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Novel Phosphodiesterase 4 Inhibitor FCPR03 Alleviates Lipopolysaccharide-Induced Neuroinflammation by Regulation of cAMP/PKA/CREB Signaling Pathway and NF-κB Inhibition

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Running Title Page

Running Title: FCPR03 inhibits neuroinflammation

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ABBREVIATIONS: cAMP, cyclic adenosine monophosphate; CNS, central nervous system; CREB, cAMP response element binding protein; IL-1β, interleukin-1 beta; IL-6, interleukin-6; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa B; PDE4, Phosphodiesterase 4; PKA, protein kinase A; TNF-α, tumor necrosis factor-α.

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Abstract

Over activation of microglia contributes to the induction of neuroinflammation, which is highly involved in the pathology of many neurodegenerative diseases. Phosphodiesterase 4 (PDE4) represents a promising therapeutic target for anti-inflammation. However, the dose-limiting side effects, such as nausea and emesis, have impeded their clinic application. FCPR03, a novel selective PDE4 inhibitor synthesized in our laboratory, shows little or no emetic potency. However, the anti-inflammatory activities of FCPR03 *in vitro* and *in vivo* and the molecular mechanisms are still not clearly understood. This study was undertaken to delineate the anti-inflammatory effect of FCPR03 both *in vitro* and *in vivo* and try to explore whether those effects were regulated by PDE4-mediated signaling pathway. BV-2 microglial cells stimulated by lipopolysaccharide (LPS) and mice intraperitoneally injected by LPS were established as in *vitro* and in *vivo* models of inflammation. Our results showed that FCPR03 dose-dependently suppressed the production of TNF-α, IL-1β and IL-6 in BV-2 microglial cells treated with LPS. Interestingly, the role of FCPR03 on the production of pro-inflammatory factors was reversed by pretreatment with Protein kinase A (PKA) inhibitor H89. In addition, FCPR03 reduced the levels of pro-inflammatory factors in the hippocampus and cortex of mice injected with LPS. Our results further demonstrated FCPR03 effectively increased production of cAMP, promoted cAMP response element binding protein (CREB) phosphorylation and inhibited nuclear factor kappa B (NF-κB) activation both *in vitro* and *in vivo*. Our findings suggest that FCPR03 inhibits the neuroinflammatory response through the

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activation of cAMP/PKA/CREB signaling pathway and NF-κB inhibition.

Introduction

Increasing evidence suggest that neuroinflammation exacerbates many neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease and depression (Blandini, 2013, Eikelenboom, et al., 2012, Heneka, et al., 2014, Maes, et al., 2009, Philips and Robberecht, 2011, Wilms, et al., 2003), mainly through microglia activation, which results in the production of various pro-inflammatory cytokines and subsequent neuronal cell death (Gonzalez, et al., 2014, Pais, et al., 2013). In recent years, the strategy for inhibiting microglia-mediated inflammation has attracted increasing attention and may provide a promising therapy for neuroinflammatory and neurodegenerative diseases.

Microglia, the resident immune cells of the central nervous system (CNS), constitutes a double-edged sword for the CNS, with both beneficial and detrimental effects. It is well-known that microglia can phagocytize invading pathogens and cellular debris (Neher, et al., 2013), secrete neurotrophic factors that regulate the microenvironment (Czeh, et al., 2011) and even repair injured tissues, but over-activated microglia releases various pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) (Rawji, et al., 2016). Multiple clinical studies reveal that microglia over-activation and elevated levels of pro-inflammatory cytokines are found in brains of patients with neurodegenerative diseases (Heneka, et al., 2014, McGeer and McGeer, 2013).

Lipopolysaccharide (LPS), a major outer membrane component in Gram negative bacteria, potently induces inflammation, activating of microglial cells and increasing

the expression of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 (Norden, et al., 2016). TNF-α can activate the nuclear transcription factor-κB (NF-κB) pathway, which in turn leads to cell apoptosis (Brenner, et al., 2015). IL-1β, an important contributor in brain inflammation, is significantly increased in patients with neurodegenerative diseases (Kaushal, et al., 2015). IL-6 upregulation may increase the levels of harmful mediators in the vascular endothelium, mediating inflammatory cascades and leading to the aggravation of neurodegenerative diseases (Rothaug, et al., 2016). It is well established that NF-κB regulates the expression of pro-inflammatory cytokines in LPS-induced microglial cells (Kopitar-Jerala, 2015), suggesting that inhibiting NF-κB may to be an effective therapeutic target for inflammatory diseases.

As the predominant modulator of the cyclic adenosine monophosphate (cAMP) signaling cascade, phosphodiesterase 4 (PDE4) represents a promising target for modulating the immune function. Numerous studies have shown that PDE4 inhibition increases the intracellular availability of cAMP and actives the cyclic adenosine monophosphate (cAMP)/ protein kinase A (PKA)/cAMP response element binding protein (CREB) signaling pathway (McGirr, et al., 2016, Wang, et al., 2016). Reports document PDE4 inhibitors are widely used pharmaceutical agents with a broad range of anti-inflammatory property. The PDE4 inhibitor rolipram is suggested to prevent leukocytes accumulation, airway hyper-responsiveness and cytokine release (Ikemura, et al., 2000). Furthermore, roflumilast inhibits lung inflammation in mildly asthmatic patients with allergen challenge (Gauvreau, et al., 2011). Recently, another selective PDE4 inhibitor, apremilast is attributed therapeutic effects in psoriatic arthritis and

plaque psoriasis (Papp, et al., 2012, Schett, et al., 2012). Recent studies also reveal that cAMP/PKA/CREB signaling is closely involved in neuroinflammatory responses (Wang, et al., 2012). Preclinical findings suggested therapeutic efficacy for PDE4 inhibitors in a wide range of neurodegenerative diseases where neuroinflammation plays an important role, including Alzheimer's disease (Gong, et al., 2004), Parkinson's disease (Blandini, 2013), major depressive disorder (Plattner, et al., 2015), and multiple sclerosis (Gonzalez-Garcia, et al., 2013).

FCPR03 or N-isopropyl-3-(cyclopropylmethoxy)-4-difluoromethoxy benzamide (**10j**), displays a high PDE4 enzymes selectivity with a low IC_{50} of 60 nM (Fig.1A). In addition, our previous studies also found that FCPR03 inhibited LPS-induced TNF-α, iNOS, and COX-2 expression in microglial cells and did not cause emesis in beagle dogs during the 180 min observation period at 0.8 mg/kg (Zhou, et al., 2016), suggesting that FCPR03 not only has a good property of anti-inflammation, but also has less emetic potency than other PDE4 inhibitors, such as rolipram. However, the underlying mechanisms are still not clearly understood. Although a good anti-inflammatory effect of FCPR03 has been preliminarily demonstrated at the cellular level, its anti-inflammatory effect is still needed to be verified from *in vivo* studies, and importantly, whether its anti-inflammatory effect is mediated through inhibition of PDE4 enzyme and the subsequent activation of cAMP/PKA/CREB signaling pathway are also unclear. In the present study, the effects of FCPR03 in LPS-induced inflammation both *in vitro* and *in vivo* were evaluated. Furthermore, whether the possible anti-inflammatory effects of this compound were associated with

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the cAMP/PKA/CREB signaling cascade and NF-κB inhibition were assessed.

