored at –80 °C until the biochemical estimations were carried out.

2.5.2. Serum corticosterone level

Measurement of serum CORT was performed by using a commercially available enzyme-linked immunosorbent assay kit (ELISA, Enzo Life Sciences, Inc., New York, USA) according to the manufacturer's instructions. Briefly, samples (diluted with sample diluents buffer in a proportion of 1:40) and standards were added to each well, respectively, and then the plate was incubated for 2 h at room temperature. After several times washing and proper color development, the optical density value was read at 405 nm in ELISA plate reader (uQuant, Bio-Tek, Vermont, USA). The sensitivity of the assay for corticosterone was 32 pg/mL.

2.5.3. Measurement of serum BDNF level

A commercial sandwich ELISA kit (ChemiKine™, Cat. No. CYT306, Millipore Corp, MA, USA) was used to measure the serum BDNF level, according to the manufacturer's instructions. Briefly, a sufficient number of microplate wells were prepared to accommodate standards and samples in duplicates. The wells were coated at 4 °C overnight with samples (diluted with sample diluents buffer in a proportion of 1:80) and different concentrations of standards (curve ranged from 7.8 to 500 pg/mL of BDNF). Then plate contents were discarded and mouse anti-BDNF monoclonal antibody solution (diluted with sample diluent buffer in a proportion of 1:1000) was added to each well. After 3-h incubation at room temperature, the plate was washed four times with wash buffer. After washing, streptavidin–HRP conjugate solution (diluted with sample diluent buffer in a proportion of 1:1000) was added to each well and incubated at room temperature for 1 h. Afterwards, the plate contents were discarded. After washing the plate four times with wash buffer, TMB/E substrate was added in each well and incubated for 20 min in dark. Then reaction stop solution was added and the amount of BDNF determined by measuring absorbance at 450 nm. The standard curve demonstrated a direct relationship between optical density and BDNF concentration.

2.6. Western blot analysis

Following decapitation, rat hippocampus was isolated rapidly and stored at −80 °C for Western blotting detection. The tissues were weighed, sonicated in RIPA lysis buffer supplemented with fresh protease and phosphatase inhibitors, and centrifuged at a speed of 12,000 rpm for 25 min. Then the protein concentration was determined by BCA assay. After denaturation and electrophoresis, the proteins were transferred onto a PVDF membrane in wet conditions. Following blocking in Tris-buffered saline Tween-20 solution with 5% (*w/v*) nonfat milk powder (1 h, RT), the protein membranes were incubated respectively in primary antibody: rabbit anti-BDNF (1:400, Santa Cruz Biotechnology, Inc., CA, USA), cAMP response element binding protein (CREB, 1:1000, Cell Signaling Tech), p-CREB (Ser133, 1:400, Cell Signaling Tech, MA, USA) and *β*-actin (1:8000, Sigma–Aldrich Inc., MO, USA) at 4 °C overnight. After washing, the membranes were incubated (1 h, RT) with anti-rabbit or anti-mouse IgG labeled with horseradish peroxidase (Jackson ImmunoResearch Inc., PA, USA) and then the membranes were washed and developed with Chemiluminescence reagents (Millipore Corp., MA, USA). The chemiluminescence signal was transformed into a digital image using FUJIFILM imaging system LAS-3000 (Fuji Photo Film Co., Ltd., Tokyo, Japan).

2.7. Measurement of extracellular monoamine neurotransmitters level from superfused brain slices

Measurements of extracellular 5-HT levels were performed as previously described[20]. Briefly, after the rats were decapitated, the brain was removed, rinsed with cold ACSF buffer (NaCl 125 mmol/L, KCl 5 mmol/L, NaH_2PO_4 1.2 mmol/L, $CaCl_2$ 2.6 mmol/L, $MgCl_2$ 1.3 mmol/L, $NaHCO_3$ 26 mmol/L, Glucose 10 mmol/L), and placed it on a glass plate over ice. Prefrontal and hippocampal sections were removed by standard dissection procedures, weighed, and cut with a sharp razor blade into slices about 1.0 mm thick. In the work described here, 24-well cell culture plate with 500 μL/well ACSF buffer was

