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Original Paper

Paeoniflorin Attenuates Inflammatory Pain by Inhibiting Microglial Activation and Akt-NF-κB Signaling in the Central Nervous System

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Key Words
Paeoniflorin • Freund's complete adjuvant • Pain • Microglia • Akt • NF-κB

Abstract
Background/Aims: Paeoniflorin (PF) is known to have anti-inflammatory and paregoric effects, but the mechanism underlying its analgesic effect remains unclear. The aim of this study was to clarify the effect of PF on Freund's complete adjuvant (CFA)-induced inflammatory pain and explore the underlying molecular mechanism. ***Methods:*** An inflammatory pain model was established by intraplantar injection of CFA in C57BL/6J mice. After intrathecal injection of PF daily for 8 consecutive days, thermal and mechanical withdrawal thresholds, the levels of inflammatory factors TNF-α, IL-1β and IL-6, microglial activity, and the expression of Akt-NF-κB signaling pathway in the spinal cord tissue were detected by animal ethological test, cell culture, enzyme-linked immunosorbent assay, immunofluorescence histochemistry, and western blot. ***Results:*** PF inhibited the spinal microglial activation in the CFA-induced pain model. The production of proinflammatory cytokines was decreased in the central nervous system after PF treatment both *in vivo* and *in vitro*. PF further displayed a remarkable effect on inhibiting the activation of Akt-NF-κB signaling pathway *in vivo* and *in vitro*. ***Conclusion:*** These results suggest that PF is a potential therapeutic agent for inflammatory pain and merits further investigation.

Introduction

Pain is an unpleasant sensory experience induced by noxious stimuli, characterized by a heightened responsiveness to several stimuli (hyperalgesia or allodynia) [1, 2]. Nearly a third of the world population experiences clinical pathological pain [3]. Pain is believed to be

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caused by aberrant neuronal responses along the pain transmission pathway from the dorsal root ganglion (DRG) to the spinal cord, thalamus, and cortex [4]. Accumulating evidence indicates that neuroinflammation may play a vital role in the pain transmission pathway. Recent studies have revealed that microglia, the immune effector cells in the central nervous system (CNS) critically contribute to the pathogenesis of inflammatory pain [5].

Microglia is resident macrophages in the CNS and exerts important functions in maintaining homeostasis in the CNS [6, 7]. Activation of microglia occurs in most pathological processes, which is often accompanied by morphologic change and production of cytotoxic molecules, and upregulation of phagocytosis and immune surface antigens [8-10]. Activation of microglia is also involved in the pain transmission pathway of acute and chronic pains such as neuropathic pain, inflammatory pain and cancerous pain [11-13]. It has been found that the activated spinal microglia release bioactive molecules including interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), nitric oxide (NO), and brain-derived neurotrophic factor (BDNF), which aggravate the range and duration of pain [14-16].

Chinese herbs are important resources to develop safe and effective candidates for the pain therapy. Paeoniflorin (PF) is a chemical compound as one of the major constituents of an herbal medicine derived from *Paeonia lactiflora* [17, 18]. In *Paeonia*, PF can form new compounds with the addition of phenolic substituents [19, 20]. PF can improve CNS diseases such as cognitive impairment, depression, and Parkinson's disease [21-25]. The combined use of PF and opioid analgesics could potentiate the analgesic effect of opioid analgesics in the treatment of cancerous pain as compared with the use of opioid analgesics alone [26, 27]. Our previous study demonstrated that PF exerted the anti-inflammatory effect by inhibiting the activation of microglia [28].

In the present study, we sought to investigate the effect of PF on microglia in inflammatory pain and discovered that Akt-NF-κB signaling pathway was a critical step in the pathogenesis of inflammatory pain. This finding suggests that PF may prove to be a viable therapeutic candidate for the treatment of inflammatory pain.

