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Antidepressant like effects of piperine in chronic mild stress treated mice and its possible mechanisms

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Abstract

In this study, we investigated the antidepressant-like effect of piperine in mice exposed to chronic mild stress (CMS) procedure. Repeated administration of piperine for 14 days at the doses of 2.5, 5 and 10 mg/kg reversed the CMS-induced changes in sucrose consumption, plasma corticosterone level and open field activity. Furthermore, the decreased proliferation of hippocampal progenitor cells was ameliorated and the level of brain-derived neurotrophic factor (BDNF) in hippocampus of CMS stressed mice was up-regulated by piperine treatment in the same time course. In addition, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactic dehydrogenase (LDH) assays showed that piperine (6.25–25 μM) or fluoxetine (FLU, 1 μM) dose-dependently protected primary cultured hippocampal neurons from the lesion induced by 10 μM corticosterone (CORT). Reverse transcription-polymerase chain reaction (RT-PCR) was used to detect the messenger ribonucleic acid (mRNA) level of BDNF in cultured neurons. Treatment with piperine (6.25–25 μM) for 72 h reversed the CORT-induced reduction of BDNF mRNA expression in cultured hippocampal neurons. In summary, up-regulation of the progenitor cell proliferation of hippocampus and cytoprotective activity might be mechanisms involved in the antidepressant-like effect of piperine, which may be closely related to the elevation of hippocampal BDNF level.

Keywords: Piperine; Hippocampal progenitor cell proliferation; Brain-derived neurotrophic factor; Antidepressant; Chronic mild stress; Primary cultured hippocampal neuron

Introduction

Major depression is a serious mood disorder that affects 17–20% of the population of the world and may result in major social and economic consequences (Kessler et al., 1994). Although significant progress has been made in the research work for treatment of depression, the therapeutic responses are still unsatisfactory and the common molecular mechanisms of antidepressants are still far from clearly understood.

Animal studies have suggested that factors involved in the pathophysiological changes of depression include elevated glucocorticoid secretion, decreased level of brain-derived neurotrophic factor (BDNF), and cAMP-response element binding protein (CREB) in the hippocampus (Duman et al., 2000). All these changes could lead to reduction of hippocampal volume and vulnerability to subsequent episodes of depression as a result of decreased neurogenesis, increased remodeling of dendrites, and loss of glial cells (for review see Manji et al., 2001; Nestler et al., 2002a,b; Coyle and Duman, 2003). Chronic stress procedure, as a widely used animal model for antidepressant screening, can induce depression-like behavior (Garcia, 2002). More importantly, chronic stress procedure decreases neurogenesis in the adult hippocampus (Czeh et al., 2001; Gould et al., 1997; Tanapat et al., 1998). Some research also indicated that classical antidepressants, fluoxetine and desipramine, protected PC12 cells from the

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lesion induced by corticosterone and up-regulated the hippocampal neurogenesis in chronically stressed mice (Li et al., 2003, 2004).

Piperine is a major alkaloid of black pepper (*Piper nigrum* Linn.) and long pepper (*P. longum* Linn.). Piperine has been used extensively as condiment and flavoring for all types of savory dishes. Piper species have been used in folk medicine for the treatment of various diseases, including seizure disorders. Piperine is suggested to have anxiolytic effect and anti-inflammatory activity. Results from previous studies demonstrated that piperine showed antidepressant-like activity in two classical behavioral models, forced swimming test and tail suspension test (Lee et al., 2005; Li et al., in press). Piperine also inhibited monoamine oxidase (MAO) activity (Kong et al., 2004; Lee et al., 2005; Li et al., in press) and increased the level of noradrenaline and serotonin in some regions of mouse brain (Li et al., in press). However, the effect of piperine on neurogenesis is not well demonstrated. In order to explore the influences of piperine on hippocampal neurogenesis in chronic mild stressed mice and the possible common mechanisms for antidepressants, the effects of piperine on the progenitor cell proliferation and BDNF level in hippocampal sections from mice exposed to chronic mild stress were measured. Furthermore, the influence of piperine on cell survival and BDNF level in corticosterone-treated cultured hippocampal neurons was also examined.

Materials and methods

Animals

Adult male Kunming mice (18–22 g) were obtained from the Experimental Animal Center of Shenyang Pharmaceutical University. Before CMS procedure, animals were housed in groups under standard conditions (12 h light/dark cycle; lights on from 0730 to 1930; 22±2 °C ambient temperature; 55±10% relative humidity). They were fed with standard diet and water ad libitum and were allowed to acclimate 7 days before they were used. All animals were randomized into 6 groups with 20 mice per group. The experiment procedures involving animals and their care were conducted in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Drug administration

Piperine with a purity of 99.8% was obtained as described in previous study (Wei et al., 2004), and dissolved in 0.9% saline after being dispersed with Tween-80. The final concentration of Tween-80 is less than 0.1%. The selective serotonin reuptake inhibitor, fluoxetine, was used as a reference control. Drugs and vehicle were administered intraperitoneally (i.p.) in a volume of 10 ml/kg. The repeated drug treatment of CMS animals was performed twice daily from day 22 to day 35. The doses of these drugs in this study were determined as referred to previous studies (Lee et al., 2005; Li et al., in press; Song et al., 2006). Previous in vitro studies have established fluoxetine has an effect on cell proliferation at 1 μM, a concentration comparable to plasma levels of therapeutic concentrations of this antidepressant (Edgar et al., 1999; Manev et al., 2001). Thus, on the basis of the plasma concentration of piperine which showed its antidepressant effects in CMS procedure, a concentration range of 6.25, 12.5 and 25 μM piperine were used in in vitro experiments.

