ETAZOLATE ABROGATES THE LIPOPOLYSACCHARIDE (LPS)-INDUCED DOWNREGULATION OF THE cAMP/pCREB/BDNF SIGNALING, NEUROINFLAMMATORY RESPONSE AND DEPRESSIVE-LIKE BEHAVIOR IN MICE

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Abstract—Increasing evidence has indicated that immune challenge by bacterial lipopolysaccharide (LPS) induces depressive-like behavior, neuroinflammatory response and upregulates phosphodiesterase-4 (PDE4), an enzyme that specifically hydrolyzes cyclic adenosine monophosphate (cAMP). However, whether the potential PDE4 inhibitor etazolate prevents the LPS-induced depressive-like behavior remains unclear. Here using a model of depression induced by the repeated administration of LPS during 16 days, and then investigated the influence of LPS on the expression of PDE4, interleukin-1β (IL-1β) and antidepressant action of etazolate in mice through forced swimming, novelty suppressed feeding, sucrose preference and open-field tests. Our results showed that etazolate pretreatment facilitated the recovery from weight loss and prevented the depressive-like behavior induced by repeated LPS administration. Moreover, the antidepressant action of etazolate was paralleled by significantly reducing the expression levels of PDE4A, PDE4B, PDE4D and IL-1β and up-regulating the cAMP/phosphorylated cAMP response-element binding protein (pCREB)/brain-derived neurotrophic factor (BDNF) signaling in the hippocampus and prefrontal cortex of mice. These results indicate that the effects of etazolate on the depressive-like behavior induced by repeated LPS treatment may partially depend on the inhibition of PDE4 subtype, the activation of the cAMP/pCREB/BDNF signaling and the anti-inflammatory responses in the hippocampus and prefrontal cortex. Crown Copyright © 2014 Published by Elsevier Ltd. on behalf of IBRO. All rights reserved.

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Abbreviations: ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; cAMP, cyclic adenosine monophosphate; FST, forced swimming test; IL-1β, interleukin-1β; LPS, lipopolysaccharide; NSF, novelty suppressed feeding; pCREB, phosphorylated cAMP response-element binding protein; PDE4, phosphodiesterase-4; PKA, protein kinase A; RIPA, radio-immunoprecipitation assay; RT-PCR, real-time polymerase chain reaction; sAPPα, soluble form α of the amyloid precursor protein; SDS, sodium dodecyl sulfate; SPT, sucrose preference test; TLR4, Toll-like receptor 4.

INTRODUCTION

Depression is a common, recurrent, and incapacitating psychiatric illness associated with significant morbidity and mortality (Nemeroff, 2007; Goldster-Dubner et al., 2010). Although the accepted monoamine-deficiency theory represents an important aspect of depression pathophysiology, the neuropathophysiology of this mood disorder remains unclear (Sümegi, 2008; Hasler, 2010). More studies suggest that serotonin levels rapidly increase after antidepressant administration in both primates and rodents several weeks before therapeutic benefits are achieved (Rutter et al., 1994; Kreiss and Lucki, 1995; Anderson et al., 2005), indicating that the monoamine-deficiency hypothesis cannot fully explain the delayed antidepressant effects. Thus, the classic monoamine hypothesis of depression has been challenged, and alternative therapeutic strategies based on novel understandings of the etiology of depression are urgently needed.

Accumulating evidence reveals a close linkage between inflammation and depression (Dantzer, 2006, 2012; O’Connor et al., 2009; Galecki et al., 2012; Leonard and Maes, 2012; Maes, 2008, 2010; Maes et al., 2012; Krogh et al., 2013; Patki et al., 2013; Zeugmann et al., 2013). In addition, depressive symptoms frequently develop in chronically infected patients and in patients with inflammatory bowel disease, chronic kidney disease, or rheumatoid arthritis (Moreau et al., 2005; Fuller-Thomson and Sulman, 2006; Wolfe and Michaud, 2009; Hedayati et al., 2010). Given that the close link between peripheral inflammation and depression (Dantzer, 2006, 2012; O’Connor et al., 2009; Galecki et al., 2012; Leonard and Maes, 2012; Maes, 2008, 2010; Maes et al., 2012), it is reasonable to predict a beneficial effect of anti-inflammatory therapy on depression-like behavior. Here, in order to induce prolonged depressive-like behavior, the animal model of depression induced by repeated lipopolysaccharide (LPS) administration was used in mice.

