Abstract. Substantial evidence from previous studies has suggested an association between major depressive disorder (MDD) and inflammation, and previous studies have associated prefrontal cortex (PFC) dysfunction with MDD. Systemic administration of bacterial lipopolysaccharide has been used to study inflammation-associated behavioral changes in rodents. However, proteomic studies investigating PFC protein expression in an LPS-induced mouse model of depression have yet to be conducted. Using two-dimensional electrophoresis coupled with matrix-assisted laser desorption ionization-time of flight-tandem mass spectrometry, PFC proteomes were comparatively assessed in LPS‑induced acute inflammation reaction mice, LPS-induced depressive-like behavior mice (Dep), and control mice. A total of 26 differentially expressed proteins were identified, two of which were selected for western blot analysis, the results of which revealed a significant increase in the expression levels of creatine kinase B and dihydropyrimidinase-like 3 in Dep mice, suggesting that changes in energy metabolism and neurogenesis occur in the PFC of Dep mice. Further investigation on these processes and on the proteins of the PFC are required in order to elucidate the pathophysiological mechanism underlying MDD.

Introduction

Major depressive disorder (MDD) is a complex heterogeneous mood disorder, and an estimated one in four women and one in six men are likely to experience depression during their lifetime (1). Previous findings have established a close association between inflammation and depression (2). As compared with euthymic individuals, patients with MDD exhibit an increased inflammatory response (3), and patients with increased inflammatory cytokine levels suffer from increased rates of depression (4). Furthermore, in rodent models, the systemic administration of the pro-inflammatory endotoxin lipopolysaccharide (LPS) triggers depressive-like behavioral alterations (5) and promotes interleukin (IL)‑1β, IL‑6, and tumor necrosis factor α (TNF-α) inflammatory cytokine production in the brain (6). LPS-induced depressive-like behavior may be observed even after the acute inflammation reaction characteristic of the disease in normalized LPS‑treated mice (6). Therefore, increases in inflammatory cytokine levels may be associated with the development of depression (7). However, the mechanisms underlying this phenomenon remain to be elucidated.

A previous study reported that the prefrontal cortex (PFC) has an important role in the pathogenesis of MDD (8). Patients with MDD exhibit gray matter density abnormalities in the right dorsolateral prefrontal cortex (9). Our previous metabolic study demonstrated the presence of amino acid metabolic dysfunction in the PFC of a rodent model of depression (10). Although PFC dysfunction has been associated with the pathophysiology of depression, the association between the PFC and depressive behavior under inflammatory conditions requires further investigation. Therefore, the present study used pro-inflammatory bacterial endotoxin LPS to induce acute an inflammatory reaction and depressive-like behavior in mice to comparatively analyze the PFC proteomes by two-dimensional electrophoresis (2-DE) and matrix-assisted laser desorption ionization-time of flight-tandem mass spectrometry (MALDI-TOF-MS/MS). Western blotting was then used to
investigate the expression levels of creatine kinase B-type (CKB) and dihydropyrimidinase-related protein 3 (DPYSL3), proteins which are involved in the pathophysiology of MDD.

Materials and methods

Animals. Male CD-1 mice (n=48; weight, 35-40 g; age, 10-14 weeks) were obtained from the animal facility at the Chongqing Medical University (Chongqing, China). The animals were housed under standard laboratory conditions (12 h light/dark cycle; temperature, 23±1˚C; humidity, 45-55%), in a single cage with a shelter, and food and water was available ad libitum. The present study was approved by the Ethics Committee of the Chongqing Medical University and all experiments were conducted in accordance with the National Institutes of Health (Bethesda, MD, USA) Guidelines for Animal Research (Guide for the Care and Use of Laboratory Animals).

Drug administration. The mice were randomly divided into three groups: A control group (Con; n=16) treated with 1 mg/kg sterile saline, an LPS-induced acute inflammatory reaction group (AIR; n=16) treated with 0.83 mg/kg LPS, and an LPS-induced depressive-like behavior group (Dep; n=16) treated with 0.83 mg/kg LPS. For the AIR and Dep mice, LPS (L-3129, serotype 055:B5; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sterile saline and intraperitoneally injected at a dose of 0.83 mg/kg to induce acute inflammatory reaction (11) and depressive-like behavior (12).