Materials and Methods

Animals

Male C57BL/6J mice were housed under climate-controlled conditions with a 12-h light/dark cycle (lights on at 8:00 A.M., lights off at 8:00 P.M.) at 25 °C and humidity of 65 % environment during the entire acclimatization for a week with free access to food and water. All experimental procedures were approved by the Ethical Committee of Jiaxing University Medical College (No. JUMC 2017-009) and performed in accordance with the Guide for the Care and Use of Laboratory Animals of the said university.

Establishment and drug treatment of the inflammatory pain model

PF was purchased from Preferred Biotech (Chengdu, China). The right hind-foot of mice was hypodermically injected with Freund's complete adjuvant (CFA, 20 μL) once to establish an inflammatory pain model. Sham surgery was performed by injecting the same amount of 0.9% normal saline (NS) to the right hind-foot as a control. After CFA or NS injection, the mice were administered intrathecally 5μL phosphate buffered solution (PBS) as a vehicle control, or 5μL of 0.5, 1, 2 μg PF daily at 10:00 AM from day 1 to day 8 after CFA injection under narcotization with intraperitoneal injection of 4% chloral hydrate at 7 mL/kg body weight. Fifty mice were equally randomized into three groups: Sham group, CFA group, and CFA+PF group (0.5, 1, and 2 μg PF).

Thermal withdrawal latency testing

Thermal hyperalgesia was measured using ZH-YLS-6BS thermal pain stimulator (Zhenghua Biolog, Anhui, China). Mice were placed in transparent Plexiglass cages. Radiant heat with an appropriate intensity was applied from underneath the platform to the plantar surface of the hind paw until mice showed positive signs of pain. The time when the mouse began licking or withdrawing its paw was recorded and defined as the hot-plate paw withdrawal latency (hPWL). Each measurement was repeated three times at 15-

min intervals and the mean force evoking reliable withdrawals were taken as the threshold. Tests were performed 1 day before modeling and 1, 2, 3, 7 and 8 days after CFA-injection.

Mechanical withdrawal threshold testing

Mechanical allodynia was examined by assessing paw withdrawal threshold (PWT) using BIO-EVF3 Electric von Frey (IITC, USA). Mice were placed in transparent Plexiglass cages on the top of an elevated metal mesh floor, and a detector was applied at the central region of the plantar surface of the right hind paw to identify the threshold of pain response directly. Each measurement was repeated three times at 10-min intervals and the mean force evoking reliable withdrawals were taken as the threshold.

Enzyme-linked immunosorbent assay (ELISA)

Ultimately, animals were anesthetized and perfused transcardially with 4% paraformaldehyde/PBS (pH 7.4) and sacrificed by decapitation. The spinal cord was immediately dissected, and the contaminating blood was washed away with PBS. The spinal cord tissues (lumbar enlargement segments, ca. L4-5) were homogenized in ice-cold PBS and centrifuged at 3600× *g* for 15 min at 4 °C, and the supernatant was used for the double antibody sandwich ELISA and Western blotting. The contents of inflammatory factors TNF-α, IL-1β, and IL-6 were measured by ELISA kits (Roche, Germany) according to the manufacturer's instructions.

Immunofluorescence histochemistry

The L4-5 spinal cord was fixed in the 4 % paraformaldehyde at 4°C for 5 h and then transferred to 30 % sucrose/PBS for 24 h. Floating transverse sections of 10 μm were used for immunofluorescence histochemistry. The sections for immunofluorescent staining were blocked in a solution containing 5% normal goat serum and 0.1% Triton X-100 for 3 h at room temperature. Then, the sections were incubated 24 h at 4°C with primary antibodies, Iba1 (rabbit polyclonal anti-Iba1, 1:2000; Wako Pure Chemicals), and Neu-N (mouse monoclonal anti-Neu-N, 1:1000; Sigma). After washing, the sections were then incubated with the fluorescent-conjugated secondary antibody (Alexa 488 and Alexa 543, 1:1000; Invitrogen, CA) for 2 h at room temperature. Fluorescent images were obtained with a confocal microscope (Olympus, Japan). Image J software (NIH, Bethesda, MD) was used to analyze cell images.