Recent findings have also suggested that cyclic adenosine monophosphate (cAMP)/cAMP response element binding (CREB)/brain-derived neurotrophic
factor (BDNF) signaling is closely involved in anti-inflammatory responses (Wang et al., 2012), depression and antidepressant actions (D’Sa and Duman 2002; Li et al., 2009, 2011; Rojas et al., 2011; Breuillaud et al., 2012; Takano et al., 2012). In addition, phosphodiesterase-4 (PDE4) inhibitor rolipram that readily produces antidepressant-like actions (O’Donnell and Frith, 1999; Zhang et al., 2002, 2006; Li et al., 2009, 2011; Shalaby and Kamal, 2012; Wang et al., 2013), which are associated with increased the level of cAMP and its downstream targets of cAMP-dependent protein kinase A (PKA), CREB, and BDNF (Manji and Duman, 2001; Li et al., 2009, 2011). Therefore, the potential PDE4 inhibitors may be an efficient alternative strategy to play antidepressant action especially in depressive disorder induced by inflammation. However, clinical development of PDE4 inhibitor rolipram was abandoned due to the side-effects. In the present study, etazolate was chosen as a potential neuroprotective strategy to play antidepressant action especially in depressive disorder induced by inflammation. However, clinical development of PDE4 inhibitor rolipram was abandoned due to the side-effects. In the present study, etazolate was chosen as a potential neuroprotective treatment on the basis of publications that revealed its ability to inhibit PDE4 enzyme (Wang et al., 1997; Drott et al., 2010; Jindal et al., 2012). Although limited pre-clinical studies have been conducted on etazolate, it has recently clinical trial results on its safety and tolerance are encouraging (Vellas et al., 2011). The aim of our study was to elucidate whether and why etazolate could prevent the depressive-like behavior, and its relationship with neuroinflammation and cAMP/PKA/CREB/BDNF signaling in repeated LPS treated mice.

**EXPERIMENTAL PROCEDURES**

**Animals**

Experiments were conducted using young, healthy male imprinting control region (ICR) mice (22–25 g), which were born and reared in the animal facility of the Ningbo University Medical School, China. The mice were housed in opaque polypropylene cages (36 cm × 24 cm × 17 cm) at five mice to seven mice per cage. The cages were maintained at 22 ± 2 °C and 60 ± 5% relative humidity under a 12-h light:12-h dark cycle (lights on at 07:00 h). Rodent chow and water were provided *ad libitum*. All experiments were conducted in a quiet room from 09:30 to 16:30 h. All experiments using animals were performed according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publications No. 85-23, revised 1985 and No. 80-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of Ningbo University Medical School (China).

**Experimental design and antidepressant treatment**

Animals were randomly divided into three groups as follows: (i) saline (0.9% NaCl for LPS) + vehicle (Saline containing 0.5% dimethyl sulfoxide for etazolate); (ii) LPS (0.83 mg/kg) + vehicle; and (iii) LPS (0.83 mg/kg) + etazolate (3 mg/kg). Intraperitoneal (i.p.) injections of 0.1 ml/10 g LPS or saline were administered once daily for 16 d to no-stressed mice 60 min after the etazolate or vehicle treatment (Fig. 1). Training or testing was conducted 1 h after etazolate or vehicle treatment. All behavioral studies were performed by two observers who were blinded to the treatment conditions. Data were pooled for statistical analysis. Animals were sacrificed 1 h after the last drug treatment. The doses of etazolate and LPS were selected according to the previous studies (Jindal et al., 2012) and our preliminary data, with some modifications.

**Forced swimming test (FST)**

FST was performed as previously described (Hedlund et al., 2005; Sarkisyan et al., 2010). In brief, individual mice were placed in a clear plastic cylinder (height: 25 cm; diameter: 10 cm) containing 10 cm of fresh water at 23 ± 2 °C. The mice were videotaped during a 6-min session. Mouse activities, such as climbing, swimming, and immobility, were then analyzed. The durations of immobility, climbing, and swimming were measured during the final 4 min of the test. Water in the tank was replaced after each swimming session. Following the swim, each mouse was towel-dried and placed under a heat lamp to dry. Climbing was defined as vigorous active movements of mice using all four paws while they were placed parallel to the wall of the swimming vessel, as well as having the head and shoulders above the water. Swimming was defined as moving all four paws in an actively swimming motion. The movement should be more vigorous than was necessary to merely maintain the head above the water. Immobility was noted when the mice remained floating in the water without struggling and only made movements that were necessary to keep their head or nose above the water. Trained observers who were blinded to the treatments evaluated the recordings of the test sessions.