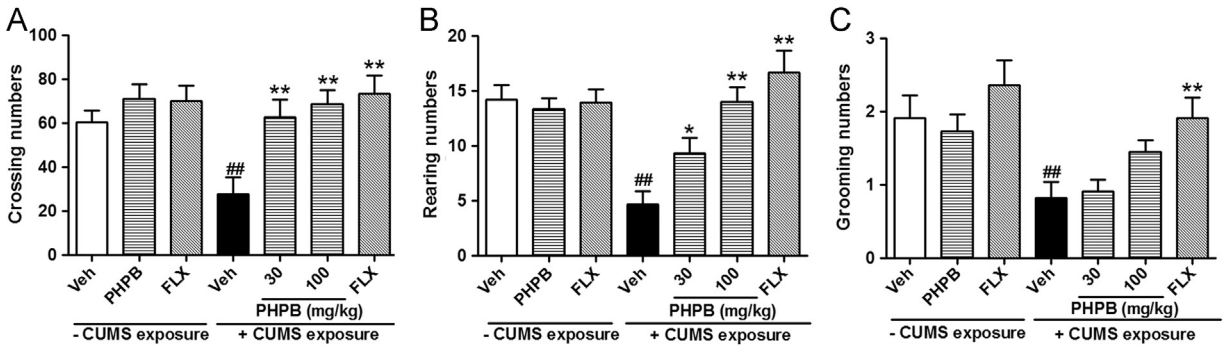

Figure 2 Effects of CUMS, PHPB and FLX on behaviors of rat crossing (A), and rearing (B) and grooming (C) were tested in the open field following the CUMS procedure. $P<0.01$ CUMS *vs*. the vehicle-treated control group; $^{*}P<0.05$, $^{**}P<0.01$ *vs*. CUMS alone, $n=11$.

used to incubate the brain slices. The plate was immersed in a water bath at 37 °C. After incubation for 30 min, pipet the supernatants 190 μL/well into a centrifugal tube and add 10 μL $HClO_4$ to a final, 0.6 mol/L, and centrifuge at a speed of 15,000 rpm for 20 min at 4 °C. Then after pipetting supernatants 120 μL into a centrifugal tube, then added 180 μL solution B($C_6H_5O_7K_3$ 20 mmol/L, K_2HPO_4 300 mmol/L, EDTA · Na_2 2 mmol/L), centrifuge at a speed of 15,000 rpm for 20 min at 4 °C. The supernatants were analyzed with HPLC-ECD (BASi LC-4C, West Lafayette, IN, USA).

2.8. *Statistical analysis*

The data were analyzed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). The data in figures were presented as mean ± SEM. Significant differences between the treatment groups were identified by one-way ANOVA followed by the Bonferroni test. Values of $P<0.05$ were considered statistically significant.

3. Results

3.1. *Effects of PHPB on depressive-like behaviors in rats*

3.1.1. *Open field test*

In order to determine whether PHPB possesses an antidepressant-like activity, we tested the number of crossing, rearing and grooming in open field test after CUMS for five weeks. As shown in Fig. 2, chronic stress significantly decreased activity as indicated by reduction in numbers of crossings ($P<0.01$ *vs*. control-vehicle), rearing ($P<0.01$ *vs*. control-vehicle) and grooming ($P<0.01$ *vs*. control-vehicle). After treatment with PHPB (30 or 100 mg/kg) or FLX (10 mg/kg), the CUMS rats significantly reversed the above mentioned behavioral alterations compared to the vehicle-treated CUMS group. The rats without CUMS exposure, chronic treatment with PHPB (100 mg/kg) or FLX (10 mg/kg) had no effect on open field performance.

