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Pectolinarin inhibited LPS-stimulated inflammation in microglial BV_2 cells via NF-κB signaling pathway

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Author contributions

Cheng BF and Wang L designed the experiments. Feng X and Dong YQ performed most of the experiments and wrote the manuscript. Jian SQ and Yu HH contributed to performing the experiments. Li JJ, Ma T and Zhang J contributed to the data analysis. Cheng BF, Wang L, Yang HJ and Zhang YD supervised and corrected the manuscript.

Competing interests

The authors declare no conflicts of interest.

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Abbreviations

Pec, pectoinarin; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor α; IL-6, interleukin 6; NO, nitric oxide; ROS, reactive oxygen species; NF-κB, nuclear factor kappa B; IKK, inhibitor of nuclear factor kappa-B kinase; PD, Parkinson's disease; AD, Alzheimer's disease; RT-PCR, real-time quantitative PCR; IL-1β, interleukin-1β; ELISA, enzyme-linked immunosorbent assay.

Citation

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Abstract

Background: Neuro-inflammation is regarded as one of the critical pathogenesis in neurodegenerative diseases, which is characterized by the activated microglial cells. Pectolinarin (Pec), a natural flavonoid that exists in many Chinese herbal medicines, has been reported to have various biological activities. However, the effects and mechanisms on neuro-inflammation are not clear. **Methods:** In this study, the inhibitory effects and mechanisms of Pec on neuro-inflammation were investigated in the LPS-stimulated microglial BV_2 cells. BV_2 microglial cells were treated with Pec or vehicle, followed by LPS. Enzyme-linked immunosorbent assay, real-time quantitative PCR, nitric oxide and reactive oxygen species assay, and western blot were performed to examine the effects of Pec on neuro-inflammatory responses. **Results:** We showed that Pec significantly inhibited the expression of tumor necrosis factor α and interleukin 6 in mRNA and protein levels induced by LPS. Moreover, the production of nitric oxide, iNOS, reactive oxygen species, and COX-2 were suppressed by Pec in LPS-stimulated microglial BV_2 cells. In addition, Pec inhibited LPS-induced inflammation via nuclear factor kappa B signaling pathway, as evidenced by the reduction of the phosphorylation of inhibitor of nuclear factor kappa-B kinase, the degradation of IκBα, and the nuclear translocation of p65. **Conclusion:** Taken together, Pec exhibited anti-inflammatory effects in LPS-stimulated microglial BV_2 cells via nuclear factor kappa B signaling pathway, which might provide therapeutic potential for neuro-inflammation and neurodegenerative diseases.

Keywords: neuroinflammation; microglial cells; pectolinarin; NF-κB

Highlights

Pectolinarin inhibited the production of TNF-α and IL-6, NO, iNOS, ROS, and COX-2 in LPS-stimulated microglial BV_2 cells. The phosphorylation of IKK, the degradation of IκBα and the nuclear translocation of p65 were inhibited by pectolinarin.

Medical history of objective

Cirsium japonicum Fisch. ex DC., a traditional Chinese herb used for nourishing blood (blood vomiting, blood in stools, blood in urine and traumatic bleeding effects), eliminating carbuncles and dispelling sores (anti-inflammatory, anti-bacterial and anti-oxidant effects), is first derived from in Hong-Jing Tao's "*the Famous Physician's Record*" (summarized in the Wei and Jin dynasties, 220–450 C.E.). Pectolinarin is a natural glycosylated flavone that exists in *Cirsium japonicum* species and has a wide range of biological activities, including anti-tumor, antioxidant, antiviral, anti-inflammatory, anti-depressant, and anti-diabetic effects.

Background

Neuro-inflammation is a defense mechanism to multiple exogenous stimuli and pathogens in the central nervous system [1]. It is regarded as the pathogenesis of several neurodegenerative diseases, such as Parkinson's disease (PD) and Alzheimer's disease (AD) [2–4]. Microglia, the main immune defense cells, constitute 10–15% of the glial cell population in the brain, which play a vital role in the innate immune response and represent the first line of defense against invading pathogens and pro-inflammatory reactions [5–8].