Open-field test (OFT). Prior to experimentation, the mice were placed in the testing room for 30 min to allow adaptation. The animals were then individually placed in the center of the open field area (44.5x44.5x45 cm) and after 30 sec adaptation, a 6 min period of free exploration was recorded using a Sony DCR-SR45E digital camera (Sony, Tokyo, Japan). The total distance was recorded during the last 5 min. Following each trial, the apparatus was wiped and cleaned with 80% alcohol. The videos of the test were analyzed using a Smart video-tracking system (PanLab Harvard Apparatus, Holliston, MA, USA).

Forced swimming test (FST). An FST was conducted as previously described (13) with minor modifications. Briefly, the mice were individually placed into transparent glass cylinders (diameter, 15 cm; height, 30 cm) containing 15 cm of water at 23±1˚C. Each mouse was required to swim for 6 min, and the total duration of immobility was recorded during the last 5 min of testing. Immobility was defined as the absence of active, escape-oriented behaviors, such as swimming, jumping, rearing, sniffing, or diving (14). The water was replaced with fresh water between each test. Following completion of each test, the mouse was gently removed from the cylinder, dried, and returned to its cage.

Tail suspension test (TST). The TST is one of the most widely-used models for assessing antidepressant-like activity in mice. The test was conducted as previously described (15) with minor modifications. Briefly, each mouse was suspended by its tail using adhesive tape placed 2 cm from the tip of the tail in an acoustically and visually-isolated suspension box (22x21x33 cm) for 6 min, and the duration of immobility was recorded during the last 5 min of testing. The mice were regarded as immobile only when they hung passively or stayed completely motionless.

Protein sample preparation. Our previous studies established a highly effective protein sample preparation protocol (16,17). Briefly, 48 mice (n=16) were decapitated under 100% diethyl ether [Chongqing Chuanlong Chemical (Group) Co., Ltd., Chongqing, China] anesthesia. PFC tissue samples were rapidly dissected from the brain and subsequently frozen in liquid nitrogen. The tissue samples of each experimental group were stored at -80˚C until use.

The PFC tissue samples from each experimental group (n=10) were removed from the liquid nitrogen and suspended in 2 ml acetone solution supplemented with 0.2% (w/v) dithiothreitol (DTT) and 10% (w/v) trichloroacetic acid. Following tissue homogenization (Eppendorf, Hamburg, Germany), the cell suspension was incubated at -20˚C overnight prior to centrifugation at 35,000 x g for 30 min at 4˚C. The supernatant was decanted, and the cell pellet was suspended in 2 ml pre-cooled acetone solution supplemented with 0.2% (w/v) DTT, and incubated at -20˚C for 1 h, prior to being centrifuged at 35,000 x g for 30 min at 4˚C. The supernatant was then decanted, and the cell pellet was dried in a fuming cupboard. Each sample was dissolved in 2 ml 2-D lysis buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) containing 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris, 65 mM DTT, 0.3 mg/ml EDTA, 35 µg/ml phenylmethylsulfonyl fluoride, 0.7 µg/ml pepstatin, 0.5 µg/ml leupeptin, and 0.5% v/v CA, and centrifuged at 40,000 x g for 60 min at 15˚C. The protein concentration was calculated using a Bradford Protein Assay kit (Bio-Rad Laboratories, Inc.). Bovine serum albumin (Sangon Biotech Co., Ltd., Shanghai, China) was used as a standard control.