Cell culture

The murine BV2 cell line was maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified incubator with 5 % CO_2. The cells were put into a 12-well plate (5.6×10^5/mL) [29]. BV2 cells cultured were equally randomized into three groups: (1) pure BV2 cell group as control group (CTL group); (2) lipopolysaccharide group (LPS group), where BV2 cells were incubated with LPS (0.1 μg/mL) for 24 h; and (3) LPS+PF group, where cells were co-incubated with LPS (0.1 μg/mL) and PF (200 μM) for 24 h. Further, cells were fixed with 4% preheated neutral paraformaldehyde at 37 ℃ for 15 min and incubated at room temperature for 50 min with Alexa-Fluor-594 conjunct phalloidine (50 mg/mL) after treatment with PBS containing 0.1% Triton X-100. The cell structure was observed under a confocal microscope. The culture media were centrifuged at 3600× *g* for 5 min at 4 °C to harvest the cell supernatant.

Western blotting

The western blotting analysis was performed as previously described [30]. Briefly, an equal amount of proteins was loaded and fractionated on 12% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and then electrophoretically transferred to the polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA), immunoblotted with antibodies, and visualized by enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL). Film signals were digitally scanned and quantitated using Image J. Densitometric analysis of target protein levels were normalized to an internal control (GAPDH or Lamine B2) and expressed as a relative value.

Statistical analysis

Data are expressed as mean ± standard error of mean (SEM). n represents the number of tested animals or cells. The one-way analysis of variance (ANOVA) was used for data analysis. Difference associated with $P<0.05$ was considered statistically significant.

Results

PF attenuates tactile allodynia after CFA-injection

There was no significant difference in hPWL and PWT between the three groups before CFA injection. From post-operative day 1, the frequency of hind paw withdrawals elicited by mechanical and thermal stimulus was significantly higher in CFA group than that in Sham group ($P < 0.05$) (Fig. 1). In contrast, repeated intrathecal administrations of PF markedly suppressed this decrease in hPWL and PWT in a dose-dependent manner ($P < 0.05$) (Fig. 1). These data indicate that PF prevented the development of tactile allodynia after CFA injection.

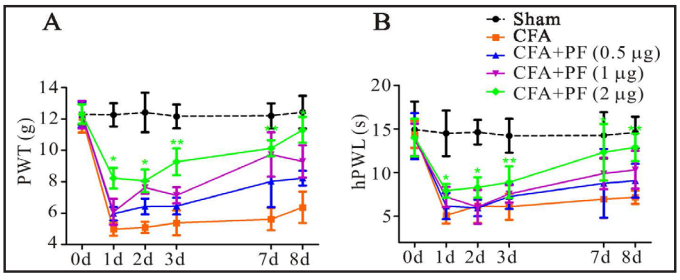

Fig. 1. The effect of PF on mechanical PWT and hPWL. (A) Mechanical PWT was determined by von Frey filament test. (B) hPWL was measured with the thermal pain stimulator. Each point and vertical line represent the mean ± SEM of the values obtained. Statistical difference was determined using unpaired two-sided Student's t-test. Note: PF, paeoniflorin; CFA, Freund's complete adjuvant; PWT, paw withdrawal threshold; hPWL, hot-plate paw withdrawal latency. *P<0.05 vs. CFA.

PF reduces proinflammatory cytokine expression after CFA injection

Knowing that proinflammatory cytokine expression is increased in the process of inflammatory pain [31], we detected TNF-α, IL-6, and IL-1β in the spinal cord to see whether PF could relieve pain by inhibiting the production of proinflammatory cytokines in the CNS. It was found that the expression of TNF-α, IL-6, and IL-1β expression induced by CFA was reduced significantly after PF treatment (Fig. 2), indicating that PF could inhibit the expression of inflammatory cytokines in the CNS.