3.1.2. *Sucrose preference test*

The sucrose preference was assessed by calculating the percentage of sucrose solution intake as total sucrose solution intake/total intake of sucrose and water. As shown in Fig. 3A, CUMS-vehicle group showed a remarkable reduction in sucrose consumption *versus* the unstressed-vehicle group ($P<0.01$ *vs*. control-vehicle). PHPB at doses of 30 mg/kg, 100 mg/kg or FLX at dose of 10 mg/kg markedly increased the percentage of sucrose consumption in the sucrose preference test compared with the CUMS-vehicle ($P<0.01$ *vs*. CUMS).

3.1.3. *Forced swimming test*

As indicated in Fig. 3B, CUMS exposure significantly increased immobility time in the forced swimming test in the stressed-vehicle rats in comparison with the control-vehicle animals ($P<0.01$ *vs*. control-vehicle), PHPB treatment (30 and 100 mg/kg) induced a noteworthy decrease in immobility time in CUMS rats compared to vehicle-treated CUMS exposed rats ($P<0.01$ *vs*. CUMS). Similar results were obtained after FLX (10 mg/kg) administration ($P<0.01$ *vs*. CUMS).

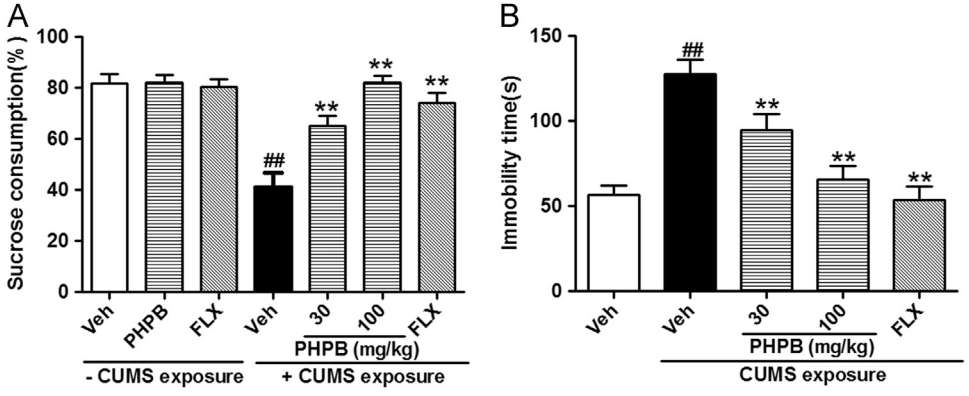

Figure 3 Effects of CUMS, PHPB and fluoxetine treatment on sucrose consumption (A) and immobility time in the forced swimming test (B). $P<0.01$ CUMS *vs*. the vehicle-treated control group; $^{**}P<0.01$ *vs*. CUMS alone, $n=11$.

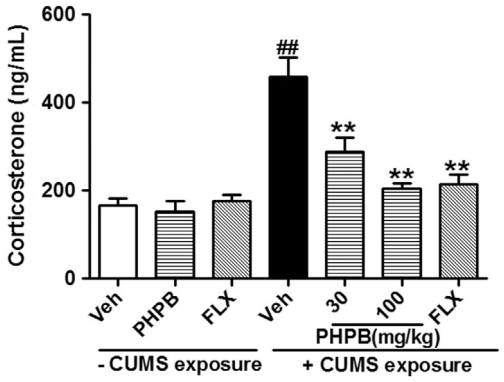

Figure 4 Effects of CUMS, PHPB and fluoxetine treatment on serum corticosterone. $P<0.01$ CUMS *vs*. the vehicle-treated control group; $^{**}P<0.01$ *vs*. CUMS alone, $n=11$.

According to the above results from behavioral tests, PHPB definitely attenuated depressive-like behaviors in CUMS-treated rats. The underlying mechanisms were needed to be clarified.

3.2. *Impact of PHPB on serum corticosterone levels in rats*

The effects of PHPB on serum corticosterone levels were then observed. The CUMS exposure caused a conspicuous elevation of serum corticosterone level (Fig. 4) compared to the non-stressed control group ($P<0.01$ *vs*. control-vehicle). Administration of PHPB or fluoxetine notably reduced the enhanced serum corticosterone level ($P<0.01$ *vs*. CUMS). In non-stressed rats, there was no big change on basal serum corticosterone levels with chronic treatment of PHPB (100 mg/kg) or FLX (10 mg/kg). These results suggested that PHPB significantly reduced the enhanced serum corticosterone level induced by CUMS, which might be involved in its antidepressant effect.