Materials and Methods

Drugs. Lipopolysaccharide (LPS) from *Escherichia coli* strain 055:B5 (Sigma-Aldrich Corp., St Louis, USA), Rolipram, 4-[3-(Cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidinone (Enzo Life Sciences, Farmingdale, NY, USA), and the PKA inhibitor H89, N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide·2HCl hydrate (Beyotime Institute of Biotechnology, Nanjing, China) were used. The drugs were prepared freshly before use. All other chemicals used were of analytical grade.

Cell culture. BV-2 microglial cells, an immortalized murine microglial cell line, were a kind gift from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). They were maintained in DMEM containing 10% FBS and antibiotics (100 IU/ml penicillin and 100 mg/ml streptomycin) at 37°C in a 5% CO_2 incubator. At about 80-90 % confluency, cells were split twice a week with 0.25% trypsin.

Cell viability assay. Cell viability was evaluated by the tetrazolium salt 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO, USA) assay (Zhou, et al., 2016). Briefly, BV-2 microglial cells (8×10^3 cells/well) were seeded into 96-well plates and pretreated with various concentrations of FCPR03 for 1 h, before stimulation with LPS (1 μg/ml) for 24 h. After treatment, the medium was removed, and cells were incubated with MTT (0.5 mg/ml) for 4 h at 37°C. The resulting formazan crystals were solubilized with DMSO, and absorbance was measured at 570 nm on a microplate reader (Synergy HT, BioTek, USA).

Animals. Male C57BL/6 mice (8 weeks, 20-25 g) were obtained from the Laboratory

Animal Centre of Southern Medical University (Guangzhou, China), and acclimated in the facility for 1 week before the experiments. They were housed at 8 per cage in standard environmental conditions (22 ± 2℃; humidity. 60 ± 5%; 12 h light/dark cycle with lights on at 7:00), with access to food and water *ad libitum*. Experimental procedures were conducted between 8:30 and 16:30. All experiments using animals were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH, 2011); this study was approved by the Laboratory Animal Ethics Committee of Southern Medical University.

Mouse model of neuroinflammation. LPS was administered intraperitoneally (i.p.) to induce neuroinflammation in mice, as previously described (Yuan, et al., 2016). Mice were randomly divided into five experimental groups (n=8): control (vehicle, 10 ml/kg), LPS (vehicle, 10 ml/kg), LPS + FCPR03 (FCPR03 0.5 and 1 mg/kg, respectively), and positive control (rolipram 1 mg/kg) groups. Drugs were intragastrically administered once daily for 7 consecutive days. At 7 days, mice were intraperitoneally injected with saline or LPS (1.2 mg/kg) 30 min after the last drug administration. Then, the animals were sacrificed by cervical dislocation 24 h after saline or LPS challenge. The hippocampus and cortex were isolated immediately and stored at -80 °C for further biochemical analysis.

Novel object recognition test. The test was carried out as described previously (Zhang, et al., 2014). On day 6 after FCPR03 administration, each mouse was allowed to move freely in a white box (60 × 60 × 15 cm) for 5 min to habituate to the surroundings. 24 h later, mice were individually placed in the center of the box

containing two identical objects located in two corners diagonal from each other. The time spent in exploring each object was recorded during a 5 min period. Exploration was defined as touching or facing the object within a 2 cm distance. 24 h after LPS injection, the food intake and body weight change were measured, then mice were tested for memory using the same procedure except that one of the objects was replaced with one of a different shape and color. The recognition index was expressed by the ratio TN/(TF + TN), where TF = time spent exploring the familiar object and TN = time spent exploring the novel object.

Assessment of cytokine and cAMP levels. TNF-α, IL-1β and IL-6 levels were determined by using ELISA kits purchased from Boster (Wuhan, China). Cytokine amounts in 100 μl samples were determined according to the manufacturer's protocol. Quantitative determination of cAMP levels was performed by ELISA (R&D systems, USA, cAMP assay kit) according to the manufacturer's instructions. Relative cAMP levels were normalized to total protein. All samples and standards were assayed in duplicate.

Immunofluorescence staining. The nuclear localization of NF-κB p65 was examined by indirect immunofluorescence assay (Kim, et al., 2015). BV-2 microglial cells (1×10^6 cells/well) were cultured on sterile 15 mm cover slips in 24-well plates for 12 h, and pretreated with FCPR03 (20 μM) for 1 h. After stimulation with LPS for 1 h, the cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100 in PBS and blocked with 5% bovine albumin V. Then, anti-NF-κB p65 monoclonal antibodies (1:100) (Cell Signaling Technology, Beverly, MA, USA) were

applied overnight at 4°C, followed by 3 h of incubation with Alexa 488-conjugated secondary antibodies at 4°C. After 3 washes with PBS, the cell nuclei were counterstained with 1 μmol/L DAPI. Finally, the cells were visualized and photographed on a Nikon Instruments C2 Confocal Microscope (Nikon). Representative images were obtained from 16 high power fields per sample.

Western blot analysis. Western blot procedures were performed as previously described (Zhou, et al., 2016). In brief, brain tissues and microglial cell lysates were prepared using a modified RIPA buffer (1×RIPA lysis buffer including 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail). Total protein concentration was measured using a BCA protein assay kit (Thermo Scientific, MA, USA). Equal amounts of protein were separated by 10-15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and proteins were transferred onto PVDF membranes (Millipore, Bedford, MA, USA). After blocking in Tris-buffered saline with 0.1% Tween-20 (TBST) (blocking buffer) containing 5% (W/V) nonfat milk for 1 h at room temperature, the membranes were incubated overnight with specific primary antibodies at 4°C. After 3 washes in TBST, incubation was carried out with appropriate secondary antibodies for 3 h at 4°C. Detection was performed with the enhanced chemiluminescence reagent (ECL, Millipore) and protein bands were revealed by autoradiography. The following primary antibodies were used: anti-phospho-CREB (Cell Signaling Technology, Beverly, MA, USA), anti-NF-κB p65 (Cell Signaling Technology, Beverly, MA, USA) and rabbit anti-GAPDH polyclonal (Abcam, Cambridge, MA, USA) and anti-histone H3 (Cell

Signaling Technology, Beverly, MA, USA) antibodies. GAPDH and H3 were used as loading controls. Densitometric analysis of protein bands was performed using the ImageJ software. To assess NF-κB p65 translocation, nuclear and cytoplasmic extracts were prepared with Nuclear and Cytoplasmic Protein Extraction Kit purchased from Beyotime Institute of Biotechnology (Nanjing, China).

Statistical analysis. Data are expressed as mean ± standard error of the mean (SEM), and were assessed by one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. Statistical Package for the Social Sciences (SPSS) version 13.0 (SPSS, USA) was used for statistical analyses. Graphs were plotted with the GraphPad Prism 5.0 software. $P<0.05$ was considered statistically significant.

Results

Effect of FCPR03 on BV-2 microglial cells viability. To rule out FCPR03 toxicity in microglia, BV-2 microglial cells were pretreated with FCPR03 and/or LPS to test cell viability using the MTT assay. Precisely, BV-2 microglial cells were pretreated with FCPR03 at 5, 10 and 20 μM, respectively, for 1 h, and incubated in presence of LPS (1 μg/ml) for 24 h. Our data showed that FCPR03 and/or LPS produced no toxicity at the tested concentrations (P>0.05). Similar to FCPR03, the positive control rolipram (20 μM) also showed no toxic effects on BV-2 microglial cells (P>0.05) (Fig. 1B).