**Novelty suppressed feeding (NSF)**

NSF is responsive to the chronic administration of classical antidepressants (Santarelli et al., 2003). The test apparatus consisted of an illuminated (1000 lx), soundproofed plastic box (45 cm × 45 cm × 20 cm), the floor of which was covered with approximately 1 cm of wooden bedding. All food was removed from the home cage 24 h before the behavioral test. Water remained

![Fig. 1. Schematic of the experimental procedure. LPS or saline and etazolate or its vehicle were intraperitoneally (i.p.) injected once per day for 16 d. Behavioral tests were performed 11 d after drug administration and were continued until day 16, on which the animals were sacrificed for biochemical assays. FST, forced swimming test; NSF, novelty suppressed feeding; SPT, sucrose preference test.](image-url)
available ad libitum. At the time of testing, a single pellet of food (regular chow) was placed on a white, circular filter paper (diameter: 10 cm) in the center of the box. A mouse was placed in a corner of the box, and its behavior was immediately recorded for 5 min. The latency until feeding (defined as the mouse biting the pellet while using its forepaws) was determined by a blind observer. Subjects were removed from the apparatus after they began eating, or a maximum latency of 5 min, and returned to their home cage where consumption of a pre-weighed food pellet was determined after 5 min.

Sucrose preference test (SPT)

SPT (Bekris et al., 2005) was used to determine anhedonia, a core symptom of major depression in humans. The test consisted of a two-bottle choice paradigm (Kentner et al., 2010; Dagyte et al., 2011). In the training course, mice were made to drink from two water bottles for 48 h prior to the test. During the test, the mice were given access to two pre-weighed bottles, one containing tap water and the other containing 1% sucrose, for 24 h. The bottles were weighed at 8:00 and 17:00 h, and the preference for sucrose over water was used as a measure of anhedonia. The sucrose preference was calculated as the ratio of the consumed sucrose solution to the total amount of liquid consumed. In addition, the normal water intake (%) was calculated as ratio of the amount of normal water to that of total solution (sucrose and water). The positions of the bottles were counterbalanced daily to prevent place preference.

Real-time polymerase chain reaction (RT-PCR) analysis

The mice were sacrificed after the final behavioral test for RT-PCR analysis. Immediately after the decapitation of the mice, their hippocampi and prefrontal cortices were rapidly dissected and immersed in liquid nitrogen. Frozen tissues were stored at −80°C until use. The tissues were homogenized, and total RNA was extracted using an RNeasy total RNA isolation kit (Qiagen, Valencia, CA, USA). Complementary DNA (cDNA) was generated from 2 μg of total RNA at a total volume of 20 μl using a SuperScript® III First-Strand Synthesis Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The primers used are as follows: PDE4A, forward primer 5′-GAAGTCTCAGGTGGCTTCCA-3′ and reverse primer 5′-CAGTCCCGTTGTCTTCCA-3′; PDE4B, forward primer 5′-GTGACGAGCTCCGGTGTTTC-3′ and reverse primer 5′-GCCGATACAACCTCCAAGGACTT-3′; and PDE4D, forward primer 5′-CCTCAGAATATGGTGCACTGT-3′ and reverse primer 5′-GTGACGAGCTCCGGTGTTTC-3′. PCR amplifications were performed as follows: 10 min preincubation at 95°C to activate the FastStart Taq DNA polymerase, 40 cycles of denaturation at 95°C for 15 s, and primer annealing and extension at 60°C for 30 s. Melting-curve analysis showed the specificity of the amplifications. mRNA levels of cytokines (PDE4A, PDE4B, and PDE4D) were normalized by subtracting the cycle threshold (Ct) values obtained using β-actin mRNA. The results are expressed as 2−ΔCt [ΔCt = Ct (PDE4 subtypes) − Ct (β-actin)]. All PCR experiments were performed in triplicate.