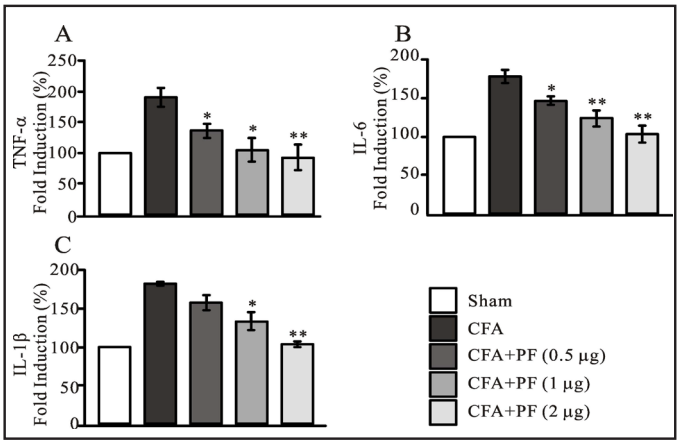

Fig. 2. The effect of PF on CFA-induced inflammation. The expression of TNF-α, IL-6, and IL-1β was inhibited by PF in a dose-dependent manner. Note: #P<0.05 vs. Sham; *P<0.05 vs. CFA.

PF inhibits microglial activation after CFA injection

Knowing that microglia activation and subsequent proinflammatory cytokine expression are responsible for enhanced pain hypersensitivity [32], we used Iba-1 as the microglial activation marker to see whether PF played a role in the activation of spinal microglia in the CFA-induced inflammatory pain mice. It was found that the fluorescence intensity in the spinal cord dorsal horn of CFA-injected mice was increased by more than 50% compared with that in Sham group, and the expression of Iba-1 was reduced after PF treatment (Fig. 3A). In addition, we also tested another microglial activation marker CD11b and found that the expression of the CD11b protein was up-regulated by 9-fold in CFA and CFA+PF groups (Fig. 3B). There was no significant change in neurons in the three groups. These results suggest that PF could relieve pain by inhibiting the activation of microglia in the CNS in the CFA-induced pain model.