FCPR03 inhibited pro-inflammatory cytokines release in LPS-stimulated BV-2 microglial cells. Since PDE4 is a potential therapeutic target for the limitation of inflammation. Here, we tried to assess whether FCPR03 inhibited the production of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) in LPS-treated microglial cells. As shown in Fig. 2A-C, treatment with LPS caused significant increase in the production of TNF-α, IL-1β and IL-6 in BV-2 microglial cells (P<0.01). However, the levels of these pro-inflammatory factors in the supernatant were dramatically attenuated by FCPR03 at the dose of 10 μM (P<0.05), and rolipram had a similar anti-inflammatory effect at 20 μM with FCPR03 (P<0.01). Moreover, the anti-inflammatory effect of FCPR03 was almost blocked by the pretreatment with PKA inhibitor H89 (Fig. 2D-F), indicating that the anti-inflammatory effect of FCPR03 may probably through upregulating cAMP/PKA signaling pathway.

Effect of FCPR03 on cAMP/PKA/CREB signaling in BV-2 microglial cells. To ensure whether the anti-inflammatory effect of FCPR03 was mediated by

cAMP/PKA/CREB signaling pathway, we assessed the intracellular cAMP level in BV-2 microglial cells with ELISA assay. We found that FCPR03 alone could significantly enhance the level of intracellular cAMP in BV-2 microglial cells at 20 μM (P<0.05) (Fig. 3A). While stimulation with LPS for 24 h dramatically decreased intracellular cAMP level in BV-2 microglial cells (P<0.05), and this effect was reversed by the pretreatment of 20 μM FCPR03 (P<0.05) (Fig. 3B). Furthermore, our data also showed FCPR03 promoted CREB phosphorylation in BV-2 microglial cells, an effect peaking at 30 min (P<0.05) (Fig. 4A), whereas the increased phosphorylation of CREB was also reversed by pretreatment with H89 (P<0.05) (Fig. 4B). On the other hand, our results also revealed that pretreatment with 20 μM FCPR03 could reverse the reduction of phosphorylated CREB induced by LPS (Fig. 4C-D). Hence, our data demonstrate that FCPR03 activates the cAMP/PKA/CREB signaling pathway, which subsequently suppresses the production of pro-inflammatory cytokines in LPS-induced BV-2 microglial cells.

Effect of FCPR03 on NF-κB p65 activation in LPS-stimulated BV-2 microglial cells. Activation of the NF-κB plays an important role in regulating the expression of pro-inflammatory cytokines. To assess whether FCPR03 inhibited the activation of NF-κB p65 under the condition of neuroinflammation, BV-2 microglial cells were treated with LPS (1 μg/ml) in presence of FCPR03 (20 μM) for 1 h, and the activation of NF-κB p65 was analyzed by Western blotting. As shown in Fig. 5A, LPS and FCPR03 did not alter the protein expression of NF-κB p65 in BV-2 cells. Whereas LPS promoted the translocation of NF-κB p65 from cytosol into nucleus, FCPR03

inhibited LPS-induced subcellular translocation. More interestingly, this effect of FCPR03 was blocked by pretreatment with H89 (P<0.05) (Fig. 5B-C). Furthermore, immunofluorescence assay verified these findings (Fig. 5D). The data suggest that inhibition of pro-inflammatory cytokines release by FCPR03 may partially depend on inhibiting NF-κB p65 translocation.

Here, we have demonstrated a significant anti-inflammatory effect of FCPR03 *in vitro*, and this effect is probably through up-regulating the cAMP/PKA/CREB signaling pathway and inhibiting the activation of NF-κB. However its anti-inflammatory effect and the responsible signaling are still needed to be studied in animal model.

Effect of FCPR03 on the levels of brain pro-inflammatory cytokines in LPS-treated mice. Since FCPR03 exhibited an appreciable anti-inflammatory effect *in vitro*, we are eager to know whether FCPR03 will have a similar effect *in vivo*. A single systemic administration of LPS in mice is widely used as an important neuroinflammatory animal model *in vivo* (Khan, et al., 2016). Hence, we adopted this model to evaluate the anti-inflammatory effect of FCPR03. Consistent with previous findings, the current study showed that the levels of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) in the hippocampus and cortex were significantly increased 24 h after parenteral administration of 1.2 mg/kg LPS (P<0.01). However, after 7 consecutive days of administration with FCPR03 or rolipram, the levels of pro-inflammatory cytokines in the hippocampus and cortex were significantly decreased, confirming the finding that FCPR03 is a promising compound which has

the property to reduce neuroinflammation (Fig. 6).

Effect of FCPR03 on the cAMP/PKA/CREB signaling pathway in LPS-treated mice. Previous data revealed that FCPR03 possessed of a potent anti-inflammatory property *in vitro* via regulating the cAMP/PKA/CREB signaling pathway. Herein, we assessed whether the anti-neuroinflammatory effects of FCPR03 in LPS-treated mice were mediated by this signaling pathway. As shown in Fig. 7A, LPS administration significantly decreased cAMP levels both in the hippocampus and cortex compared with the control group ($P<0.01$). As expected, FCPR03 (1 mg/kg) or rolipram (1 mg/kg) increased cAMP levels both in the hippocampus and cortex ($P<0.05$). CREB phosphorylation in the hippocampus and cortex were dramatically decreased after LPS administration ($P<0.01$); meanwhile, these effects were reversed by pretreatment with FCPR03 ($P<0.05$) (Fig. 7B-C). In line with *in vitro* data, these results suggest that the anti-neuroinflammatory effects of FCPR03 may involve the cAMP/PKA/CREB signaling pathway in LPS-treated mice.

Effect of FCPR03 pretreatment on LPS-induced NF-κB activation in hippocampus and cortex. To further confirm the suppressive effect of FCPR03 on LPS-induced NF-κB activation, western blot was performed to monitor NF-κB p65 protein expression in the hippocampus and cortex. As shown in Fig. 8A-B, the NF-κB p65 protein levels in the whole cells showed no significant difference both in the hippocampus and cortex. However, cytoplasmic NF-κB p65 protein levels were markedly decreased in the LPS group, both in the hippocampus and cortex ($P<0.05$) (Fig. 8C-D), while the protein levels in nucleus were increased markedly in both brain

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regions (P<0.01) (Fig. 8E-F), indicating that NF-κB p65 was activated and translocated into the nucleus 24 h after exposure to LPS. Pretreatment with FCPR03 (1 mg/kg) inhibited the translocation of NF-κB p65. Our results demonstrate that FCPR03 contributes to the attenuation of neuroinflammatory response probably through inhibiting NF-κB activation.

FCPR03 alleviated the sickness behaviors and cognitive deficits in female mice treated by LPS. It has been reported that the response of mice with different sex to LPS was different (Sorge, RE, et al., 2011). Female mice were more resistant to LPS than male mice, and this effect may be related to the secretion of estrogen (Hughes EL, et al., 2013). Having known that FCPR03 had a potent anti-inflammatory property in male mice, we then performed another experiment to explore the anti-inflammatory effect of FCPR03 in female mice. As shown in Fig. 9, after 7 consecutive days administration with 1 mg/kg FCPR03 or rolipram, the levels of cytokines in the hippocampus and cortex were significantly decreased (P<0.05). However, consistent with previous studies, we noticed that the response of female mice to LPS was weaker than that in male mice. Hence, we further investigated the alterations of sickness behaviors and cognitive deficits in female mice. Our results showed that FCPR03 significantly ameliorated the loss of appetite and cognitive deficits in female mice treated by LPS (P<0.05). We also found that the body weight in mice administrated with FCPR03 was slightly higher than that in LPS group, even there was no significant difference observed. Taken together, our results indicate that FCPR03 possesses a potent anti-neuroinflammatory property in both female and male mice.