In a resting stage, microglia survey the microenvironment in real-time with their ramified processes and secrete various neurotrophic factors to help the development and maintenance of neuronal. When the microglia cells were activated, the shape of them could be changed from highly ramified morphology into an ameboid shape. In addition, a series of cellular and molecular events happened. Microglia would secrete a high level of pro-inflammatory factors and cytotoxic mediators, such as tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), nitric oxide (NO), COX-2 and iNOS [9]. Therefore, regulation of microglial activation might represent a potential therapeutic strategy for neuro-inflammation.

Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria, which is a strong stimulator of microglial activation [10]. LPS recognizes and binds with LPS-binding protein and glycosylphosphatidylinositol-anchored protein CD14, interacts with toll-like receptor 4, and activates downstream signaling pathway. Activated toll-like receptor 4 activates the NF-κB signal pathway, which is the most frequently used to investigate the mechanism of inflammatory responses in microglia [11]. In response to external stimulation, IκB family members can be phosphorylated and degraded, and the NF-κB moved from the cytoplasm into the nucleus, leading to the expression of various pro-inflammatory mediators [12].

Traditionally, Chinese herbal medicine has been widely used to treat various diseases with little side effects, including neurodegenerative diseases [13–17]. Pectolinarin (Pec) is a glycosylated flavone that was first isolated from a known medicinal Chinese herb, *Linaria vulgaris* subsp. *chinensis* (Bunge ex Debeaux) D. Y. Hong. Pec has been widely reported due to its presence in many Chinese herbs, such as *Cirsium japonicum* Fisch. ex DC., *Kickxia ramosissima* (Wall.) Janch., *Lantana camara* L., and *Picnomon acama* (L.) Cass. [18, 19]. Pec has a wide range of biological activities, including anti-tumor, antioxidant, antiviral, anti-inflammatory, anti-depressant, anti-diabetic, and hepatoprotective effects [20–24]. As for anti-inflammatory effects, previous studies have shown Pec inhibited the acid-induced writhing in mice in a dose-dependent manner, suppressed inflammation in fibroblast-like synoviocytes by inactivating PI3K/Akt pathway [25, 26]. However, the effects of Pec on neuro-inflammation are still largely unknown.

In this study, the anti-inflammatory effects of Pec in LPS-stimulated murine microglial cell line BV_2 were investigated, and the underlying mechanisms were further elucidated.

Materials and methods

Materials

Pectolinarin was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). LPS was obtained from Sigma Co., Ltd. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium was obtained from Hyclone Co., Ltd. (Shanghai, China). Fetal bovine serum and penicillin-streptomycin (P/S) were supplied from Gibco Co., Ltd. (Gaithersburg, MD, USA). COX-2, iNOS, p65, p-p65, IKKβ, p-IKKα/β, IκB-α, GAPDH, lamin B1 antibodies, and relative secondary antibodies were obtained from Cell Signaling Technology Inc. (Boston, MA, USA).

Cell culture and treatment

Murine microglial cell line BV_2 was provided by the National Infrastructure of Cell Line Resource (Wuhan, China). BV_2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% P/S in a humidified chamber under 37 °C and 5% CO_2 atmosphere. In the subsequent experiments, the cells were pretreated with the indicated concentrations of Pec for 1 h prior to the addition of LPS.

Cell viability

Cell Counting kit (CCK8, Beyotime, Shanghai, China) was used to detect cell viability in 96-well plates. Cells were plated in each well at a density of 1×10^5 cells/mL and treated with Pec for 24 h. After treatment, 10 μL of CCK8 was added into the cell culture medium, and then the plate was incubated for 1 h at 37 °C. The plate was detected for absorbance at 490 nm by a microplate reader, and the results were calculated by the following Equation (1):

$$\text{Viability} = (A_{(\text{experiment})} - A_{(\text{blank})})/(A_{(\text{control})} - A_{(\text{blank})}) \times 100\% \quad (1)$$

NO assay

Cells were firstly treated with Pec for 2 h and then stimulated by LPS (1 μg/mL) for 24 h. Then, the cell supernatant was added in a new 96-well plate, mixed with equal volumes of Griess reagent I and II (Beyotime, Shanghai, China), and then detected the absorbance at a wavelength of 540 nm within 10 min. Sodium nitrite was used as a standard in the assay.