Chronic mild stress procedure

In the present study, the CMS procedure was performed as described in our previous research work (Song et al., 2006). Initially, the animals were given 1% sucrose solution for a 48-h period in their home cages with no food or water following food and water deprivation for 18 h. Subsequently, they were given sucrose for 1 h per day on five consecutive days. The sucrose intake was measured at the end of the training in order to group the mice. The mice in the experimental groups were then subjected to CMS for 5 weeks. The CMS procedure consisted of a variety of unpredictable mild stressors including one period (2 h) of paired caging, one period (3 h) of tilted cage (45°), one period of food and water deprivation (18 h), one period of (1 h) restricted access to food (5 micropellets), one period of (1 h) exposure to an empty bottle, one 21 h period with wet cage (200 ml water in 100 g sawdust bedding) and one period with 36 h of continuous light. Thus, stressors were presented both during the mice' active (dark) period and during the inactive (light) period. These stressors were randomly scheduled over a one-week period and repeated throughout the 5-week experiment. In contrast to other previous procedures in rats, nociceptive stressors were excluded, and only environmental and social disturbances were applied (Pardon et al., 2000). The non-stressed control animals were housed in normal conditions.

Open field test

Twenty-four hours after the last stressor and drug exposure, the open field test was performed. The open field consisted of a base (100×100 cm) and black walls (20 cm) divided into 25 (5×5) identical sectors (20×20 cm) by white stripes. The squares were subdivided into peripheral and central sector, where the central sector included the 9 central squares (3×3) and the peripheral sector contained the squares close to the wall. The animals were placed in the central sector and their activity video-recorded for 5 min for further analysis. The open field arena was thoroughly cleaned between each test. The room was lit by a dim red light. No stressor was applied to the animals for at least 24 h before the test. Open field activity was scored manually. Motility was scored when an animal crossed a sector border with both its hind-limbs. The following activities were scored: the number of peripheral sectors crossed (peripheral activity), number of central squares crossed (central activity), and the overall activity, in both peripheral and central areas (total activity) during the 5-min test.

Sucrose consumption and body weight gain

Sucrose intake (1% sucrose solution) and body weight were measured once a week, on separate days, during a 1-h window

after 18 h of food and water deprivation. Consumption was measured by weighing the pre-weighed bottle at the end of the test. The intake was expressed in relation to the animals' body weight (g/kg). Baseline was measured less than 1 week before the start of CMS. The food and water deprivation period preceding sucrose intake measurement may be considered as a further stress applied on top of the CMS protocol. However, control mice were also exposed to the food and water deprivation, as a part of the test.

Plasma corticosterone level

Separate groups of animals were used for the measurement of plasma corticosterone level of CMS stressed mice. Chronic treatment with piperine (2.5–10 mg/kg i.p.) was given during the last 2 weeks of the 5-week CMS procedure and the animals were sacrificed at weekly intervals. The mouse blood samples were collected and kept on ice and then centrifuged immediately at 2000 ×*g* at 4 °C for 15 min. The obtained plasma was kept at −80 °C until analysis. Corticosterone levels were measured using a commercially available radioimmunoassay (RIA) kit (ICN Biomedicals, Costa Mesa, CA, USA).

Hippocampal frozen section preparation

For labeling with bromodeoxyuridine (BrdU; Sigma, St. Louis, MO), mice were administered BrdU (4×75 mg/kg, i.p. every 2 h) 4 days after the last drug and stressor treatment. The 4-day time point was chosen because a similar paradigm has been used in a previous study of chemical-induced seizures on hippocampal neurogenesis (Parent et al., 1997). Twenty-four hours after the last BrdU injection, mice were sacrificed and transcardially perfused (cold saline for 5 min following by 4% cold paraformaldehyde for 15 min). After perfusion, all brains were post-fixed overnight in paraformaldehyde (with shaking) at 4 °C and stored at 4 °C in 30% sucrose. Serial sections of the brains were cut (30 μm sections) through the entire hippocampus on a microtome and were stored at −20 °C.