2-DE. 2-DE was conducted as previously described (18). Isoelectric focusing (IEF) was performed using 17 cm ReadyStrip™ IPG Strips (pH 3-10; Bio-Rad Laboratories, Inc.), and 350 µl rehydration buffer containing 0.1 mg protein were loaded onto the strips. Each strip was rehydrated for 12 h (at 30 V) using 350 µl rehydration solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, 0.2% Bio-Lyte, and 0.001% bromophenol blue (Bio-Rad Laboratories, Inc.) (19). IEF was performed on a Protein IEF cell (Bio-Rad Laboratories, Inc.) as follows: 50 V for 12 h, 250 V for 30 min, 1,000 V for 1 h, 1,000-10,000 V over a 5 h step-up period, and 10,000 V for 6 h. Following IEF, the IPG strips were equilibrated in the reduction buffer containing 0.375 M Tris-HCl (pH 8.8), 6 M urea, 20% glycerol, and 2% SDS. The first equilibration was performed using equilibration buffer supplemented with 2% DTT, and then the second equilibration was performed using equilibration buffer supplemented with 2.5% indole-3-acetic acid. The second dimension was performed by running the strips on 1-mm thick 10% SDS-PAGE using a Protein™ IIxi Multi-Cell (Bio-Rad Laboratories, Inc.). Six gels (three gels per group) were simultaneously run at 12.5 mA/gel for 30 min and then 25 mA/gel for 5.0-5.5 h at 20˚C. The protein spots
were visualized by silver staining as previously described by Yan et al. (20).

**Gel image analysis.** Following visualization, images were scanned using an Epson 10000XL scanner (Epson Co., Ltd., Beijing, China) at an optical resolution of 300 dpi. Image analysis and spot detection were conducted using PDQuest 8.0.1 (Bio-Rad Laboratories, Inc.) with Gaussian spot modeling. For quantitative comparison of the spots across the gels, replicate images of the gels were created. To correct for the variability in silver staining, the individual spot volumes were normalized by dividing the optical density (OD) of each spot value by the sum the total OD of all spots in the respective gels. This method controlled for differences in sample loading and color intensities among the gels. Automated and manual spot matching were also performed. Integrated intensities demonstrating a $\geq 1.5$-fold change were applied to determine the statistical differences in protein expression between the two groups (17).

**MALDI-TOF/TOF.** Protein samples were separated by 2-DE and visualized with silver staining. In-gel protein digestion was performed as previously described by Zhou et al. (21) with minor modifications. The protein spots of interest were excised from the gels and destained. Following reduction and alkylation, the gel sections were digested overnight with Sequencing Grade Modified Trypsin (Promega Corporation, Madison, WI, USA). The digested peptides were extracted using 100 µl 60% CAN (Merck Millipore, Darmstadt, Germany) supplemented with 0.1% TFA (Merck Millipore) and concentrated in a SpeedVac® vacuum concentrator (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The peptides were re-dissolved in a matrix solution and spotted on a MALDI target plate (Applied Biosystems Life Technologies, Foster City, CA, USA). The peptides were then analyzed using the 4800 Plus MALDI TOF/TOF Analyzer (Applied Biosystems Life Technologies) in default mode.

**Western blotting.** To investigate the differential protein expression in the PFC, two proteins, creatine kinase B-type (CKB) and dihydropyrimidinase-related protein 3 (DPYSL3) were selected for western blotting. $\beta$-tubulin was used as a loading control (1:1,000). The PFC samples from each group (n=6) were homogenized using a standard lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, sodium fluoride, EDTA, and leupeptin (Beyotime Institute of Biotechnology, Haimen, China), prior to protein extraction. A bicinchoninic acid assay was used to analyze the protein concentrations. A total of 30 µg protein samples were separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. The membranes were blocked using 5% (w/v) skimmed milk for 1 h at room temperature, and then incubated overnight at 4°C with anti-CKB rabbit monoclonal antibody (1:3,000; cat. no. ab92452; Abcam, Cambridge, UK) and anti-DPYSL3 rabbit monoclonal antibody (1:1,000; cat. no. ab126787; Abcam). The membranes (Merck Millipore) were washed in Tris-buffered saline with 0.05% Tween 20.
Table I. Differentially expressed proteins in the prefrontal cortex, as determined by 2-DE and MALDI-TOF-MS/MS.