3.3. *PHPB increased BDNF levels in serum and hippocampus in CUMS rats*

It has been reported that BDNF plays an important role in the pathophysiology of depression[12,13]. Thus, the effects of PHPB on BDNF levels in serum and hippocampus in CUMS rats were explored. As shown in Fig. 5A, CUMS exposure had a noticeable impact on serum BDNF concentrations ($P<0.01$ *vs*. control-vehicle). The stress-induced decreases in serum BDNF levels were notably reversed in rats treated with PHPB (100 mg/kg) or FLX (10 mg/kg) ($P<0.05$ *vs*. CUMS). On the other hand, PHPB (100 mg/kg) and FLX (10 mg/kg) treatments had no influence on serum BDNF level in normal control rats.

The BDNF levels in hippocampus of stressed rats (*vs*. non-stressed vehicle control rats) were also decreased significantly by the Western blot analysis ($P<0.01$ *vs*. control-vehicle, Fig. 5B). The reduction was ameliorated by 100 mg/kg PHPB treatment ($P<0.05$ *vs*. CUMS). Similar results were obtained with the fluoxetine (10 mg/kg) administration ($P<0.01$ *vs*. CUMS). These results indicated that PHPB increased BDNF levels in serum and hippocampus in CUMS rats, which might contribute to its antidepressant effect.

3.4. *PHPB enhanced phosphorylation of CREB in CUMS rats*

It's well established that the BDNF expression is closely related to the activation of cAMP response element binding protein (CREB).

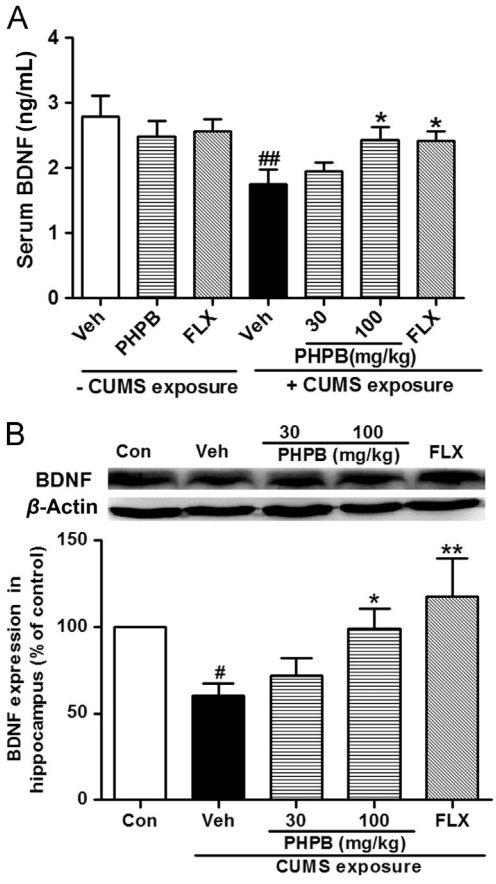

Figure 5 Effects of CUMS, PHPB and fluoxetine treatment on BDNF level. (A) The content of serum BDNF of different groups. $P<0.01$, CUMS *vs*. the vehicle-treated control group; $^{*}P<0.05$ *vs*. CUMS alone, $n=11$. (B) The expression of BDNF in the hippocampus. Quantification of the protein levels of BDNF were determined by Quantity One software. $P<0.05$, CUMS *vs*. the vehicle-treated control group; $^{*}P<0.05$, $^{**}P<0.01$ *vs*. CUMS alone, $n=4$. Band 1: vehicle group (distilled water); Band 2: CUMS+vehicle (distilled water); Band 3: CUMS+PHPB (30 mg/kg); Band 4: CUMS + PHPB (100 mg/kg); Band 5: CUMS+FLX (10 mg/kg).