Discussion

Neuroinflammation, which is characterized by over-activation of microglia (Wes, et al., 2016), is closely bound up with the pathogenesis and progression of many neurodegenerative diseases (Dendrou, et al., 2016, Raison, et al., 2006), and inhibition of inflammation mediated by activated microglia is considered to be a promising approach for the treatment of these disorders (Heneka, et al., 2014, Gonzalez, et al., 2014). In the present study, we show for the first time that FCPR03, a selective PDE4 inhibitor, potently inhibits neuroinflammatory responses both *in vitro* and *in vivo*. Furthermore, our data demonstrate that the anti-neuroinflammatory effect of FCPR03 is probably mediated through the activation of cAMP/PKA/CREB signaling pathway and NF-κB inhibition.

Pro-inflammatory mediators, such as TNF-α, IL-1β and IL-6 are produced following microglia activation (Rawji, et al., 2016). LPS induces inflammation by activating of microglia and produces various pro-inflammatory cytokines (Smith, et al., 2012). Based on these properties, microglial cell treated with LPS is widely used as an *in vitro* cell model of neuroinflammation as well as a tool for the evaluation of potential anti-neuroinflammatory compounds (Gupta and Kaur, 2016, Huang, et al., 2016, Pfluger, et al., 2016). Thus, this cell model was used in the present study to evaluate the anti-neuroinflammatory effect of FCPR03.

Pro-inflammatory cytokines exacerbate the pathology of neurodegenerative diseases and even deteriorate the process of many brain injuries, such as traumatic brain injury, and stroke as well. Selective PDE4 inhibitors, such as roflumilast,

cilomilast and apremilast, were reported to curb inflammatory responses both *in vitro* and *in vivo* (Buenestado, et al., 2012, McLean, et al., 2009). In the present study, we sought to investigate the anti-inflammatory effects of the novel PDE4 inhibitor FCPR03 both *in vitro* and *in vivo*. In line with other PDE4 inhibitors, such as apremilast (Perez-Aso, et al., 2015) and GSK256066 (Grundy, et al., 2016), FCPR03 can markedly inhibit the release of pro-inflammatory cytokines and exhibit a potent anti-inflammatory property. Combined with our previous enzymatic and side effect studies on FCPR03 (Zhou, et al., 2016), these data suggest that FCPR03, a PDE4 inhibitor with better selectivity and less emetic potency, has considerable anti-inflammatory activity.

cAMP, an ubiquitous second messenger, is a critical regulator of inflammatory responses, including the migration and recruitment of leukocytes, cytokine secretion, and the activation and proliferation of T cells (Mosenden and Tasken, 2011). PDE4 inhibitors, such as rolipram, produce anti-inflammatory, cognition-enhancing and antidepressant-like effects via enhancing cAMP-mediated signaling. However, the serious side effects, such as nausea and emesis, have impeded its further clinical application (Li, et al., 2009). Inflammatory stimulants, including LPS, interferon (IFN)-g and TNF-α remarkably reduce intracellular cAMP level in microglial cells, leading to changes in morphology and promoting the production of pro-inflammatory cytokines, whereas these changes could be reversed by PDE4 inhibitors (Ghosh, et al., 2012). In accordance with this result, we found that pretreatment with FCPR03 prevented the decrease of intracellular cAMP and phosphorylated CREB levels in

LPS-treated microglial cells. Furthermore, for the sake of investigating whether PKA mediates the anti-inflammatory effect of FCPR03, cells were pretreated with PKA inhibitor H89 (Zeng, et al., 2016). Interestingly, the current data revealed that these effects of FCPR03 were mostly reversed by H89. Therefore, it is possible that the anti-inflammatory effect of FCPR03 is mediated through cAMP/PKA/CREB signaling cascade.

Transcription factors, including NF-κB, are important regulators in the transcription of these inflammatory cytokines. Activating NF-κB induced by LPS stimulates the expression of proinflammatory cytokines that aggravate inflammation, while inhibition of NF-κB activation suppresses the expression of various genes, such as TNF-α, IL-1β and IL-6 (Zhou, et al., 2016). In normal microglial cells, NF-κB is retained in the cytoplasm, whereas NF-κB will translocate into nuclear after the activation of microglia challenged by LPS. A recent article which points out that NF-κB is activated after the treatment of LPS, and the activity of NF-κB is regulated by cAMP/PKA/NF-κB axis (Li, et al., 2016). Pretreatment with FCPR03 can significantly suppress NF-κB activation, this effect was mostly blocked by pretreatment with H89, suggesting that the inhibition of pro-inflammatory cytokine release by FCPR03 is probably dependent on NF-κB inhibition which is regulated by cAMP/PKA signaling pathway.

Since hippocampus and cortex play important role in the regulation of memory, cognition, and emotion, we mainly focused on the neuroinflammation in these brain regions. Our previous study showed that lentiviral miRNA-mediated long-form

PDE4D knockdown could attenuate memory deficits in a mouse model of Alzheimer's disease induced by hippocampal $A\beta_{42}$ injections, and this effect was partially produced by inhibiting the neuroinflammation in CNS (Zhang, et al., 2014). Though male mice are typically more responsive to TLR4 agonists than females, our results showed that the pro-inflammatory cytokines levels in hippocampus and cortex were significantly decreased in both male and female mice. What's more, FCPR03 could dramatically alleviate sickness behaviors and improve the cognitive deficits induced by LPS. In addition, the reduction of cAMP and pCREB in hippocampus and cortex were reversed by pretreatment with FCPR03. As current available PDE4 inhibitors are limited by the emetic potency, FCPR03 is a promising candidate compound for inhibiting neuroinflammation and attenuating many neurodegenerative diseases associated with neuroinflammation.

To further explore the anti-inflammatory mechanisms of FCPR03, we investigated the effect of FCPR03 on the activation of the NF-κB. NF-κB plays a key role in inflammatory process and suppression of NF-κB could inhibit LPS-induced inflammation (Seeley and Ghosh, 2016). Our data clearly show that pretreatment with FCPR03 could significantly inhibit the activation of NF-κB both *in vitro* and *in vivo*, suggesting that the anti-neuroinflammatory effect of FCPR03 is probably associated with inhibiting the activation of NF-κB.

In summary, FCPR03 inhibited neuroinflammation induced by LPS in BV-2 microglial cells through activating cAMP/PKA/CREB pathway, inhibiting the translocation of NF-κB and thereby reducing the production of TNF-α, IL-1β and IL-6.

Furthermore, FCPR03 exerted significant anti-neuroinflammatory effect in LPS-treated mice. In our study, we mainly focused on the alterations of inflammatory indicators and the signaling pathways involved in the role of FCPR03, as inflammation participates in the behavioral changes in animals under the condition of stress, behavioral tests, such as assessments on learning and memory, depression and anxiety are deserved to be investigated in the future. Additionally, it is interesting to determine the sex-specific differences in PDE4 expression/activity and TLR4 activation in this model, and whether there is any difference in their response to FCPR03 needs to be studied. On the other hand, the data presented here are obtained from LPS-induced cell and animal models, but the anti-inflammatory effect of FCPR03 is still needed to be confirmed in other animal models, such as in Alzheimer's disease and depressive animal models. Taken together, these results reveal that FCPR03 possesses anti-neuroinflammatory property, strongly suggesting its potential as a candidate drug for the treatment of diseases associated with neuroinflammation.

Authorship Contributions

Participated in research design: Zheng-Qiang Zou, Yu-Fang Cheng，Hai-Tao Wang，Jiang-Ping Xu.