Immunoblot analyses

Western blot analysis was performed as previously described (Wang et al., 2012; Zhang et al., 2013). In brief, brain tissues were homogenized in a radio-immunoprecipitation assay (RIPA) lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS); Upstate, Temecula, CA, USA) containing protease and phosphatase inhibitors (Pierce Biotechnology, Rockford, IL, USA) and then centrifuged at 15,000g for 30 min. Samples (80 μg protein each) were separated using SDS–polyacrylamide gel electrophoresis (PAGE) and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (0.22 μm; Millipore, Temecula, CA, USA). The samples were then incubated overnight with rabbit anti-phosphorylated cAMP response-element binding protein (anti-pCREB) (Ser133) (1:1000; Millipore, Temecula, CA, USA), anti-CREB (1:1000; Millipore, Temecula, CA, USA), anti-BDNF (1:500; Millipore, Temecula, CA, USA), anti-PDE4A (1:500; Fab Gennix, Frisco, TX, USA), anti-PDE4B (1:500; Fab Gennix, Frisco, TX, USA), anti-PDE4D (1:500; Fab Gennix, Frisco, TX, USA), interleukin-1β (IL-1β) (1:800; Millipore, CA, USA) and anti-β-actin antibodies (1:1000; Cell Signaling, Billerica, MA, USA) at 4°C. Afterward, the membranes were incubated with Alexa Fluor 700 conjugated goat anti-rabbit antibody (1:10000; Invitrogen, Eugene, OR, USA) for 60 min. Detection and quantification of specific bands were performed using a fluorescence scanner (Odyssey Infrared Imaging System, Li-COR Biotechnology, Lincoln, NE, USA). For band stripping, the membranes were incubated with a stripping buffer (Chemicon, Temecula, CA, USA) for 15 min. All samples were analyzed at least in triplicate.

cAMP assay

The samples extracted by the RIPA lysis buffer were diluted with 0.1 N HCl to a final protein concentration of 1 mg/ml. cAMP levels were determined by ELISA (Assay Designs, Ann Arbor, MI, USA). The cAMP concentration is expressed as pmol/ml.

Statistical analyses

All measurements were performed by an independent investigator blinded to the experimental conditions. Data are expressed as the means ± standard error of means (SEM). Data were analyzed by a one-way analysis of variance (ANOVA) followed by Newman–Keuls multiple comparison tests using the GraphPad Prism software (Version 5.0, Prism software for PC, GraphPad), with the exception of the data of the body weights, which were analyzed by a two-way repeated measures ANOVA. Statistical significance was considered when p < 0.05.
RESULTS

Effects of etazolate on repeated LPS administration-induced depressive-like behavior

Compared with the saline plus vehicle-treated mice, mice subjected to repeated administration of LPS plus vehicle for 11 d exhibited a significant depression-like behavior characterized by increased immobility time and decreased climbing time during the FST (Fig. 2). In the FST, the duration of immobility was significantly longer in the LPS plus vehicle-treated group than in the saline plus vehicle group [F(2,27) = 5.073, p < 0.05; Fig. 2a]. However, the mice in the LPS plus etazolate group showed significantly decreased immobility time during the final 4 min of FST compared with those in the LPS plus vehicle-treated group [F(2,27) = 5.073, p < 0.05; Fig. 2a]. This result indicates that etazolate abrogated the repeated LPS administration-induced depressive-like behavior. In addition, statistical analysis of the FST behavioral data showed significant differences in the climbing behavior of mice among the three experimental groups [F(2,27) = 4.802, p = 0.0164; Fig. 2b]. The mice in the LPS plus vehicle-treated group showed significantly decreased climbing behavior during the FST compared with those in the saline plus vehicle group (p < 0.05). The mice in the LPS plus etazolate group also exhibited significantly restored climbing behavior during the final 4 min of FST compared with those in the LPS plus vehicle group (p < 0.05). This result also indicates that etazolate administration significantly prevented the depressive-like behavior induced by repeated LPS treatment. However, all treatments did not cause significant differences in the swimming behavior of the mice during the FST [F(2,27) = 0.5978, p = 0.5571; Fig. 2c], suggesting that the behaviors changes were not associated with general motor activity.