Determination of ROS production

ROS production induced by LPS stimulation was determined with ROS Assay kit (Beyotime, Shanghai, China) following the instruction. Cells were treated and reacted with ROS detection reagents for 1 h. Cells were washed with PBS and observed using fluorescence microscopy (Leica Co., Ltd., Wetzlar, Germany).

Enzyme-linked immunosorbent assay (ELISA)

The BV_2 cells were stimulated by LPS (1 μg/mL) with or without Pec for 24 h, the cell supernatant was collected and centrifuged at 1,000 r/min for 5 min. Then, the supernatant was diluted with the sample dilution buffer at the appropriate ratio. The levels of TNF-α and IL-6 were examined by using ELISA kit (Neobioscience Technology Co., Ltd., Shenzhen, China) according to the manufacturer's instruction.

Real-time quantitative PCR (RT-PCR)

Cells were stimulated by LPS (1 μg/mL) with or without Pec for 6 h, total RNA of the cells was extracted by using Trizol (Life Technologies Co., Ltd., Shanghai, China) according to its protocol. Total RNA was reverse-transcribed using an All-In-One RT master mix (Applied Biological Materials Inc., Nanjing, China). Real-time quantitative PCR was performed by using AceQ Universal SYBR® qPCR master mix (Vazyme Biotech Co., Ltd., Nanjing, China) and an ABI 7500 sequence system. The primer sequences are shown in Table 1.

Western blot

After treatment, the cells were washed twice with cold PBS (pH 7.4) and lysed by RIPA lysis buffer for 5 min. Then, the samples were followed by centrifuge at 13,000 rpm for 10 min at 4 ˚C. Nuclear proteins of BV_2 cells were extracted with the nuclear/cytoplasmic protein extraction kit (Beyotime, Shanghai, China). The supernatant was collected, and concentrations were measured by Bradford assay (Biorad Co., Ltd., Hercules, CA, USA). For western blot, proteins were separated by electrophoresis on 10–15% SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The membranes were blocked with 5% skim milk for 1 h at room temperature and incubated with the indicated antibodies overnight at 4 ˚C. Subsequently, the membranes were washed with TBST three times and incubated with the secondary antibody for 1 h at room temperature. The protein bands were visualized using High Sensitivity ECL kit (Wanlei bio, Shanghai, China) by LuminesCent image analyzer (Amersham Imager 600, GE Healthcare, Little Chalfont, UK). Grayscale of each band was performed using Image J software (NIH, Bethesda, MD, USA).

Statistical analysis

The experiment data were presented as mean ± SD. Statistical analysis was carried out with one-way ANOVA followed by Turkey's test using GraphPad Prism 6.0. A P value < 0.05 was defined statistically significant. All experiments were performed at least three times.

Results

Effects of Pec on cell survival

Before determining the effects of Pec on anti-inflammation, we first examined the cytotoxicity of Pec (Figure 1A) on BV_2 microglial cells. The effect of Pec on BV_2 cell viability was evaluated by CCK8 assay. BV_2 cells were treated with vehicle (DMSO) or Pec (0.1, 1, 10, 50, 100 μM) for 24 h. As shown in Figure 1B, we found that Pec at the indicated concentrations did not affect the viability of BV_2 cells. The results indicated that the concentrations selected for further study were non-cytotoxic to BV_2 cells.

Pec suppressed the production of TNF-α and IL-6 in LPS-stimulated BV_2 cells

Inflammatory cytokines such as TNF-α and IL-6 are involved in the inflammatory process in LPS-induced BV_2 cells. We investigated whether Pec inhibited the secretion of TNF-α and IL-6. Pretreatment with or without Pec (50 μM and 100 μM) for 1 h and then treat with LPS (1 μg/mL), TNF-α and IL-6 expression was measured by RT-PCR. As shown in Figure 2C, 2D, the mRNA expression of TNF-α and IL-6 was significantly inhibited by pretreatment with Pec. On the other hand, the culture medium was collected to detect the protein level of

Table 1 Primer sequences

Genes	Forward primers	Reverse primers
GAPDH	TCGGTGTGAACGGATTTGGC	GCCGTTGAATTTGCCGTGAG
TNF-α	CAGGCGGTGCCTATGTCTC	CGATCACCCCGAAGTTCAGTAG
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC

TNF-α, tumor necrosis factor α; IL-6, interleukin 6.