Conducted experiments: Zheng-Qiang Zou，Jia-Jia Chen.

Contributed new reagents or analytic tools: Zhong-Zhen Zhou.

Performed data analysis: Zheng-Qiang Zou, Hong-Fang Feng, Hai-Biao Guo.

Wrote or contributed to the writing of the manuscript: Zheng-Qiang Zou， Yu-Fang Cheng, Hai-Tao Wang, Wenhua Zheng, Jiang-Ping Xu.

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FOOTNOTES

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Figure Legends

Fig 1. Chemical structure of FCPR03 and BV-2 microglial cells viability after pretreatment with FCPR03. Chemical structure of FCPR03 (A). BV-2 microglial cells were pretreated with various concentrations of FCPR03 (5, 10 and 20 μM) or rolipram (20 μM) for 1h before stimulation with LPS (1 μg/ml) for 24 h. Cell viability was detected by the MTT assay (B). Values are mean ± SEM (n=6/group).

Fig 2. The inhibition effects of FCPR03 on TNF-α, IL-1β and IL-6 production in LPS-induced BV-2 microglial cells. BV-2 microglial cells were pretreated with various concentrations of FCPR03 (5, 10 and 20 μM) or rolipram (20 μM) for 1 h at presences of the selective PKA inhibitor H89 (10 μM) for 40 min, before stimulation with LPS (1 μg/ml) for 24 h. Then, the levels of TNF-α, IL-1β and IL-6 in cell culture supernatants were measured with commercial ELISA kits. Data are expressed as mean ± SEM (n=6/group). $^{\#\#}P<0.01$ compared with the control group; $^{*}P<0.05$, $^{**}P<0.01$ compared with the LPS group; $^{+}P<0.05$ compared with LPS + FCPR03 (20 μM) group.

Fig 3. FCPR03 increased the level of intracellular cAMP in BV-2 microglial cells. BV-2 microglial cells were treated with various concentrations of FCPR03 for 1 h (A), cells were pretreated with various concentrations of FCPR03 for 1 h, then incubated with or without LPS (1 μg/ml) for 24 h (B), intracellular cAMP concentrations were measured by ELISA. Results are shown as the mean ± SEM (n=6/group). $^{\#}P<0.05$ compared with the control group; $^{*}P<0.05$ compared with the LPS group.

Fig 4. FCPR03 promoted the phosphorylation of CREB in BV-2 microglial cells. BV-2 microglial cells were pretreated with 20 μM of FCPR03 for the indicated times (A) or various concentrations of FCPR03 (0, 5, 10 and 20 μM) for 30 min (B). BV-2 microglial cells were pretreated with LPS (1 μg/ml) for the indicated times (C). Following the treatment of FCPR03 (20 μM) for 30min, BV-2 microglial cells were incubated with LPS (1 μg/ml) for another 90 min (D). Levels of phosphorylated CREB (pCREB) and total CREB were determined by Western blot. The corresponding quantification data are shown in each panel. Data are expressed as mean ± SEM (n=3/group). $^{\#}P<0.05$ compared with the FCPR03 (20 μM) group; $^{*}P<0.05$ compared with the control group.

Fig 5. Effect of FCPR03 on NF-κB p65 activation in LPS-stimulated BV-2 microglial cells. NF-κB p65 protein levels in whole cells (A), cytosolic (B) and nuclear (C) were analyzed by Western blot. NF-κB p65 subunit translocation was assessed by immunofluorescence (D). Scale bars = 20 μm. The corresponding quantification data are shown in each panel. GAPDH and histone H3 were used as internal controls. Data are expressed as mean ± SEM (n=3/group). $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with the control group; $^{*}P<0.05$ compared with the LPS-treatment group; $^{+}P<0.05$ compared with the LPS + FCPR03 (20 μM) group.

Fig 6. Effects of FCPR03 on production of LPS-induced pro-inflammatory cytokines in hippocampus and cortex. After 7 consecutive days pretreatment with FCPR03 or rolipram, mice were injected intraperitoneally with saline or 1.2 mg/kg LPS. Brains were removed 24 hours later, with the hippocampus and cortex dissected and

homogenized. Levels of the pro-inflammatory cytokines TNF-α (A), IL-1β (B) and IL-6 (C) were quantified by ELISA kits. Data are expressed as mean ± SEM (n=6-8/group). $^{\#\#}P<0.01$ compared with the control group; $^{*}P<0.05$, $^{**}P<0.01$ compared with the LPS group.

Fig 7. Effects of FCPR03 on the cAMP/PKA/CREB signaling pathway in LPS-induced mice. 24 h after LPS administration, the entire hippocampal and cortical extracts were homogenized, and cAMP levels were determined by ELISA (A). Western blot was used to assess pCREB and CREB protein levels in the cortex (B) and hippocampus (C). The corresponding quantification data are shown in each panel. Data are expressed as mean ± SEM (n=3/group). $^{\#\#}P<0.01$ compared with the control group; $^{*}P<0.05$, $^{**}P<0.01$ compared with the LPS group.

Fig 8. Effects of FCPR03 on LPS-induced NF-κB activation in the hippocampus and cortex. 24 h after LPS administration, the total NF-κB p65 protein levels in cortex and hippocampus were detected (A-B), then cytosolic and nuclear proteins in the mouse hippocampus and cortex were separated. Cytosolic NF-κB p65 protein levels in the cortex and hippocampus (C-D) as well as nuclear NF-κB p65 protein amounts in the cortex and hippocampus (E-F) were analyzed by Western blot. The corresponding quantification data are shown in each panel. Data are expressed as mean ± SEM (n=3/group). $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with the control group; $^{*}P<0.05$, compared with the LPS group.

Fig 9. FCPR03 alleviated the sickness behaviors and cognitive deficits in female

mice-treated by LPS. After 7 consecutive days pretreatment with FCPR03 or rolipram, mice were injected intraperitoneally with saline or 1.2 mg/kg LPS. 24 h after LPS injection, food intake (A) and body weight change (B) were measured. Whereafter, the novel recognition test (C) was carried out. Then the hippocampus and cortex were dissected and homogenized. Levels of the pro-inflammatory cytokines TNF-α (D), IL-1β (E) and IL-6 (F) were quantified by ELISA assay. Data are expressed as mean ± SEM (n=6-8/group). $^{\#\#}P<0.01$ compared with the control group; $^{*}P<0.05$ compared with the LPS group.