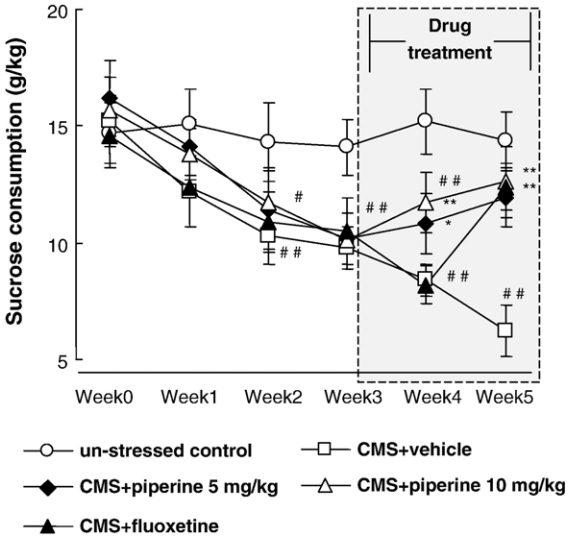

Fig. 1. Effects of piperine on the sucrose consumption of mice exposed to chronic mild stress (CMS) (mean±S.E.M., $n=12$). Chronic treatment with piperine (2.5–10 mg/kg, i.p.) was given during the last 2 weeks of the 5-week chronic mild stress procedure. $^{\#}P<0.05$ compared with un-stressed control group; $^{*}P<0.05$ compared with CMS+vehicle group.

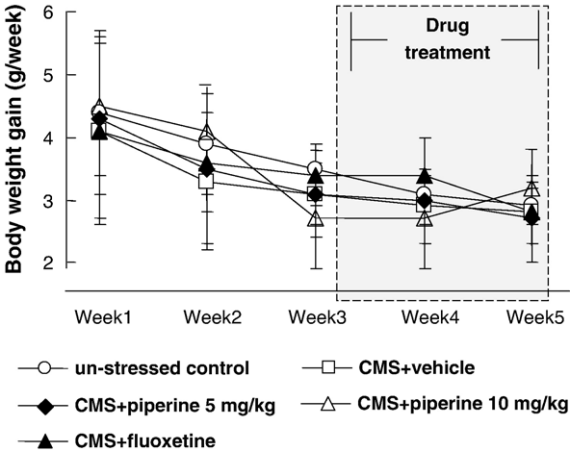

Fig. 2. Effects of piperine on the body weight gain of CMS stressed mice (mean±S.E.M., $n=12$). Chronic treatment with piperine (2.5–10 mg/kg, i.p.) was given during the last 2 weeks of the 5 week chronic mild stress procedure.

Immunohistochemical determination

Free-floating sections were used in the determination of BrdU labeling. After DNA denaturation and several PBS rinses, sections were incubated for 30 min in 2 N HCl and then 10 min in boric acid. After washing in PBS, sections were incubated for 30 min in 3% H_2O_2 to eliminate endogenous peroxidases. After blocking with 3% normal goat serum in 0.01% Triton X-100, cells were incubated with anti-mouse BrdU or BDNF (1:400; Boehringer–Mannheim, Indianapolis, IN) overnight at 4 °C. Sections were then incubated for 1 h with secondary antibody (biotinylated goat anti-mouse; Vector Laboratories, Burlingame, CA) followed by amplification with an avidin–biotin complex, and cells were visualized with diaminobenzidine (DAB). Finally, BrdU-positive cells or BDNF level was observed and pictures were taken with microscope (Olympus BX60, Japan). The expression of BDNF proteins in the hippocampus was analyzed using the image analysis system (MetaMorph/CoolSNAP fx/AX70, Germany) and was measured by inspecting the average gray value and average optical density.

Primary cultures of hippocampal neurons

Pregnant Sprague–Dawley rats were supplied by the Experimental Animal Center of Shenyang Pharmaceutical University. Primary hippocampal neuron cultures were established from 18-day fetuses of Sprague–Dawley rats. Hippocampi were dissected, minced, and digested in calcium- and magnesium-free Hanks' balanced salt solution (HBSS) containing 0.25% trypsin and 6 g/l glucose, then resuspended in DMEM, high glucose type, supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml

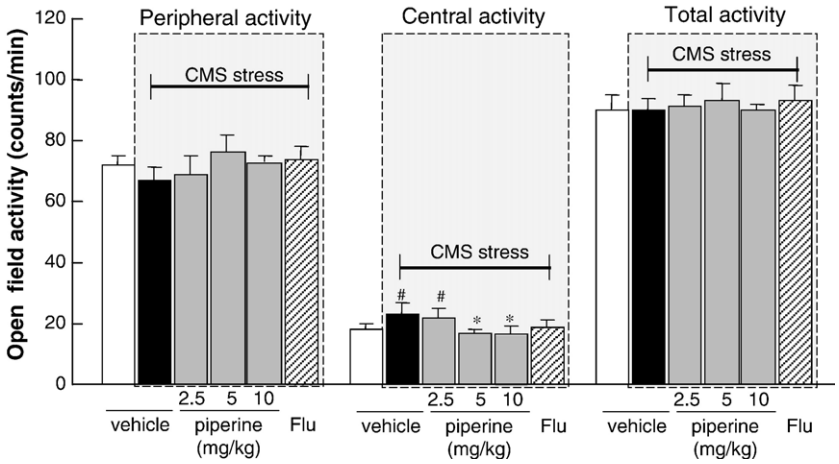

Fig. 3. Effects of piperine on peripheral activity, central activity and total activity of CMS stressed mice in open field test (mean±S.E.M., $n=12$). Chronic treatment with piperine (2.5–10 mg/kg, i.p.) was given during the last 2 weeks of the 5-week chronic mild stress procedure. $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with un-stressed control group; $^{*}P<0.05$, $^{**}P<0.01$ compared with CMS+vehicle group.