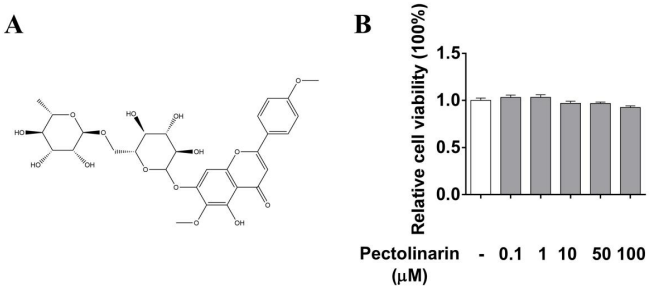

Figure 1 Effects of Pec on cell survival. (A) Chemical structure of Pec. (B) BV_2 cells were stimulated with different concentrations of Pec for 24 h and the cell viability was determined by CCK8 assay. All data were presented as means ± SD of three times.

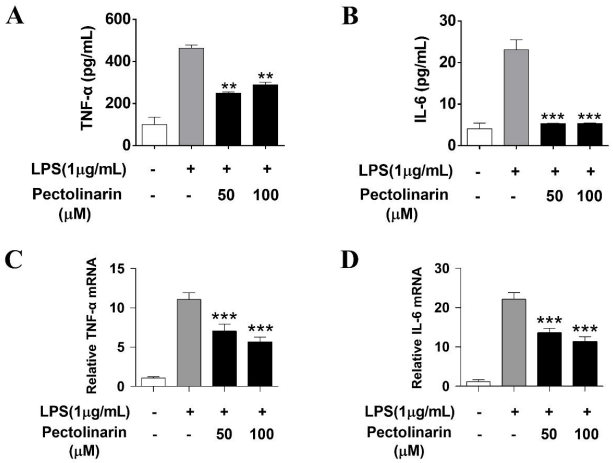

Figure 2 Effects of Pec on the production of pro-inflammatory cytokines (TNF-α and IL-6) in LPS-induced BV_2 cells. (A, B) The protein levels of TNF-α and IL-6 were determined by ELISA kits. (C, D) The mRNA levels of TNF-α and IL-6 were measured by RT-PCR. All data were presented as the mean ± SD of three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. LPS-treated group. LPS, lipopolysaccharide; TNF-α, tumor necrosis factor α; IL-6, interleukin 6.

TNF-α and IL-6 by ELISA. The results showed that Pec suppressed LPS-induced production of TNF-α and IL-6 at the protein level in BV_2 cells (Figure 2A, 2B).

Pec inhibited oxidative stress induced by LPS in BV_2 cells

In addition to pro-inflammatory cytokines release, many oxidative stress markers were also involved in inflammatory process in BV_2 cells, such as NO, inflammatory enzymes iNOS, ROS, and COX-2. LPS significantly augmented NO and iNOS production, while Pec treatment decreased the expression of NO and iNOS in BV_2 cells (Figure 3A–3C). Then, we investigated ROS production and COX-2 expression. As shown in Figure 4A, Pec significantly inhibited LPS-induced ROS production. Pec also significantly reduced the increase in COX-2 expression compared to LPS treatment (Figure 4B, 4C). These data indicated that Pec inhibited oxidative stress reactions by reducing the production of NO, iNOS, ROS, and COX-2.