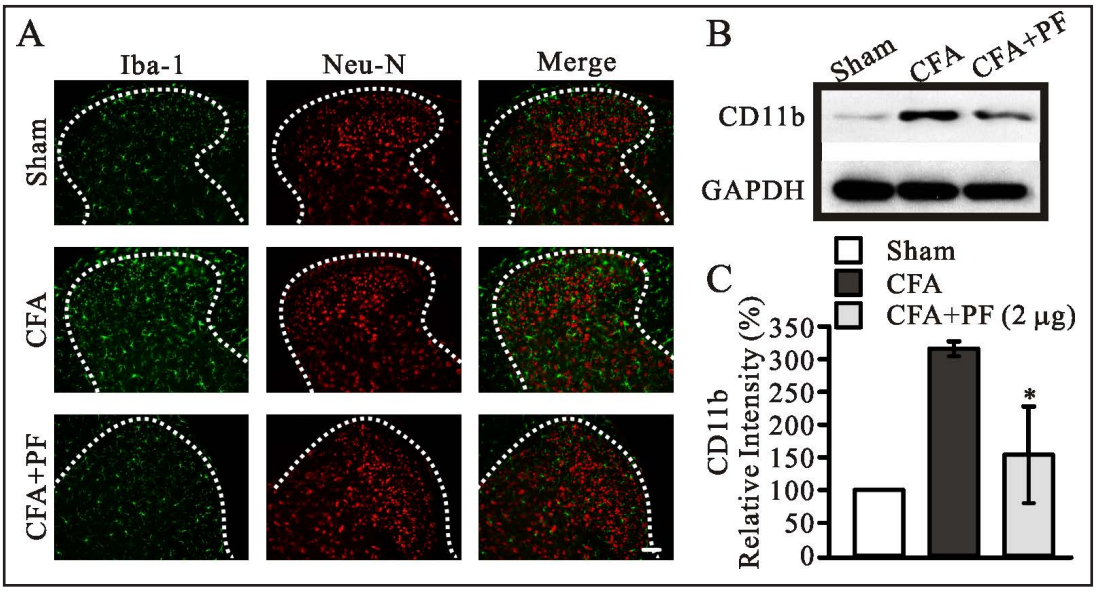

Fig. 3. The effect of PF on microglia activation in the spinal cord. (A) Spinal microglia and neuron were observed by immunohistochemistry using the microglia marker Iba-1 and the neuronal marker Neu-N, respectively. Dotted lines showed the dorsal horn of spinal cord. Scale bar is 200 μm. (B) The western blotting analysis was used to test the expression of CD11b in the spinal cord. (C) Graphic representation of the relative expression of CD11b normalized to GAPDH. The data represent the means ± SEM. Note: *P<0.05 vs. CFA.

PF inhibits microglial activation and proinflammatory cytokine expression in vitro

BV2 cells were used to test the effect of PF. It is found that BV2 cells were classified as ramified in the resting state and turned to the hypertrophied type after 24-h 0.1 μg/mL LPS treatment (Fig. 4A). Knowing that microglia activation was also associated with F-actin rearrangement, we further tested the pattern of F-actin by using phalloidin staining. In the control group, LPS-stimulation caused relatively actin polymerization, as represented by concentrated phalloidin staining in the periphery of the plasma membrane. In contrast, actin aggregation induced by LPS-stimulation was markedly decreased after PF treatment (Fig. 4B).

Knowing that microglia activation produces more proinflammatory cytokine [16], we used ELISA kit to test whether PF could attenuate the release of proinflammatory cytokines, and found that the expression of TNF-α, IL-6, and IL-1β in the culture medium was increased after LPS-treatment, and reduced after PF intervention (Fig. 4C). Taken together, these data indicate PF could inhibit LPS-induced microglial activation and proinflammatory cytokine production.

Akt-NF-κB signal pathway may be involved in the analgesic mechanism of PF

Knowing that Akt-NF-κB pathway worked as a modulator in many pain models [33, 34], we detected the expression of phosphorylated Akt (p-Akt) in the spinal cord under the same experimental conditions and found that its expression was significantly increased in CFA-induced pain modeled mice, while PF treatment inhibited the increase of p-Akt (p-Akt relative intensity of CFA was 150.38±2.36%, and CFA+PF was 118.91±7.41% , Figs. 5A, C, D). Likewise, PF exhibited the similar effect *in vitro* (p-Akt relative intensity of CFA was 180.31±20.60%, and CFA+PF was 142.27±12.99%, Figs. 5A, F).

The expression of phosphorylated NF-κB-p65 and its nuclear translocation were increased during pain [35]. After CFA injection, the expression of NF-κB-p65 in the spinal cord was increased significantly compared with that in Sham group mice (NF-κB-p65 relative intensity was 234.25±20.03% in CFA group, and 127.25±23.22% in CFA+PF group, Figs. 5A,

D). Meanwhile, the expression of NF-κB-p65 in the nucleus was also increased in CFA-injected mice(the relative expression of NF-κB-p65 in CFA group was 186.05±36.03%, Figs. 5B, F). But the expression and distribution of NF-κB-p65 returned to normal after PF treatment (the NF-κB-p65 relative intensity was 126.86±40.77% in CFA+PF group, Figs. 5A, B, F). Likewise, PF exhibited the similar effect *in vitro* (Figs. 5A, B, D, F).