streptomycin, 25 μg/ml trypsin, and 6 g/l glucose. The tissues were dissociated mechanically with a pipette, and cells were inserted directly into 96-well plates or 60-mm culture dishes at 60,000 cells/cm^2, the substratum being previously coated with poly-L-lysine (0.1 mg/ml in 100 mM borate buffer, pH 8.4) overnight. Cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. On the third day, the cultures were transferred to DMEM containing 5% FBS and 10 μM cytosine arabinoside (AraC) for 2 days to halt the proliferation of non-neuronal cells. The cells were divided into six equal groups: control, 10 μM CORT, CORT (10 μM)+FLU (1 μM), CORT (10 μM)+piperine (6.25, 12.5 and 25 μM), $n=8$ per group in both LDH activity and MTT assay.

Assays for neuronal survival

Total neuron survival was quantified by observing the growth of neurons, and counting the number of neurons in the same groups and by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, the viable neurons in culture plates containing lines on the bottom in a 1×1 mm^2 area were observed and counted. Neurons that died in the intervals between examination points were usually absent, and the viability of the remaining neurons was assessed by morphological criteria, whereby neurons with intact neurites of uniform diameter and soma with a smooth round appearance were considered viable, and neurons with fragmented neurites and vacuolated cell bodies were considered nonviable.

For MTT assay, the cells were incubated with 0.5 mg/ml MTT for 4 h at 37 °C. Living cells converted the yellow MTT dye into an insoluble blue formazan product. The dye was solubilized by DMSO, and the absorbance intensity (490 nm; A_{490}) of each solution was measured in a 96-well plate reader.

For LDH assay, the cell culture medium was collected and LDH activity was determined with an LDH assay kit (Biovision, CA, USA). The reaction mixture was monitored at 340 nm with a spectrophotometer and the LDH activity was calculated from the decrease of NADH absorbance resulting from the conversion of pyruvate to lactate.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cultured hippocampal neurons with the use of TRIzol reagent (Invitrogen, San Diego, CA, USA). Single-stranded cDNA was synthesized from 1 μg total RNA by the use of the first-strand cDNA Synthesis Kit (Promega, Madison, WI, USA). The synthesized cDNA as a template in the PCRs was stored at −20 °C until use. The PCRs were performed in 25 μl volume, which included 1 μl cDNA sample, 2.5 mM $MgCl_2$, 0.25 mM dNTP, 1×PCR buffer, 1 μl of each specific primer (BDNF: forward 5′-GAC AAG GCA ACT TGG CCT AC-3′, reverse 5′-CCT GTC ACA CAC GCT CAG CTC-3′, product size: 356 bp; GAPDH: forward 5′-ACA TTG TTG CCA TCA ACG AC-3′, reverse 5′-ACG CCA GTA GAC

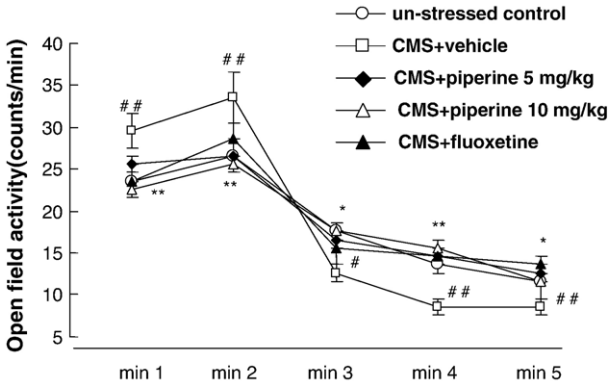

Fig. 4. Effects of piperine on the open field activity of CMS stressed mice (mean±S.E.M., $n=12$). The open field test was performed 24 h after last stressor exposure. Chronic treatment with piperine (2.5–10 mg/kg, i.p.) was given during the last 2 weeks of the 5-week chronic mild stress procedure. $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with un-stressed control group; $^{*}P<0.05$, $^{**}P<0.01$ compared with CMS+vehicle group.

Table 1
Effects of piperine on the plasma corticosterone level of CMS stressed mice (mean±S.E.M., $n=8$)