Similar findings were obtained in the NSF (Fig. 3a) and SPT (Fig. 3c) tests. The effects of drug treatment on the latency until the first time food was eaten during the NSF are shown in Fig. 3a. A significant difference was found between the groups [F(2,27) = 5.226, p = 0.0121; Fig. 3a]. Repeated LPS-treated significantly increased the latency to feed (p < 0.05). Nevertheless, the effect of LPS on latency was significantly shortened in the presence of etazolate compared with that of the LPS plus vehicle treatment (p < 0.05). In addition, no significant differences in the total food consumption of a pre-weighed food pellet between treatment groups [F(2,27) = 0.3976, p = 0.6758; Fig. 3b], demonstrating that LPS-challenge induces sickness response may have no significantly influence on depressive-like phenotype in NSF. Moreover, after 14 d of exposure to LPS, sucrose preference was significantly decreased in the LPS plus vehicle group compared with that in the saline plus vehicle group [F(2,27) = 13.45, p < 0.01; Fig. 3c]. Meanwhile, the total water consumption showed negligible changes [F(2,27) = 0.6068, p = 0.5524; Fig. 3d]. However, sucrose preference was significantly prevented by etazolate pretreatment compared with the LPS plus vehicle (p < 0.05). These data can be interpreted to indicate that etazolate blocks anhedonia associated with peripheral LPS challenge.

Effects of etazolate on LPS-induced up-regulation of PDE4 subtype expression in the hippocampus and prefrontal cortex of mice

We used RT-PCR to initially determine the mRNA expression levels of PDE4A, PDE4B, and PDE4D in the hippocampus and prefrontal cortex of mice after repeated treatment with LPS alone or in combination with etazolate. A one-way ANOVA showed significant changes in the mRNA levels of PDE4A [hippocampus: F(2,12) = 35.67, p < 0.01; Fig. 4a; cortex: F(2,12) = 6.692, p = 0.0112, Fig. 4d], PDE4B [hippocampus: F(2,12) = 8.068, p = 0.006, Fig. 4b;
cortex: $F(2,12) = 9.398$, $p = 0.0035$, Fig. 4e, and PDE4D [hippocampus: $F(2,12) = 5.397$, $p = 0.0213$, Fig. 4c; cortex: $F(2, 12) = 13.73$, $p = 0.008$, Fig. 4f] in the hippocampus and prefrontal cortex of the mice. Compared with the saline plus vehicle group, the group subjected to repeated LPS treatment showed significantly increased mRNA levels of PDE4A [hippocampus: $p < 0.01$, Fig. 4a; cortex: $p < 0.05$, Fig. 4d], PDE4B (hippocampus: $p < 0.01$, Fig. 4b; cortex: $p < 0.01$, Fig. 4e), and PDE4D (hippocampus: $p < 0.05$, Fig. 4c; cortex: $p < 0.01$, Fig. 4f) in the hippocampus and prefrontal cortex. However, this LPS-induced upregulation of the mRNA levels of PDE4A, PDE4B, and PDE4D were significantly reversed when LPS was combined with etazolate in both the hippocampus and prefrontal cortex.

Consistent with the previously described changes in mRNA levels, immunoblot analysis showed significant changes in the protein expression levels of PDE4A [hippocampus: $F(2,9) = 9.437$, $p = 0.0035$, Fig. 4e, and PDE4D [hippocampus: $F(2,9) = 5.964$, $p = 0.0224$, Fig. 4c; cortex: $F(2, 9) = 18.66$, $p = 0.0006$, Fig. 4f] in the hippocampus and prefrontal cortex. The LPS-treated mice displayed significantly upregulation of PDE4A (hippocampus: $p < 0.01$, Fig. 5b; cortex: $p < 0.01$, Fig. 5f), PDE4B (hippocampus: $p < 0.01$, Fig. 5c; cortex: $p < 0.05$, Fig. 5g), and PDE4D (hippocampus: $p < 0.05$, Fig. 5d; cortex: $p < 0.01$, Fig. 5h) in the hippocampus and prefrontal cortex compared with the saline plus vehicle group. This LPS-induced upregulation of the protein expression of PDE4A, PDE4B, and PDE4D were also significantly prevented by the combination with etazolate in both the hippocampus and prefrontal cortex.

**Effects of etazolate on LPS-induced down-regulation of cAMP, pCREB, CREB, and BDNF levels in the hippocampus and prefrontal cortex of mice**

The cAMP/CREB/BDNF signaling is vital in the mediation of antidepressant activity (D’Sa and Duman, 2002). As
shown in Fig. 6, the mice treated with LPS (for 16 d) displayed significantly decreased cAMP in the hippocampus [F(2,12) = 5.183, p < 0.05; Fig. 6a] and prefrontal cortex [F(2,12) = 6.350, p < 0.05; Fig. 6b] compared with the saline plus vehicle group. However, the LPS-induced decrease in cAMP levels in the hippocampus (p < 0.05; Fig. 6a) and the prefrontal cortex (p < 0.05; Fig. 6b) were significantly reversed in the presence of etazolate.