The effects of PHPB on phosphorylation of CREB, the active form of CREB, in CUMS rats were investigated. As shown in Fig. 6, the phosphorylated protein levels of CREB (p-CREB) in the hippocampus of rats exposed to CUMS were obviously decreased compared to non-stressed vehicle-control rats ($P<0.01$ *vs*. control-vehicle). But, there was no difference in total CREB levels among groups. Administration of PHPB (100 mg/kg) increased the ratio of p-CREB/CREB in the hippocampus ($P<0.05$ *vs*. CUMS). Similar results were obtained after treatment of FLX 10 mg/kg ($P<0.01$ *vs*. CUMS). These results indicated that PHPB increased BDNF expression might mediate by phosphorylation of CREB.

3.5. *PHPB normalized the levels of 5-HT and 5-HIAA in the brain of CUMS rats*

The levels of monoamines, 5-HT and/or noradrenaline (NE) in brain, especially at synapse during depression, are very important. The effects of PHPB on the levels of 5-HT and 5-HIAA in the brain of CUMS rats were examined. As shown in Fig. 7, both 5-HT and its metabolite 5-HIAA levels in prefrontal cortex and

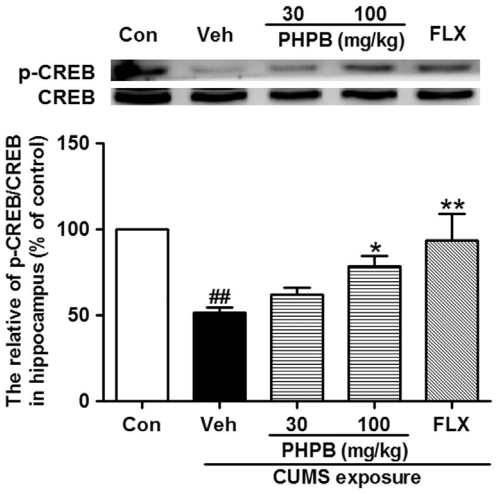

Figure 6 Effects of CUMS, PHPB and fluoxetine treatment on the CREB phosphorylation in the hippocampus. The hippocampal extracts were subjected to Western blot with antibodies against phosphorylation CREB (p-CREB) and total CREB. The upper panel is representative blotting images. $P<0.01$, CUMS *vs.* the vehicle-treated control group; $^{*}P<0.05$, $^{**}P<0.01$ *vs.* CUMS alone, $n=5$. Band 1: vehicle group (distilled water); Band 2: CUMS+vehicle (distilled water); Band 3: CUMS+PHPB (30 mg/kg); Band 4: CUMS+PHPB (100 mg/kg); Band 5: CUMS+fluoxetine (10 mg/kg).

hippocampus of rats exposed to CUMS were significantly decreased compared to control group ($P<0.01$ *vs.* control, respectively). However, treatment of PHPB (100 mg/kg) could reverse the reduction of 5-HT and 5-HIAA in both prefrontal cortex and hippocampus of stressed rats. PHPB showed no effect on NE level in both CUMS and control rats. These results demonstrated that PHPB could elevate the levels of 5-HT and 5-HIAA in the brain during depression in CUMS rats.

4. Discussion

PHPB is a new drug candidate for anti-ischemic stroke and is currently in phase II/III clinical trial. It is a prodrug of D,L-NBP that has been used in clinic more and more frequently for treatment of stroke. Recently, it has been also showed to benefit for dementia such as vascular dementia, Alzheimer's disease and so on. PHPB, as a prodrug, has better physical and chemical properties and better oral bioavailability about 3 times high compared to D,L-NBP[21].