Group	Dose (mg/kg)	Corticosterone level (ng/ml)					
		Week 0	Week 1	Week 2	Week 3	Week 4	Week 5
Non-stressed	_	49.6±6.3	47.8±5.4	53.2±4.9	51.3±7.1	49.7±6.3	48.4±8.8
Chronic mild stressed	_	47.8±8.5$^{\#\#}$	165.3±14.3$^{\#\#}$	153.1±11.8$^{\#\#}$	121.8±11.7$^{\#\#}$	93.4±12.9$^{\#}$	75.6±9.4$^{\#}$
Flu+stress	10	51.2±7.6$^{\#\#}$	159.5±13.4$^{\#\#}$	151.2±13.9$^{\#\#}$	127.2±10.6$^{\#\#}$	85.8±9.3$^{\#}$	59.7±6.4$^{\#}$ **
	2.5	52.5±6.6$^{\#\#}$	166.2±15.2$^{\#\#}$	154.3±13.1$^{\#\#}$	125.1±11.9$^{\#\#}$	96.7±12.1$^{\#}$	73.7±8.2$^{\#}$
piperine+stress	5	48.3±7.9$^{\#\#}$	171.4±16.6$^{\#}$	149.8±17.4$^{\#\#}$	119.6±14.2$^{\#\#}$	88.3±11.5$^{\#}$	68.5±8.4$^{\#}$ *
	10	46.8±5.7$^{\#\#}$	165.3±12.9$^{\#\#}$	151.3±13.1$^{\#\#}$	124.7±11.9$^{\#\#}$	82.3±10.2$^{\#}$ *	61.2±6.9 **

Chronic treatment with piperine (2.5–10 mg/kg, i.p.) was given during the last 2 weeks of the 5-week CMS procedure. The animals were sacrificed at weekly intervals. The blood samples of mice were collected and corticosterone levels were measured by radioimmunoassay. Chronic treatment with piperine (2.5–10 mg/kg, i.p.) was given for 2 weeks. $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with un-stressed control group; $^{*}P<0.05$, $^{**}P<0.01$ compared with CMS+vehicle group.

TCC ACG AC-3′, product size: 216 bp, and 0.4 μl (2 units) Taq DNA polymerase (Promega, Madison, WI, USA). Amplified reaction was performed with a thermocycler for a single 3-min initial denaturation at 94 °C followed by 33 cycles (BDNF) or 26 cycles (GAPDH) under the conditions: 94 °C (20 s), 55 °C (20 s), and 72 °C (20 s) and final extension at 72 °C for 4 min. The PCR products were separated on 1.5% agarose gels containing ethidium-bromide (EB) and quantified by densitometry. The BDNF PCR product was normalized to that of the GAPDH PCR product in each sample.

Statistical analysis

All results are expressed as the mean±S.E.M. values. The statistical analyses were carried out using the SIGMA-STAT system (Version 3.1) for Microsoft Windows. Statistical significance between groups of three or more was analyzed by two-way analysis of variance (ANOVA) or one-way ANOVA among the groups followed by Dunnett's test. A Student's *t*-test was used for the analysis of significant differences between the two groups. All tests were two-tailed. The *P* values of less than 0.05 were considered to be statistically significant.

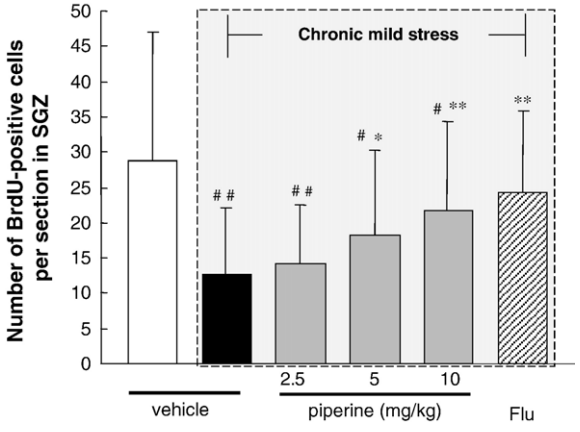

Fig. 5. Effects of piperine on BrdU positive cells in SGZ of hippocampus of chronic mild stressed mice. Chronic treatment with piperine (2.5–10 mg/kg, i.p.) was given during the last 2 weeks of the 5-week chronic mild stress procedure. Mice exposed to mild stress received injections of BrdU 4 days after the last stressor or drug treatment and were killed 24 h after the last BrdU injection. Data are expressed as means±S.E.M. ($n=12$). $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with un-stressed control group; $^{*}P<0.05$, $^{**}P<0.01$ compared with vehicle+CMS group.

Results

Sucrose consumption and body weight gain

In the sucrose solution-training phase (baseline phase), sucrose consumption did not differ significantly among the groups. CMS gradually reduced the consumption of the sucrose solution. As compared to the 15.2 g/kg intake in the baseline test, 5 weeks later the sucrose intake was reduced to 6.2 g/kg in the CMS stressed animals (Fig. 1). Treatment with piperine and fluoxetine caused a gradual recovery of the sucrose intake, while in another separate test, unstressed mice treated with piperine or fluoxetine presented no significant variation in their preference for sucrose consumption (data not shown). Stressed animals with fluoxetine treatment for 2 weeks showed significant improvement in sucrose consumption compared with that of stressed animals administered with vehicle. The results demonstrated that 2 weeks of fluoxetine treatment was needed for a complete recovery. In contrast to the gradual onset of action of fluoxetine, piperine appeared to exert significant antidepressant effects after a shorter time. For the piperine-treated stressed group, some apparent effects appeared as early as after 1 week of drug treatment. At the end of 4 weeks and thereafter the amounts of sucrose solution taken by the stressed animals receiving piperine were significantly higher than that of the vehicle-treated stressed animals. It