Pec inhibited LPS-stimulated inflammatory response via NF-κB pathway

NF-κB plays a crucial role in the development of inflammation and regulates the expression of inflammatory cytokines and mediators. Thus, the effects of Pec on NF-κB pathway in LPS-induced BV_2 cells were investigated. As shown in Figure 5A–5C, pretreatment with Pec inhibited the degradation of IκBa and the phosphorylation of IKKα/β compared with the LPS-induced group. In addition, the level of p65 was measured by western blot. For the total protein of p65, LPS stimulation increased phosphorylation of p65. Pretreatment with Pec dramatically decreased the level of phosphorylated p65 (Figure 6A, 6C). For the nuclear translocation of p65, the level of p65 in the nucleus was significantly elevated with the treatment of LPS, whereas pretreatment with Pec obviously reduced the p65 nuclear translocation (Figure 6A, 6B). These results suggested that Pec inhibited inflammatory response in LPS-stimulated BV_2 cells via NF-κB signaling pathway.

Discussion

In recent years, several monomers have been indicated for their potential neuroprotective effects in various neurodegenerative diseases [27–30]. Pec is widely distributed in medicinal plants. It is reported to have effects of antioxidant, anti-tumor, anti-cancer, and antiviral. Moreover, Pec showed anti-inflammatory effects in animal models, which resulted in potent inhibiting like-wise carrageenan-induced mouse paw edema, arachidonic acid-induced mouse ear edema, and passive cutaneous anaphylaxis [26]. However, the effects of Pec on neuro-inflammation and the potential mechanisms are not clear. In this study, we showed for the first time an anti-inflammatory role of Pec on LPS-stimulated neuroinflammation via NF-κB signaling pathway in microglial BV_2 cells.

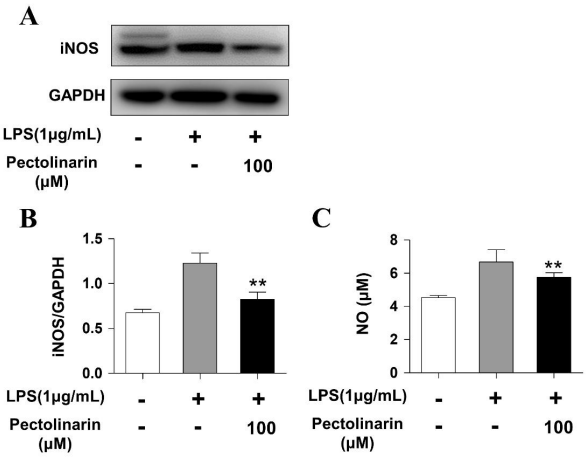

Figure 3 Effects of Pec on the production of NO and iNOS in LPS-induced BV_2 cells. (A, B) The iNOS expression was determined by western blot. GAPDH was used as an internal control. (C) The production of NO was measured by Griess reagents. All data were presented as the mean ± SD of three independent experiments. $^{**}P < 0.01$ vs. LPS-treated group. LPS, lipopolysaccharide; NO, nitric oxide.

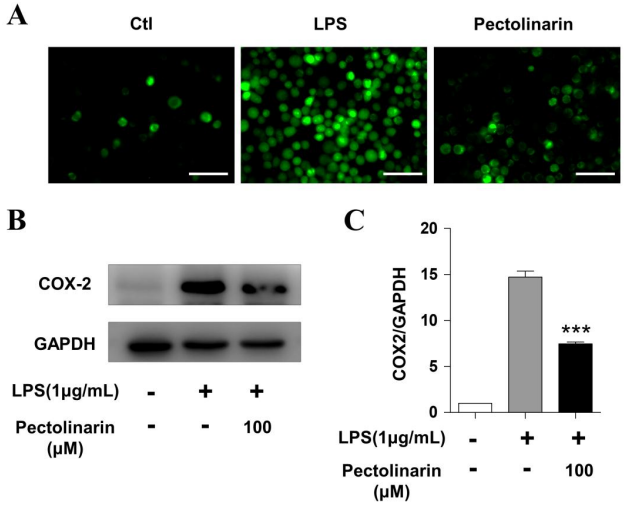

Figure 4 Effects of Pec on the production of ROS and COX-2 in LPS-induced BV_2 cells. (A) Representative pictures of ROS production (scale bar = 100 μm). (B, C) The COX-2 expression was determined by western blot. GAPDH was used as an internal control. All data were presented as the mean ± SD of three independent experiments. $^{***}P < 0.001$ vs. LPS-treated group. LPS, lipopolysaccharide.