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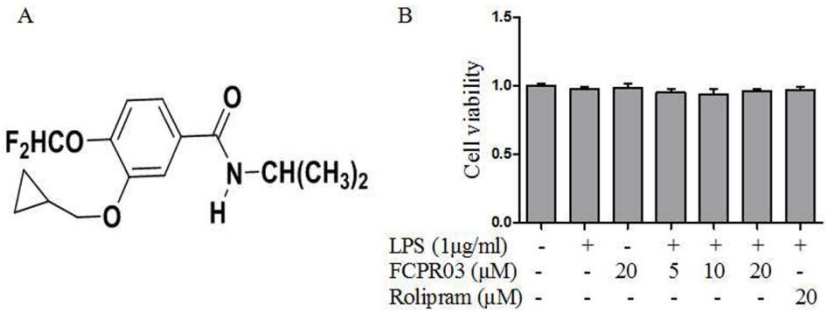

Fig. 1

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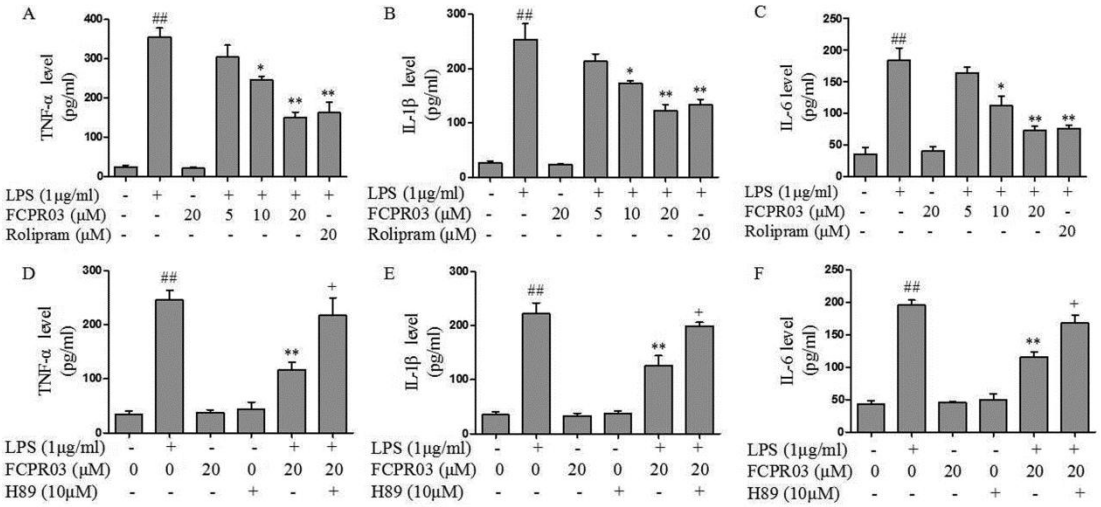

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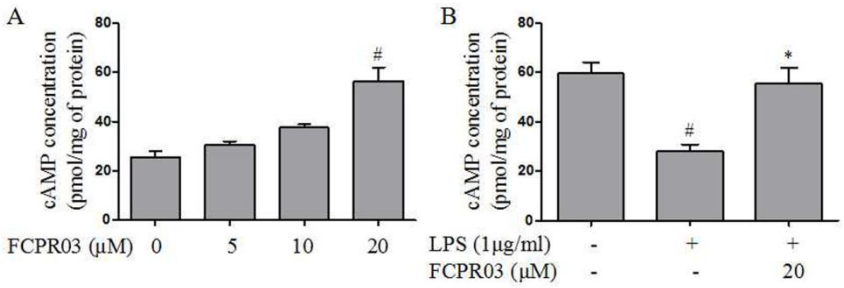

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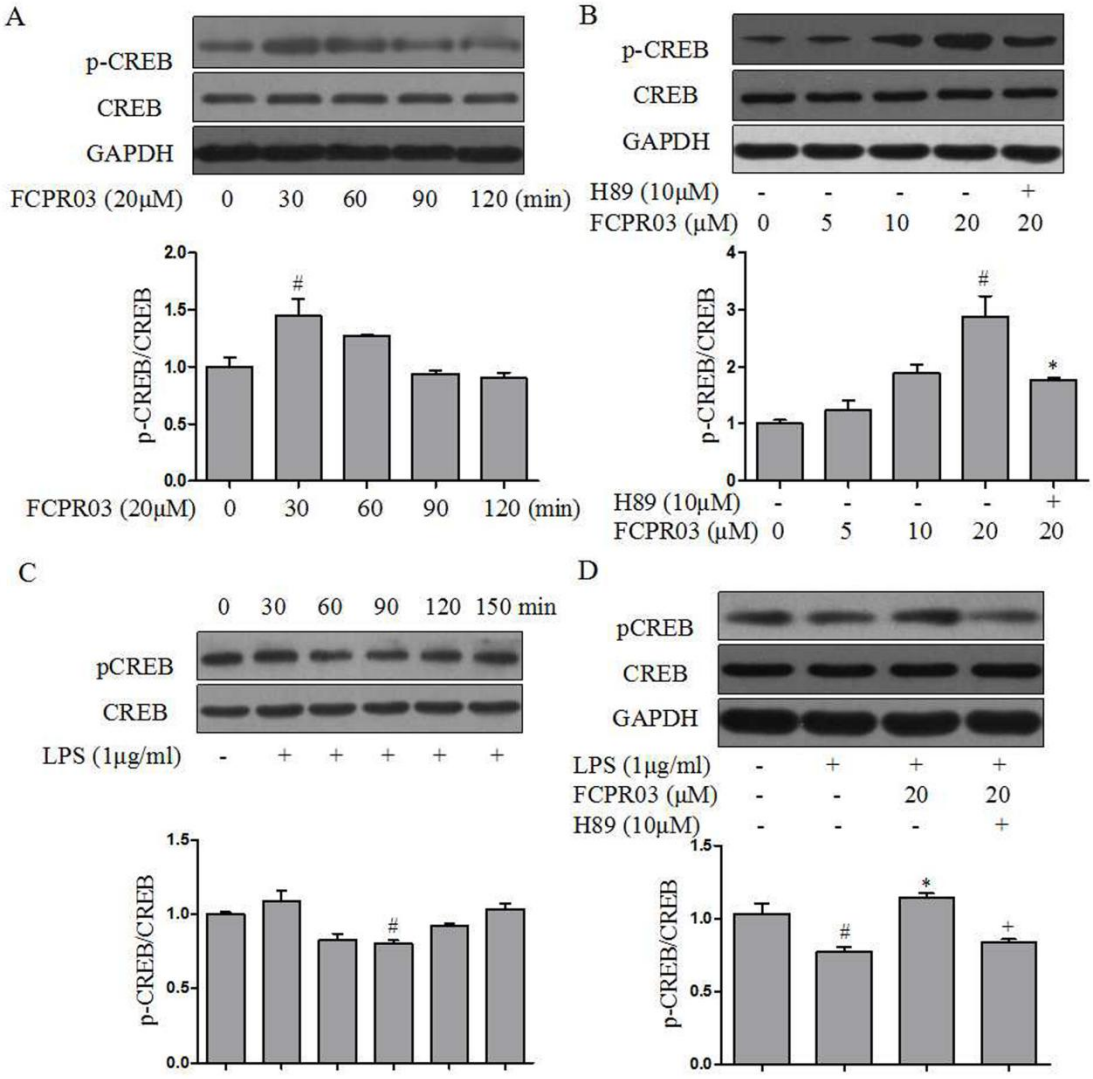