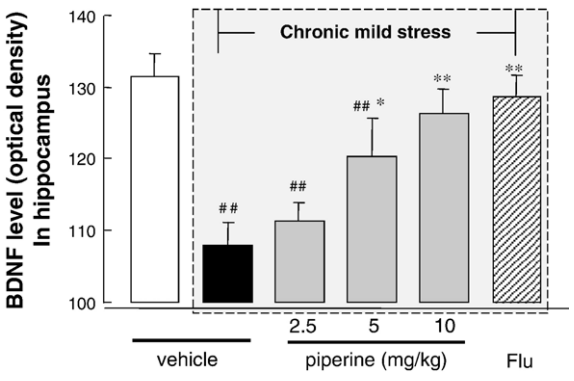

Fig. 6. Effects of piperine on BDNF level of hippocampus of chronic mild stressed mice. Chronic treatment with piperine (2.5–10 mg/kg, i.p.) was given during the last 2 weeks of the 5-week chronic mild stress procedure. Data are expressed as means±S.E.M. ($n=12$). $^{\#\#}P<0.01$ compared with un-stressed control group; $^{*}P<0.05$, $^{**}P<0.01$ compared with vehicle+CMS group.

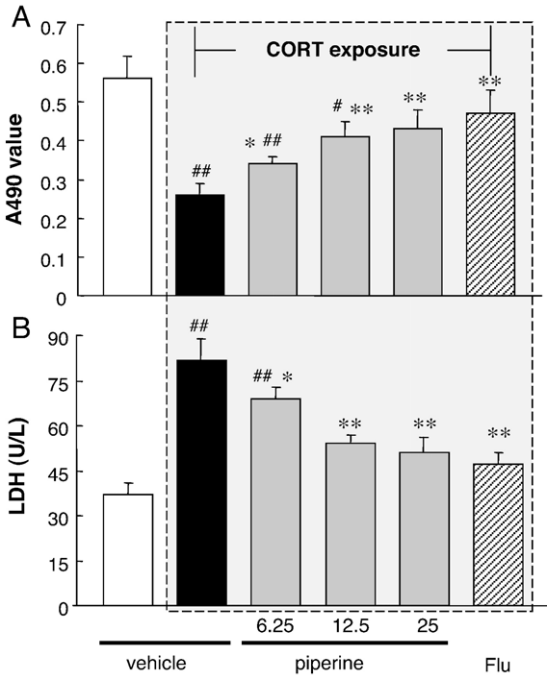

Fig. 7. Protective effect of piperine on primary cultured hippocampal neurons against the lesion induced by CORT. Cells were exposed to CORT 10 μM in the absence or presence of piperine (6.25, 12.5 or 25 μM) or fluoxetine (1 μM) for 72 h, cell viability was measured using a colorimetric MTT assay (A) and LDH assay (B). Data are expressed as means±S.E.M. ($n=8$). Data analysis was performed using Dunnett's t-test. $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with vehicle group; $^{*}P<0.05$, $^{**}P<0.01$ compared with vehicle+CORT group.

appears therefore that piperine might be more efficacious than fluoxetine in restoring the sucrose intake in CMS stressed animals. The results of present study also demonstrated no difference in body weight gain among groups (Fig. 2).

Open field test

The CMS stressed animals showed no differences in total activity compared with non-stressed control animals in 5-min open field test (Fig. 3). However, the first 2-mi locomotor activity of CMS mice was higher than that of non-stressed control group, while locomotor activity in the last 3 min was lower (Fig. 4). Furthermore, there was a tendency to higher activity in the center squares for the CMS mice (Fig. 3). After 2 weeks treatment with piperine or fluoxetine, the above-mentioned behavioral changes in locomotor activity were reduced. In another experiment performed with non-stressed animals, chronic treatment with piperine or fluoxetine showed no effects on open field performance of the animals at the dose range used in the present study.

Plasma corticosterone level

As shown in Table 1, the plasma corticosterone level of the CMS treated mice was significantly higher than that of the un-stressed control. This elevation persisted for at least 35 days although it declined during the CMS processing. These results indicated that the stressed animals might show an impaired feedback regulation in the hypothalamic–pituitary–adrenal (HPA) axis after exposure to CMS procedure. Repeated treatment with FLU (10 mg/kg) or piperine (5, 10 mg/kg) for 2 weeks significantly decreased the elevated corticosterone level.

BrdU assay of hippocampal progenitor cell proliferation

Bromodeoxyuridine (BrdU) was administered to vehicle- or drug-treated animals, and 24 h later mice were anesthetized and perfused for immunohistochemical analysis. BrdU-positive cells were observed along the subgranular zone (SGZ) of dentate gyrus. After 5 weeks of CMS procedure, the number of BrdU-positive cells was decreased in CMS stressed animals compared with un-stressed control group. The effect of various doses of piperine administered for 14 days on the progenitor cell proliferation in the dentate gyrus is shown in Fig. 5. The higher doses (5 and 10 mg/kg) of piperine significantly increased the number of positive cells in the dentate gyrus compared with the CMS control group ($P<0.05$), whereas the lower dose (2.5 mg/kg) had no significant effect. Fluoxetine administration also significantly increased the number of BrdU-positive cells compared with the CMS control group ($P<0.05$).