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2-DE, two-dimensional electrophoresis; MALDI-TOF-MS/MS, matrix-assisted laser desorption ionization-time of flight-tandem mass spectrometry; CI, confidence interval; MW, molecular weight; AIR, LPS-induced acute inflammatory reaction group; Dep, LPS-induced depressive-like behavior group; Con, control group; Ckb, creatine kinase B-type; Gstp1, glutathione S-transferase P1; Akr1b1, aldose reductase; Carkd, ATP-dependent (S)-NAD(P)H-hydrate dehydratase; Guk1, guanylate kinase; Uba1, ubiquitin-like modifier-activating enzyme 1; Ak1, adenylate kinase isoenzyme 1; Atp6v1b2, brain isofrom V-type proton ATPase subunit B; Dlat, mitochondrial dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex; Enol1, α-enolase; Got1, cytoplasmic aspartate aminotransferase; Syn2, synapsin-2; Dps3, dihydrolipoyllysine-residue acetyltransferase-related protein 3; Tuba1b, tubulin α-1B chain; Mapre2, microtubule-associated protein RP/EB family member 2; Actb, cytoplasmic actin 1; Tprg1l, tumor protein p63-regulated gene 1-like protein; Msra, mitochondrial peptide methionine sulfoxide reductase; Gstol, glutathione S-transferase omega-1; Pdia4, protein disulfide-isomerase A4; Acadvl, mitochondrial long-chain specific acyl-CoA dehydrogenase; mKIAA0038, MKIAA0038 protein; Dnm1l, dynamin-1-like protein; Sept3, neuronal-specific septin-3; Sept11, septin-11; Hspa4l, heat shock 70 kDa protein 4L.
Statistical analysis. Statistical analyses were conducted using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). The results from the western blotting were analyzed using a one-way analysis of variance followed by a Bonferroni's test. Body weight and behavioral test results were compared using the Student's t-tests. P<0.05 was considered to indicate a statistically significant difference.

Results

LPS decrease body weight and mobility. LPS-induced changes in body weight and mobility were measured 6 and 24 h post-injection by assessing changes in body weight loss and OFT (22). The weights of the Dep mice were significantly reduced, as compared with their weights prior to injection (Fig. 1A), and the AIR mice exhibited a significant decrease in the total distance travelled in the OFT test, as compared with the control group (Fig. 1B).

The Dep mice exhibited a significant increase in immobility in the TST and FST conducted 24 h post-LPS injection (Fig. 1C and D). This was the time point when typical acute sickness behavior was no longer apparent (22). These results suggest that acute activation of the peripheral innate immune system by LPS induces depressive-like symptoms in mice that are not biased by acute sickness behaviors.

Differentially expressed protein identification by 2-DE and MALDI-TOF-MS/MS were conducted to examine whether PFC protein expression was altered by LPS. Wide-range pH 3-10 strips were used to investigate the differential protein expression in the AIR and Dep mice, as compared with the Con
mice. A total of ~1,780 protein spots were visualized by silver staining (Fig. 2). To identify the differential proteins in the 2-DE gels, a 1.5-fold change was used as a threshold (Table 1). The functional classification of differentially expressed proteins was energy metabolism, neurogenesis, cytoskeleton, signal transducer, redox homeostasis, nuclein metabolism and molecular chaperones (Table 1).

**Western blot analysis.** CKB and DPYSL3 were selected for analysis by western blot analysis. The expression levels of CKB and DPYSL3 were significantly increased in the Dep mice, as compared with the Con and AIR mice (Fig. 3).

**Discussion**

Accumulating evidence suggests that inflammation may have an important role in the pathophysiology of MDD. Meta-analysis demonstrated that significantly higher blood expression levels of pro-inflammatory cytokines, such as TNFα and IL-6, were present in drug-free depressive patients, as compared with healthy controls (23). Numerous conditions such as cardiovascular disease (24), type 2 diabetes (25) and obesity (26) are characterized by chronic inflammation and high prevalence of depression. The bacterial endotoxin LPS is widely used in preclinical research as a tool to study neuroinflammation, and numerous studies have identified the mechanisms by which LPS is able to induce inflammatory responses in the brain (27). It is generally accepted that neuroinflammation induces depressive-like behavior in rats (28). In the present study, the systemic administration of LPS resulted in acute inflammatory reaction and depressive-like behavior in CD1 mice. These results suggest that the experimental animals exhibited negative side effects and depressive-like behaviors, such as weight loss, a decrease in the total distance travelled in the OFT, and an increase in immobility time both in the TST and FST. These results were concordant with those of O’Connor et al (22). Based on these results, the LPS-induced model may be established as a reliable animal model of depression, and may be used to better understand the pathophysiological mechanisms underlying depression (29,30).