Similarly, repeated treatment with LPS also significantly decreased the pCREB [hippocampus: F(2,9) = 11.53, p < 0.01; Fig. 7b] and BDNF [hippocampus: F(2,9) = 10.29, p < 0.01, Fig. 7d; cortex: F(2,9) = 5.916, p < 0.05, Fig. 7f] in the hippocampus and prefrontal cortex compared with the saline plus vehicle group. In addition, etazolate significantly prevented the LPS-induced down-regulation of pCREB [hippocampus: p < 0.05, Fig. 7b; cortex: p < 0.05, Fig. 7f] and BDNF [hippocampus: p < 0.05, Fig. 7d; cortex: p < 0.05, Fig. 7f] in the hippocampus and prefrontal cortex. By contrast, all treatments did not affect the CREB levels in either brain region [hippocampus: F(2,9) = 0.0004, p = 0.9996, Fig. 7c; cortex: F(2,9) = 0.3225, p = 0.7324, Fig. 7g].
Fig. 5. Etazolate prevents the up-regulation of PDE4A, PDE4B, and PDE4D protein expression in the hippocampus (b–d) and prefrontal cortex (f–h) of mice induced by LPS. Representative immunoblots of the PDE4 subtypes in the hippocampus (a) and prefrontal cortex (e), respectively. Results are presented as the mean ± SEM of five animals/group and were evaluated by a one-way ANOVA followed by Newman–Keuls tests. *p < 0.05, **p < 0.01 vs. saline plus vehicle group, #p < 0.05, ##p < 0.01 vs. LPS plus vehicle group.
Effects of etazolate on LPS-induced up-regulation of cytokine expression in the hippocampus and prefrontal cortex of mice

It has been shown in the previous study that LPS administration increases pro-inflammatory factors in the brain (Qin et al., 2007; Fan et al., 2013); this may cause neuroinflammation and depressive-like behavior in mice. To clarify this, we examined the expression of IL-1β in the hippocampus and prefrontal cortex. As shown in Fig. 8, levels of IL-1β expression in the hippocampus [F(2,6) = 10.35, p = 0.0114; Fig. 8b] and prefrontal cortex [F(2,6) = 89.11, p < 0.0001; Fig. 8d] were changed by drug treatment. Post-hoc Newman–Keuls analyses indicated a significant increase in IL-1β levels in rats treated with LPS + vehicle, compared to those treated with saline + vehicle in hippocampus (p < 0.05) and cortex (p < 0.01); the effect of LPS was significantly prevented by etazolate (p < 0.05 for hippocampus and p < 0.01 for cortex).

Effects of etazolate on LPS-induced body weights loss

Sixteen days after continuous administration of LPS, the body weights tended to be decreased, and were statistically different from LPS plus vehicle treatment group in the saline plus vehicle group and etazolate pretreatment group [F(2,216) = 15.62, p < 0.01, Fig. 9]. LPS treatment led to a slower gain of body weights compared to the saline plus vehicle group; this was significant during days 2–16 (Fig. 9). However, repeated pretreatment with etazolate significantly altered the body weights loss by preventing LPS-induced body weights loss during days 6–12.