BDNF level in hippocampus

BDNF has been reported to be important for neuronal survival during development. Moreover, the expression of BDNF in the hippocampus is known to be down regulated in response to acute or repeated stress. In the present study, the average BDNF level decreased in the hippocampus of mice exposed to chronic mild stress ($P<0.05$, Fig. 6) compared with un-stressed control animals, which was shown as the decreased gray value. After 2 weeks repeated administration of piperine (2.5, 5 or 10 mg/kg) or fluoxetine (10 mg/kg), the reduction of BDNF expression was reversed ($P<0.01$, Fig. 6).

MTT and LDH assay

After the treatment of neuron with CORT for 72 h, the A490 nm values significantly decreased and LDH activity markedly increased compared with control ($P<0.01$, Fig. 7), indicating that a high concentration of CORT inhibits the proliferation and survival of cultured hippocampal cells. Fluoxetine (1 μM) and piperine (6.25, 12.5 and 25 μM) reversed the changes of cell viability, which was shown as the significantly increased A490 nm values and decreased LDH release (Fig. 7). Results of the present study revealed that piperine at concentration of 6.25–25 μM could protect the cultured hippocampal neurons from the damage induced by CORT.

BDNF mRNA level in cultured hippocampal neuron

BDNF mRNA level in primary cultured hippocampal cells was determined by RT-PCR and the corresponding bands in electrophoresis were semiquantitatively calculated (Fig. 8). Piperine (6.25, 12.5 and 25 μM) reversed the reduction of

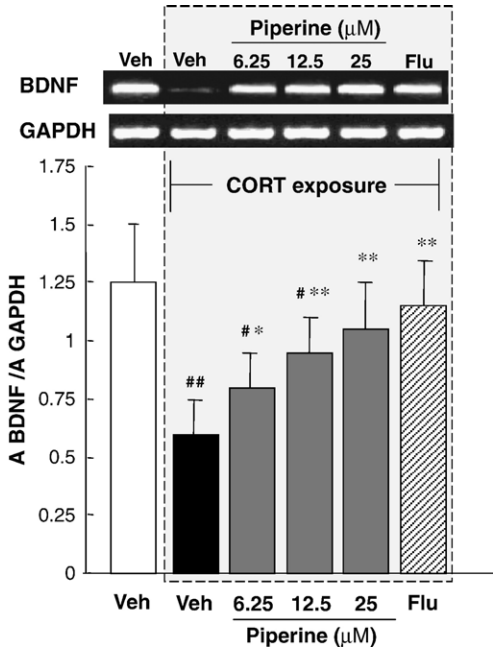

Fig. 8. Gel image of RT-PCR product for BDNF mRNA induced by 10 μM CORT in cultured hippocampal neurons. Cells were exposed to CORT 10 μM in the absence or presence of piperine (6.25, 12.5 or 25 μM) or fluoxetine (1 μM) for 72 h, the total RNA was extracted for RT-PCR and the products were electrophoresed in 2% agarose gel. The results were calculated as the intensity of the lane of each transcript over the intensity of the corresponding GAPDH band and expressed as the mean±S.E.M. ($n=8$). $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with vehicle group; $^{*}P<0.05$, $^{**}P<0.01$ compared with vehicle+CORT group.

BDNF mRNA level induced by CORT exposure compared with the vehicle-treated group ($P<0.01$). The present results suggested that the neuroprotective effect of piperine is associated with the increase in expression of BDNF.

Discussion

The CMS model of depression involves the presentation of a series of varied and unpredictable environmental stressors, such as food and water deprivation, wet cages and light–dark reversal. Following such exposure, animals have been reported to exhibit a persistent reduction in responsiveness to pleasurable stimuli, measured by a decrease in their consumption of 1% sucrose solution (D'Aquila et al., 1994). In addition, a number of behavioral and physiological changes have been reported, including decreased sexual behavior and body weight, alterations in locomotor activity, corticosterone hypersecretion and decreased rapid eye movement (REM) sleep (Papp et al., 1994; Grønli et al., 2005; Bekris et al., 2005). Among these behavioral and physiological changes produced by chronic stress, decreases in sucrose consumption have been advocated as a reliable behavioral measure that may be associated with anhedonia of depression. Reductions in sucrose consumption produced by CMS procedure have been shown to be reversed by chronic treatment with either tricyclic antidepressants or SSRIs (Willner, 1997). In the present study, 2 weeks chronic treatment with piperine, at the dose range from 2.5 to 10 mg/kg, reversed CMS induced reduction of sucrose intake indicating that piperine possessed an antidepressant-like effect in this animal model of chronic mild stress.