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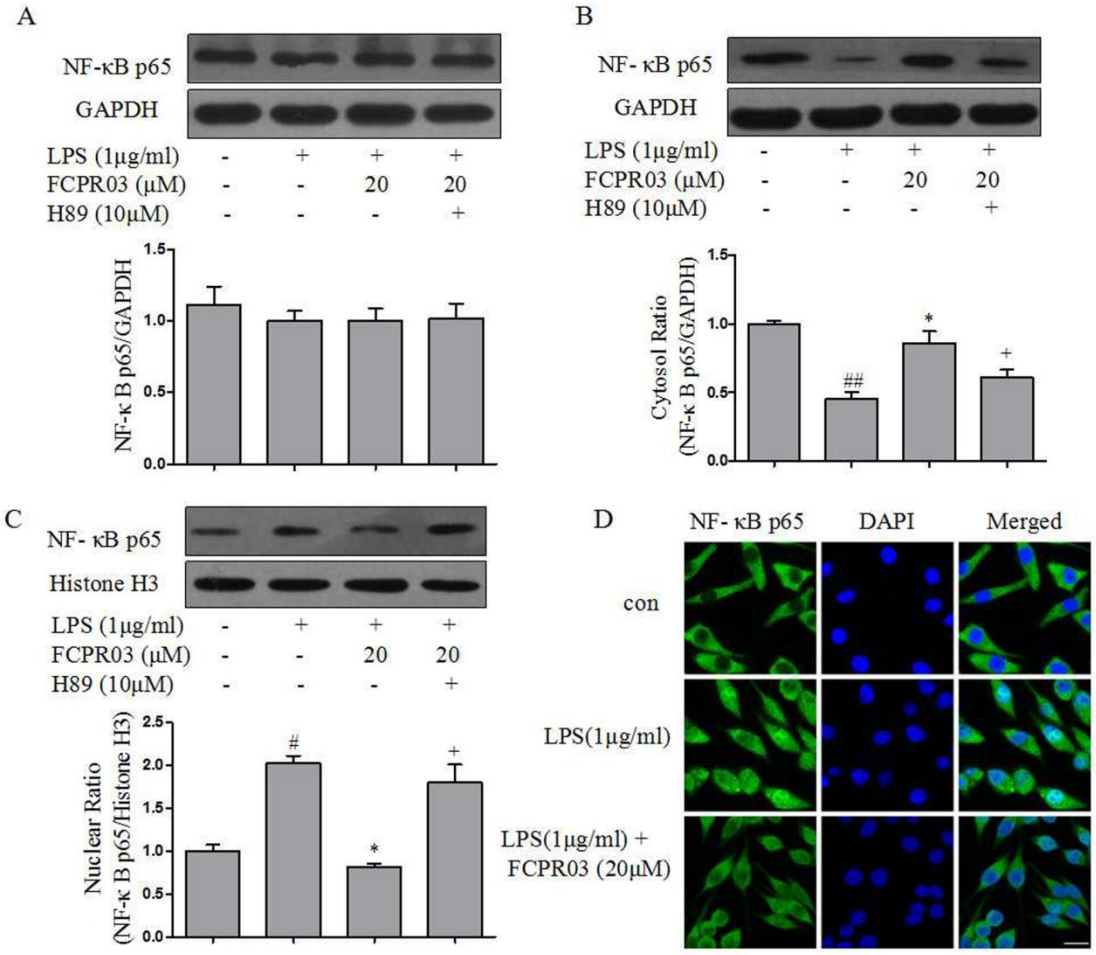

Fig. 5

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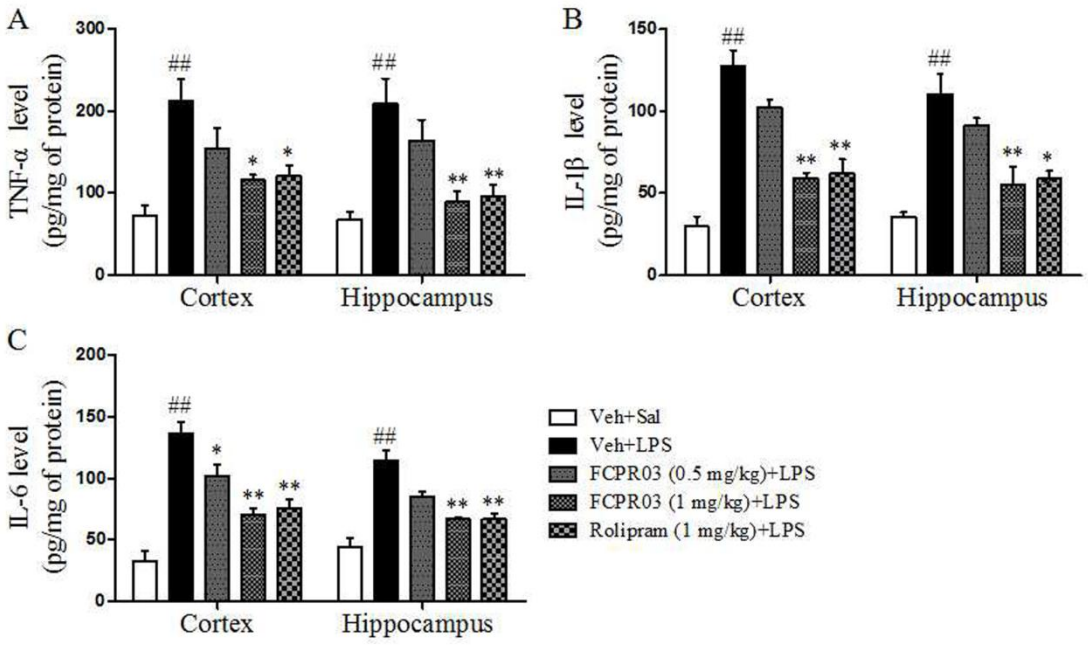

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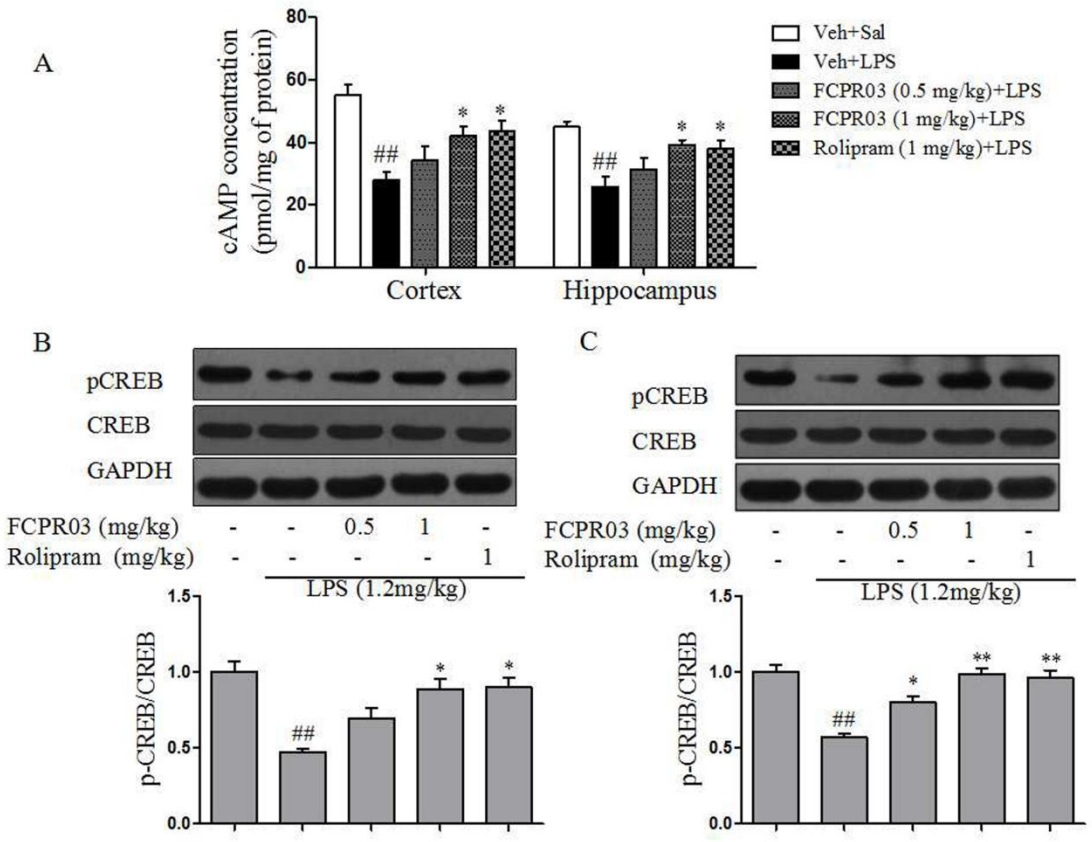