DISCUSSION

The LPS-induced model is a well documented animal model of depression and used to understand the pathophysiology of depression (Ji et al., 2013; Lawson et al., 2013). However, in contrast with the LPS administered intravenously (i.v.) and intracerebroventricular (i.c.v.) in previous studies (Tonelli et al., 2008; Park et al., 2011; Lawson et al., 2013), the intraperitoneal (i.p.) injections of 0.1 ml/10 g LPS (0.83 mg/kg) or saline were administered once daily for 16 days to no-stressed mice in current study. It was noted that in this study, in order to prolong the depressive-like behavior duration induced by administration of LPS, the protocol of LPS administration was selected according to our preliminary data. The behavioral data of this investigation showed that mice subjected to LPS exhibited increase duration of immobility in FST. Subsequently, we tested the first feeding latency of the mice, which is an indication of increased anxiety levels in the NSF (Santarelli et al., 2003). Our present study shown that repeated LPS-treated significantly increased the latency to feed in NSF. Moreover, LPS treated mice also showed a reduce preference and consumed less amount of sucrose solution as compared to untreated mice in SPT. Furthermore, our data also demonstrated that 16 days after continuous administration of LPS, the body weights tended to be decreased, and were statistically different from LPS treatment alone group in the vehicle control group, indirectly showing a sickness response induced by LPS. However, no significant differences in the total food consumption of a pre-weighed food pellet between treatment groups in NSF and the total water consumption showed negligible changes in SPT. In addition, all treatments did not cause significant differences in the swimming behavior of the mice during the FST, suggesting that the behaviors changes may not associated with sickness response and general motor activity. However, given that the peripheral administration of LPS induces sickness behavior that peaks 2–6 h later and gradually wanes (Dantzer et al., 2008), further study need to clarify that whether the depressive-like behavior overlap with sickness effect induced by LPS treatment. We obtained another interesting finding was that peripheral immune challenge with LPS, precipitates the inflammatory response in the hippocampus and prefrontal cortex in the induction of IL-1β transcription. Consistent with the previous study...
Fig. 7. Etazolate prevents the changes of pCREB, CREB, and BDNF protein expression in the hippocampus (b–d) and prefrontal cortex (f–h) of mice induced by LPS. Representative immunoblots of the hippocampus (a) and prefrontal cortex (e), respectively. Results are presented as the mean ± SEM of four animals/group and were evaluated by a one-way ANOVA followed by Newman–Keuls tests. *p < 0.05, **p < 0.01 vs. saline plus vehicle group, #p < 0.05, ##p < 0.01 vs. LPS plus vehicle group.
evidence that IL-1β signals can be relayed from the periphery to the brain by humoral pathways (Ching et al., 2007). The present work addressed the profile of cytokine expression in these brain regions may be causally related to behavioral symptoms observed. Our above data confirmed that repeated LPS administration has been suggested as an animal model of depression.

The critical finding of this study was that PDE4A, PDE4B, and PDE4D were clearly up-regulated and cAMP/pCREB/BDNF signaling pathway was significantly down-regulated in the hippocampus and prefrontal cortex of mice induced by repeated LPS treatment. Substantial evidence suggests that the cAMP signaling is crucial in pathophysiological depressive-like behavior as well as in the action of antidepressants (Li et al., 2009, 2011; Marsden, 2013; Niciu et al., 2013). Additionally, modulation of neuronal survival and synaptic plasticity by cAMP signaling is dependent mainly on activation of PKA, CREB and BDNF (Dworkin et al., 2009; Li et al., 2009, 2011). Thus, changes in the cAMP-mediated signaling in the hippocampus and prefrontal cortex could affect the efficacy of antidepressant treatments. To the best of our knowledge, this study is the first to provide evidence for the reduced cAMP/pCREB/BDNF signaling after repeated LPS treatment. Our results also further suggest that PDE4A, PDE4B and PDE4D subtypes are involved in the regulation of repeated LPS treatment-affected cAMP/pCREB/BDNF signaling in mouse brain. As a critical controller of this signaling, PDE4 was hypothesized to be involved in the depressive-like behavioral effects regulated by cAMP signaling (Li et al., 2009, 2011; Wang et al., 2012). PDE4 consists of four PDE4 genes (PDE4A, PDE4B, PDE4C, and PDE4D), which encode more than 20 variants, have been identified (Richter et al., 2013). PDE4A and PDE4D are highly expressed in the cortex, olfactory bulb, hippocampal formation, and brainstem, whereas PDE4B is mainly expressed in the amygdala, striatum, and hypothalamus (Miró et al., 2002; Zhang et al., 2005; Zhang, 2009). By contrast, PDE4C exhibits a
distribution different from those of PDE4A and PDE4D, and appears to be limited to the thalamus and cerebellum (Zhang et al., 2005; Zhang, 2009). Therefore, repeated administration of LPS induced the up-regulation of PDE4 subtypes, which suppresses the downstream cAMP-mediated signaling and may explain the depressive-like behavior in mice. In addition, given that the onset and progression of the inflammatory response are sensitive to changes in the steady-state level of the cAMP (Moore and Willoughby, 1995), the repeated LPS treatment induced the inflammatory response and may partially explain the depressive-like behavior in mice.