The CMS stressed animals showed an interesting variety in locomotor activity performance in the open field test. When the mice were placed in a novel environment, an increase in spontaneous activity in the CMS mice was observed, especially in the first 2 min of the test, while their activity was lower in the next 3 min. Decreased locomotor activity has been used as an index of high emotionality in rats (Royce, 1977; Walsh, 1976; Katz et al., 1981), but not all agree with this. Harris et al. (1997) showed more activity in the open field after CMS, while others have shown enhanced locomotor activity only during the first minute, but an unchanged total activity (Duncko et al., 2001). Pijlman et al. (2003) have shown that physical stress (repeated mild foot shocks) caused inactivity, whereas emotional stress (witnessing the foot shocks) caused hyperactivity. Other changes induced by stress in open field behavior also show divergent results (Denenberg, 1969; Katz et al., 1981; Meerlo et al., 1996; Westenbroek et al., 2003). The present results also demonstrated that 5-week CMS procedure caused no significant alterations in animals' body weight, which was inconsistent with previous works represented an obviously decreasing body weight of CMS stressed animals. The reason for these disparate results on body weight gain might be due to the variety in animal species, stress schedule and stimuli intensity.

It has been well demonstrated that chronic stress produces atrophy and dendritic arborization of CA3 pyramidal neurons (Magarinos et al., 1999). This dendritic remodeling is hypothesized to be related to the prolonged hypothalamo–pituitary–adrenal (HPA) axis activation and the resulting elevation of excitatory amino acids and corticosteroid activation during stress (McEwen, 1999; Sapolsky, 1996), and can be reversed by antidepressant treatment (Watanabe et al., 1992). In present study, the CMS induced elevation of plasma corticosterone level was reversed by chronic piperine administration. Moreover, chronic treatment with piperine significantly ameliorated the reduced proliferation of hippocampal progenitor cells caused by CMS procedure. The results indicated that the antidepressant effects of piperine might be partly related to its modulating in HPA activity and thereby the resulting neurogenesis.

Piperine is a monoamine oxidase inhibitor, and thus can increase the levels of brain monoamines, such as serotonin (5-HT) or norepinephrine (NE). In our previous study, at the dose of 10 mg/kg, piperine increased the concentrations of 5-HT and NE in some brain regions of mice. It has been reported that 5-HT or NE up-regulates neurogenesis. Moreover, our research results also demonstrated that piperine could up-regulate the BDNF level of stressed mice. BDNF plays an important role in adult neurogenesis. Studies in rat forebrain explants reveal that BDNF can promote cell survival in the subventricular zone in both young and senescent rats (Goldman et al., 1997; Kirschenbaum and Goldman, 1995). Moreover, newly generated neurons have been found in various rat brain regions after chronic intracerebroventricular infusion of BDNF (Pencea et al., 2001). Heterozygous BDNF knockout (BDNF+/−) mice, with reduced BDNF protein expression in the hippocampus, exhibit attenuated proliferation and survival of BrdU-labeled cells in the dentate

gyrus (Lee et al., 2002). Riluzole, a drug that increases endogenous BDNF production, also enhances proliferation of precursor cells in the granule cell layer, many of which differentiate into neurons. This effect is blocked by intraventricular infusion of BDNF-specific antibodies (Katoh-Semba et al., 2002). These findings suggest that BDNF is an important modulator of progenitor cell proliferation, differentiation and survival. Previous studies had demonstrated that fluoxetine up-regulated BDNF expression (Molteni et al., 2006; De Foubert et al., 2004; Altar et al., 2003; Dias et al., 2003). In this study, piperine and fluoxetine reversed the chronic stress-induced decrease of hippocampal BDNF level in the same time course of the up-regulation of progenitor cell proliferation. Given the broad range of trophic and protective effects of BDNF, one of the mechanisms by which piperine induces progenitor cell proliferation in the SGZ may involve up-regulation of BDNF. Moreover, piperine (12.5 and 25 μM) increased the A490 nm values, decreased LDH release significantly and up-regulated BDNF mRNA level compared with the corresponding CORT-treated hippocampal neurons. A previous study (Lee et al., 2006) demonstrated that piperine, at low concentrations (0.5–10 μM), reduced the 1-methyl-4-phenylpyridinium (MPP^+) induced viability loss in PC12 cells by suppressing the changes in the mitochondrial membrane permeability, leading to the release of cytochrome *c* and subsequent activation of caspase-3. In contrast, piperine at higher concentrations (50–100 μM) showed cytotoxicity (Lee et al., 2006; Unchern et al., 1997). These results suggest that piperine could increase cell viability and protect the cells in a specific dose range, even though the mechanisms for cytoprotectivity of piperine in previous works were quite different from that of our present study. So, increase of the 5-HT, NE, and downstream of BDNF level is at least part of the mechanism enhancing neurogenesis, cytoprotectivity and antidepressant effect of piperine, besides its effects on HPA axis.

In summary, piperine possesses a certain antidepressant-like property and the mechanism may be related to its cytoprotective and neuroproliferating actions. The results also showed that the effects of piperine might be mediated, at least partly, through up-regulating the level of BDNF. Furthermore, excessive cell death and reduced neurogenesis in hippocampus might be the key pathogeneses for depressive disorder. Drugs with neuroprotective activity and neurogenesis potential, could reverse the above neuropathological changes in hippocampus and may provide a new strategy for antidepressant treatment.

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