In the present study we demonstrated the anti-depressive effects of PHPB in rats subjected to CUMS, a widely used animal model of depression. The depressive behaviors in this animal model are similar to those exhibited by depressed patients[22]. Therefore, it can be used for the evaluation of antidepressant in preclinical study. The behavioral data of our study are in agreement with the previous reports[23,24]. Open field test is widely used to evaluate locomotor and exploratory behaviors in experimental animals[25]. According to our results, CUMS rats exhibited decreased crossing and rearing numbers, which indicated these animals were becoming respectively less-exploratory and apathetic. PHPB produced a significant amelioration on locomotor behaviors in CUMS rats. The sucrose preference test is an indicator of anhedonia-like behavioral change, which mimics a key symptom of human depression[26,27]. In this study, stressed rats showed a remarkable reduction in sucrose solution consumption compared to the rats without CUMS exposure and treatment with PHPB significantly reversed this behavioral change, suggesting the antidepressant-like effect of PHPB in CUMS model of depression. The FST is considered as a kind of "behavior despair" model similar to what exhibited by depressed patients[28]. The data in our study indicated that rats subjected to chronic stress exhibited increased duration of immobility and decreased swimming episodes in FST, which were the depression-like behaviors. However, chronic administration of PHPB prominently decreased the duration of immobility and increased the swimming episodes in stressed rats, which indicated the antidepressant-like effects of PHPB. Additionally, in this study the fact that no noteworthy difference in spontaneous locomotor activity was observed in open field test among three unstressed groups (vehicle, PHPB 100 mg/kg and FLX 10 mg/kg), suggesting that changes in behaviors in the FST were induced by stress rather than the action of antidepressant.

It is gratifying that administration of PHPB significantly ameliorated all these behavioral alterations associated with CUMS-induced depression. Moreover, PHPB had the beneficial effects against CUMS-induced changes in serum corticosterone (CORT) level and neurotrophin, BDNF expression in serum and hippocampus. A growing body of studies suggested that the development of depressive disorders was associated with a sustained activation of the HPA axis, which is a crossroad between central and peripheral pathways, causing the increase of the serum CORT level[29,30]. In human being, various stress events could trigger HPA axis activation. Briefly, hypothalamus releases corticotrophin in response to a stressor, which in turn activates the secretion of ACTH from the pituitary, and subsequently stimulates the secretion of cortisol from the adrenal cortex. It has been considered that enhanced activity of the HPA axis is mainly resulted from a long-lasting impairment of HPA axis feedback inhibition in depressive animal models and in depressed patients[31,32]. The dysfunction of the HPA axis negative feedback system is characterized by the failure in suppressing excessive secretion of serum CORT[9,33]. In this study, we found that the rats exposed to CUMS showed a prominent increase in serum CORT level. The data was in accordance with previous reports that CUMS exposure increased serum CORT level[34]. Treatment with PHPB and FLX remarkably decreased the serum CORT level in stressed rats.

Although disruption of HPA axis negative feedback system may contribute to the generation of depressive symptoms, central nerve nutritional disorder is also very important for depression[35]. The level of BDNF reduced notably during depression. It was reported that the serum BDNF level was lower in depressive patients, suggesting blood BDNF level as a useful biological marker in antidepressant therapies[14,36,37]. In this study, we detected the serum BDNF level and found that the CUMS rats showed a rapid decrease in serum BDNF level compared to the control rats. However, PHPB treatment normalized the CUMS-induced decrease in serum BDNF level which was in accordance with previous report[38]. Besides serum BDNF, chronic stress application markedly reduced BDNF levels in hippocampus tissue[39]. In fact, it is generally accepted that down-regulated hippocampal BDNF is an underlying cause for the development of depression. Antidepressants could reverse depression-induced BDNF downregulation in the hippocampus[40]. Consistently, in our present study, PHPB treatment reversed the CUMS-induced decrease in hippocampus BDNF level, which might be a mechanism for antidepressant-like effect of it.