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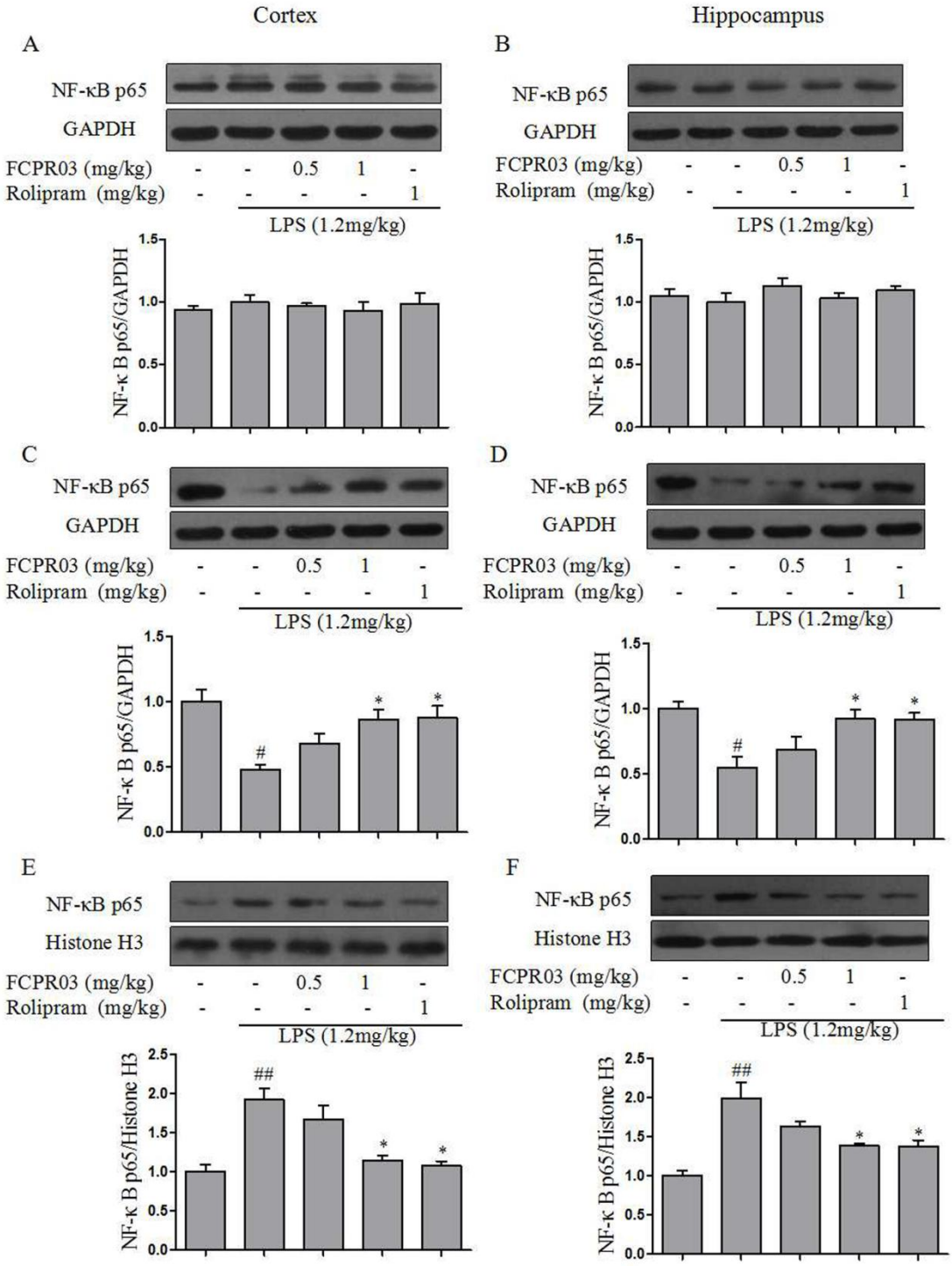

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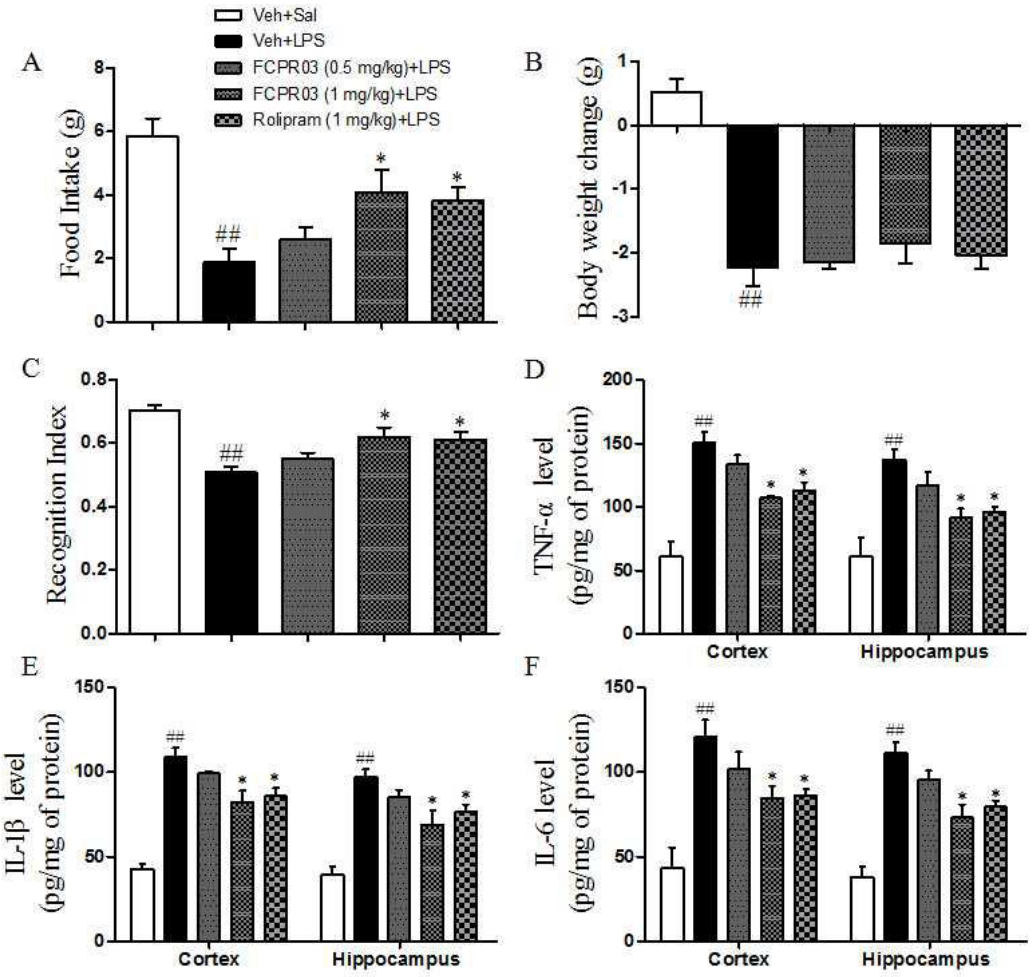

Fig. 9