Knowing that IκBα binds to NF-κB molecule in cytoplasm to inhibit the activation and nuclear translocation of NF-κB molecule and the production of NF-κB-p65 molecule is usually accompanied by the degradation of IκBα molecule [36, 37], we detected the expression of IκBα molecule in the spinal cord and found that IκBα expression was decreased in CFA-injected mice. Compared with Sham group, the expression of IκBα was significantly increased after PF intervention. We also found the similar results in BV2 cells *in vitro* (the relative expression of IκBα was 29.54±17.63% in CFA group, and 105.18±27.99% in CFA+PF group, Figs. 5A, E). These results suggest that the Akt-NF-κB signal pathway might be involved in the analgesic effect of PF.

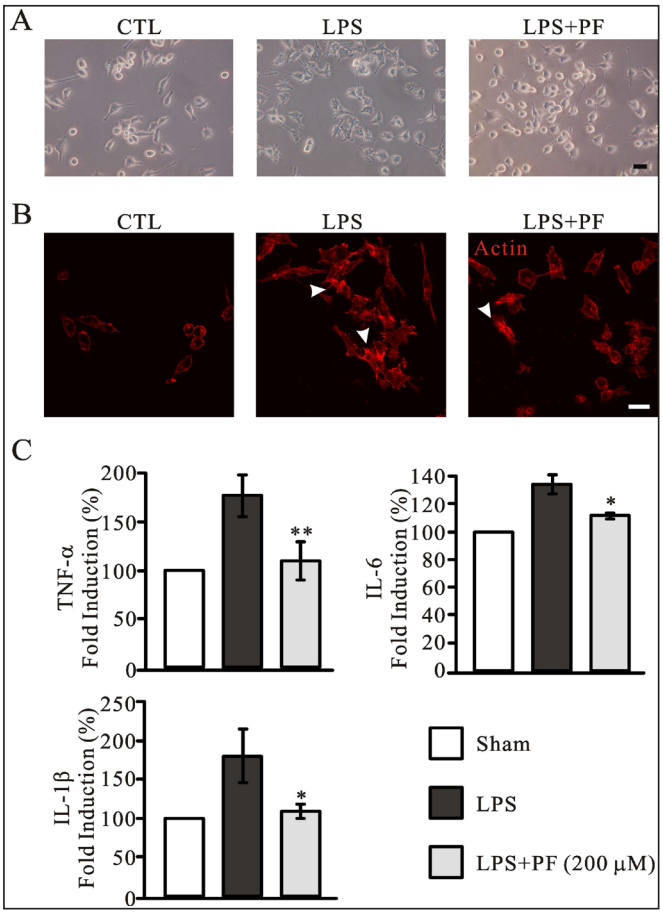

Fig. 4. The effect of PF on microglia activation in vitro. (A) BV2 cells were observed under the bright filed to see their morphological changes after 200 μM PF treatment. (B) PF inhibited LPS-induced actin polymerization. Cells were stained with phalloidin (red). F-actin aggregation was in the periphery of BV2 cells (white arrows). Scale bars: 20 μm. (C) PF inhibited the expression of proinflammatory cytokine TNF-α, IL-6, and IL-1β in vitro. Note: #P<0.05 vs. Sham; *P<0.05 vs. LPS.

Discussion

Inflammatory pain is the most common type of pain mostly caused by trauma and microbial infection [36]. Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used for analgesic treatment in clinical practice. But as NSAIDs often cause severe gastrointestinal adverse effects, it is necessary to find new analgesic drugs with minimal adverse effects. The research showed that PF had good anti-inflammatory and analgesic effects, especially when it was used in combination with morphine [26, 27]. Nevertheless, the analgesic mechanism of PF is poorly understood yet. In this study, we found that the release of inflammatory factors was reduced after intrathecal injection of PF in the CFA-induced pain mouse model, and PF-treatment could inhibit microglia activation in the CNS.