The present study used a proteomic approach to examine PFC protein expression in an LPS-induced mouse model of depression. A total of 26 proteins were identified that may have roles in the pathogenesis of MDD. Based on the MOTIF database (http://www.genome.jp/tools/motif/) and previous studies (16), a biological function classification on the differentially expressed proteins in the PFC was established. Energy metabolic-associated proteins constitute the majority of the differentially expressed proteins. In a previous study, we demonstrated that energy metabolic signaling pathways were significantly altered in the PFC of a chronic unpredictable mild stress rat model of depression, as determined by KEGG analysis (17). As determined by positron emission tomography of glucose metabolism, energy metabolic dysfunction in the PFC has been associated with depression (31). CKB was one of the energy metabolic-associated proteins of the present study, and is a cytosolic CK of the brain. There are two cytosolic CKs [brain-type CK (CKB), and muscle-type CK (CKM)], and two mitochondrial CKs [ubiquitous mitochondrial CK (uMtCK) and muscle-specific sarcomeric mtCK) (32). CKs regulate ATP regeneration via the transfer of high-energy phosphate from creatine phosphocreatine to adenosine diphosphate (33,34). The phosphocreatine/CK energy circuit is important for the maintenance of normal energy homeostasis (35,36), and has a number of integrated functions, such as temporary energy buffering and energy transfer, as well as regulating metabolic capacity (37). The results from the western blot analysis indicated that the expression levels of CKB were significantly elevated in the Dep mice, suggesting that LPS-induced depressive-like behaviors in mice may be associated with changes in the energy metabolism of the PFC.

In 2000, a previous study proposed for the first time a neurogenic theory of depression, and demonstrated that impaired adult hippocampal neurogenesis (AHN) triggers depression, and restoration of AHN lead to the alleviation of depressive symptoms (38). Previously, we reported that alterations in hippocampal neurogenesis may have a role in mediating the pathogenesis of depression (16). However, to the best of our knowledge, few studies have investigated neurogenesis in the PFC. The results of the present study demonstrated that DPYSL3, which is a neurogenesis-associated protein, was one of the differentially expressed proteins of the PFC. DPYSL3 is also known as collapsin response mediator protein 4 (CRMP4), which belongs to the cytosolic phosphoprotein family, and is involved in neurite and axonal outgrowth (39-42), neuronal differentiation (39,43,44), axonal guidance (45) and regeneration (46). Previous studies demonstrated that DPYS13 interacts with cytoskeletal proteins tubulin and actin, suggesting DPYS13 is involved in cell assembly and migration (40,47). DPYS13 is also involved in developmental neurogenesis, neuroregeneration following nerve lesion, and growth cone collapse during neuronal cell injury, by organizing filamentous actin into tight bundles (43,46,48). Immunocytochemistry revealed that CRMP-4 is transiently expressed in post-mitotic neurons during rat brain development (49). Therefore, DPYS13 was selected for further investigation. The results of the present study demonstrate that the expression levels of DPYS13 were significantly elevated in the Dep mice, suggesting that LPS-induced depressive-like behavior in mice may be associated with changes in neurogenesis in the PFC.

In conclusion, the present study has demonstrated that LPS is able to induce depressive-like behavior independently of changes in motor activity. Using a 2-DE-MALDI-TOF-MS-based proteomic approach, 26 differentially expressed proteins were identified in the PFC of the Dep mice, as compared with the Con mice. The expression levels of CKB and DPYS13 were significantly elevated in the Dep mice, as determined by western blotting. These results suggest that energy metabolic dysfunction and neurogenesis are significantly altered in the PFC of Dep mice. Investigation into these processes and proteins in the PFC is crucial to obtain a further understanding of the pathophysiological mechanisms underlying MDD.

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