It has been known that the BDNF expression is closely related to the activation of CREB (active form of CREB is phosphorylated

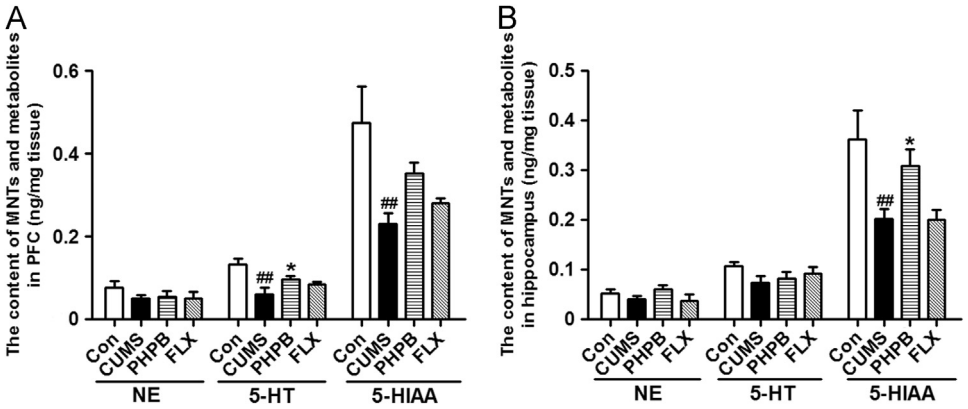

Figure 7 Effects of PHPB on the content of monoamine neurotransmitters and metabolites outside of the neurons in PFC and hippocampus. (A) The level of monoamine neurotransmitters in PFC. (B) The level of monoamine neurotransmitters in hippocampus. $P<0.01$ *vs*. control group; $^{*}P<0.05$ *vs*. CUMS alone, $n=5$–7.

CREB, p-CREB)[41]. We demonstrated that CUMS exposure resulted in a reduction of p-CREB/CREB levels in hippocampus, whereas, administration of PHPB increased the p-CREB without affecting total CREB levels. It has been reported that l-3-*n*-butylphthalide (D,L-NBP, active form of PHPB *in vivo*) promoted the proliferation, survival, and differentiation of newborn neural cells mediated by PKA/Akt-pCREB-BDNF pathway[41]. So we speculate that the above pathway could be also an important target of PHPB for treatment of depression.

It has been well known that brain monoamine neurotransmitters are closely associated with the pathological changes in depression, especially noradrenaline (norepinephrine, NE) and 5-hydroxytryptamine (serotonin, 5-HT) in patients with major depression or animal depressive models[42,43]. The results in our study showed chronic stress induced depressive-like behaviors of rats and reduced extracellular monoamine neurotransmitters: NE, 5-HT and its metabolite 5-HIAA levels in the brain. However , administration of PHPB could reverse the lower 5-HT and 5-HIAA, especially in prefrontal cortex (PFC). Meanwhile, the monoamine uptake analysis *in vitro* showed that PHPB 10 μmol/L had only about 20% inhibition rate for synaptosomal uptaking of 5-HT, in comparison, the 84% inhibition rate for positive control FLX, and PHPB has no inhibitory effect on monoamine oxidase (MAO-A/B) (data not shown). Therefore, the changes of activity of neurons in PFC during CUMS might be involved. Our unpublished data showed treatment with PHPB or fluoxetine could prevent the CUMS-induced decrease of burst-firing frequency (The difference was close to statistically significance). Taking these results together, PHPB might reverse the reduced extracellular 5-HT level in PFC *via* building up the impaired burst-firing patterns in CUMS-exposed rats.

All above results demonstrated that PHPB is a multi-target drug candidate for the treatment of depression. Though PHPB is different from FLX in chemical structure and mechanisms, the anti-depression effects of both compounds showed in this study were similar. It indicated that the mechanisms of PHPB need to be studied further. In addition, the effects of PHPB on 5-HT uptake at the synaptic junction are also needed to be explored.

However, compared with other antidepressants such as FLX etc., PHPB would be a new or good choice. Because many patients suffer from ischemic stroke and/or AD simultaneously, they may accompany by depression. In addition, unlike FLX, PHPB has weak effects on ion channels, which might make PHPB have fewer side effects in central nervous and cardiovascular systems.

In summary, our data demonstrated that PHPB was able to rescue depressive-like symptoms induced by CUMS in rats and the mechanisms were related to modulating the hyperactivation of the HPA axis activity, improving hippocampal nutritional disturbance and facilitating release 5-HT in PFC. Therefore, to treat depression might be a new indication of PHPB.

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