More evidence has shown that PDE4 inhibitor rolipram reduce the production of pro-inflammatory cytokines and modulate the activity of cAMP-mediated signaling and thus regulate CREB phosphorylation and the downstream effectors (Li et al., 2009, 2011; Reneerkens et al., 2009; Cheng et al., 2010; Wang et al., 2012), showing that potential PDE4 inhibitors may be suitable to antagonize depression. Unfortunately, the development of PDE4 inhibitor rolipram for therapeutic purposes has been hindered by side effects, such as emesis (Robichaud et al., 2001, 2002; Dyke and Montana, 2002). It has been demonstrated that a pyrazolopyridine compound, etazolate, is able to inhibit PDE4, increase cAMP levels (Wang et al., 1997), regulate the soluble form α of the amyloid precursor protein (sAPPα) release (Siopi et al., 2013) and modulate the GABA receptors (Moss et al., 1992). In addition, preclinical studies as well as pharmacokinetic and safety profiles in clinical studies (Phase I and Phase IIb) have established that etazolate is a well-tolerated drug and devoid of major side effects (Vellas et al., 2011). Furthermore, no emesis effect was observed clinically with etazolate treatment, which is main advantage of it over other PDE4 inhibitors. In this regard, antidepressant-like effect of etazolate with conventional antidepressants may be modulated through cAMP signal transduction pathway (Fujimaki et al., 2000; Santarelli et al., 2003). Thus, the potential PDE4 inhibitor etazolate was expected to reverse the depressive-like behavior induced by repeated LPS administration. Our present study clearly demonstrated that etazolate was highly effective in preventing the depressive-like behavior induced by LPS treatment in mice. In addition, the up-regulation of cAMP and pCREB activities as well as BDNF levels in the hippocampus and prefrontal cortex was coincident with the reduced depressive-like behavior produced by etazolate.

Our data also clearly show that repeated systemic administration of LPS leads to a strong neuroinflammatory response of the central nervous system with accompanying behavioral changes. We found that etazolate significantly blocked the LPS-induced IL-1β production in hippocampus and prefrontal cortex of mice. Several reasons may account for this. First, pharmacological manipulation of PDE4 and cAMP levels producing by etazolate provokes an antiinflammatory response. This is supported by PDE4 inhibitor rolipram reverses neuroinflammatory and apoptotic responses mediated by cAMP/CREB signaling (Wang et al., 2012). Second, activation of the Toll-like receptor 4 (TLR4) complex, a receptor of the innate immune system, may underpin the pathophysiology of depression (Gárate et al., 2013). In addition, cAMP-specific PDE4 critically regulates LPS-TLR4-induced inflammatory cytokine expression (Gobejishvili et al., 2013). Third, the neuroinflammatory environment is also accompanied by the decrease of an endogenous neuroprotector, sAPPα (Siopi et al., 2011), generated by the activity of α-secretases. Since cAMP has been previously linked to sAPPα production (Mallet et al., 2003), the sAPPα maybe also involved in the antiinflammatory effect of etazolate.

Additionally, earlier studies have reported a possible relationship between the GABA and cAMP (Shalaby and Kamal, 2012). Consistent with this, inhibition of PDE4 by rolipram significantly increases the GABA levels and produces antidepressant-like effects in mice exposed to chronic mild stress (CMS) (Shalaby and Kamal, 2012). However, it has been shown that only chronic treatment with PDE4 inhibitor modulates the GABA content (Shalaby and Kamal, 2012). This implies that the multiple properties of etazolate might be cross-linked. Further investigations are required to establish and clarify the detailed mechanism(s) of etazolate for antidepressant action and to find out a correlation between cAMP signaling and GABA in major depression.

In conclusion, our results emphasized that etazolate blocked the hyperactivation of the PDE4 expression, neuroinflammatory response and increased the level of cAMP, pCREB and BDNF. These observations suggest that the modifications of the behavioral deficits induced by LPS seem to depend on reducing PDE4 activity and restoring cAMP/pCREB/BDNF signaling.

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**CONTRIBUTORS**

Chuang Wang, Qinwen Wang, Wenhua Zhou and Xin Zhao designed the study and wrote the protocol. Chuang Wang, Jiejie Guo and Peipei Lin wrote the first draft of the manuscript and undertook the statistical analysis. Jiejie Guo, Peipei Lin, Xingyu Zhou, Huihui Huang, Junfang Zhang and Xiaofei Wei performed the drug treatments, the behavioral tests and the biochemical analysis. All authors contributed to and approved the final manuscript.

**DISCLOSURE/CONFLICT OF INTEREST**

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