Microglia is resident macrophages in the CNS. In the model of CFA-induced inflammatory pain, a marked pain threshold was observed on the first day after CFA injection. PF-treatment could significantly increase the threshold of mechanical pain and thermal pain, decrease the

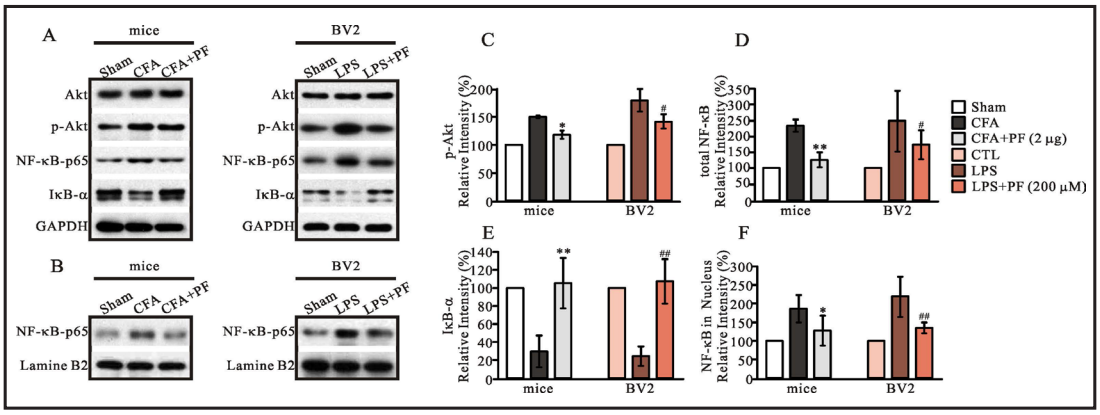

Fig. 5. The Akt-NF-κB signaling pathway is involved in the analgesic effect of PF in vivo/vitro. (A) Western Blotting analysis for the expression of Akt, p-Akt, NF-κB-p65, and IκBα in the spinal cord and in BV2 cells. (B) Nuclear extracts from each group were tested by Western blotting. Lamine B2 was used as loading control. Graphic representation of the relative expression of (C) p-Akt, (D) NF-κB-p65, (E) IκBα in the spinal cord, and (F) nuclear extracts. The data represent the means ± SEM. Note: *P<0.05, **P<0.01 vs. CFA; #P<0.05, ##P<0.01 vs. LPS.

release of TNF-α, IL-6, and IL-1β, and inhibit microglia activation and Akt-NF-κB expression. PF attenuated microglial activation and TNF-α, IL-6, IL-1β production directly *in vitro*. These results indicate that PF plays an analgesic role by inhibiting microglia in the CNS.

Knowing that inflammation is the main contributing factor of inflammatory pain, we observed that analgesic effect of PF and explored its inhibitory mechanism. It was found in previous studies that PF could inhibit the inflammatory response of dendritic cells by blocking the signal pathway of toll-like receptor 4/5 (TLR4/5) [37], and PF inhibited the synthesis of TNF-α, NO, prostaglandin E2 (PE2) and other proinflammatory factors in the mononuclear macrophage system to relieve inflammation [38]. PF also had an inhibitory effect on inflammation in the CNS. It was found that Aβ1-42-induced microglia inflammation could be effectively inhibited by PF. *In vivo* experiments also confirmed that the release of TNF-α and NO in the midbrain of the mouse Alzheimer's model was inhibited, and the memory and learning function of the mice were improved [39]. We found that PF could inhibit the activation of microglia, and cytokine synthesis, *in vivo* and *in vitro*, suggesting that the anti-inflammatory effect of PF is an important mechanism of analgesia.

In summary, PF could inhibit the activation of microglia through the Akt-NF-κB signaling pathway, and therefore may prove to be a potential therapeutic drug for the treatment of inflammatory pain. Further detailed studies are needed to better clarify the underlying mechanism.

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Disclosure Statement

The authors report no conflict of interests in this work.

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