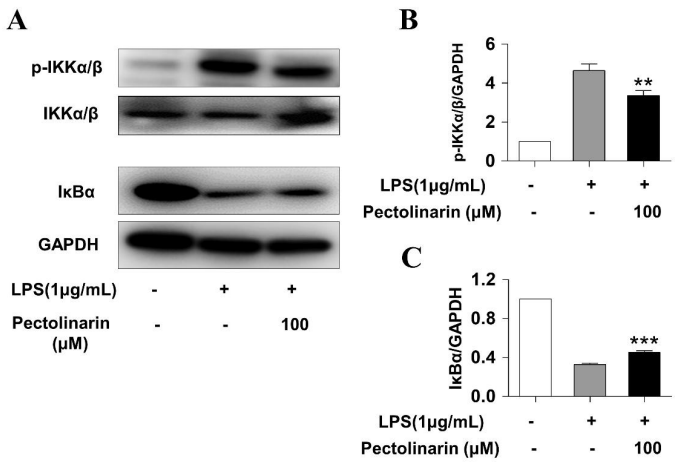

Figure 5 Effects of Pec on NF-κB signaling pathway in LPS-induced BV_2 cells. Cells were pretreated with different concentrations of Pec for 1 h, treated with 1 μg/mL LPS for 30 min. (A–C) IKKβ, IKKα/β phosphorylation, IκBα and GAPDH expression were determined by western blot. GAPDH was used as an internal control. All data were presented as the mean ± SD of three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. LPS-treated group. LPS, lipopolysaccharide; IKK, inhibitor of nuclear factor kappa-B kinase.

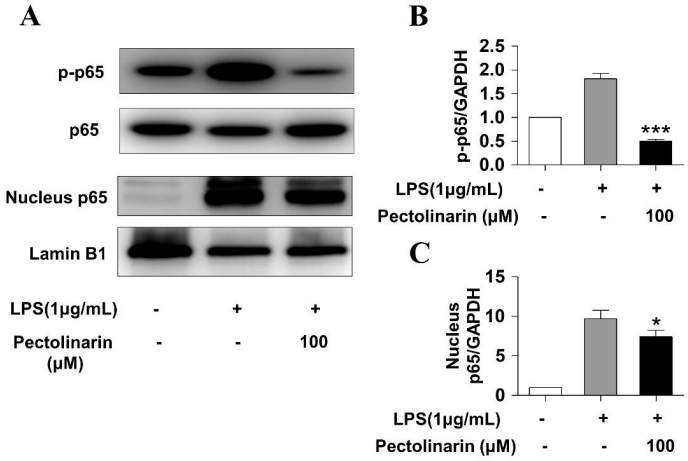

Figure 6 Effects of Pec on NF-κB signaling pathway in LPS-induced BV_2 cells. Cells were pretreated with different concentrations of Pec for 1 h, treated with 1 μg/mL LPS for 30 min. (A–C) Total p65, p65 phosphorylation, nucleus p65 and Lamin B1 expression were determined by western blot. The non-phosphorylated form of targeted protein and Lamin B1 were used as loading control. All data were presented as the mean ± SD of at least three independent experiments. $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. LPS-treated group. LPS, lipopolysaccharide.

Microglia are one of the major immune cells in the central nervous system and are responsible for resistance to infection, removal of cellular debris and microbes, and maintenance of tissue homeostasis. Microglia activation is a common feature of various neurodegenerative diseases such as PD and AD [31, 32]. Accumulating evidence suggests that over-activated microglial cells were the symbol of neuro-inflammation [33]. It is reported that microglial cells can be over activated by LPS and release a variety of inflammatory cytokines [34]. Therefore, targeting the pro-inflammatory cytokines secreted by microglial activation might be a promising therapeutic strategy to prevent or relieve neuro-inflammation. In general, over-activated microglial cells produce inflammatory cytokines such as TNF-α, IL-6, and IL-1β [35]. In this study, the results showed that the expression of TNF-α and IL-6 in LPS-stimulated microglial cells could be suppressed with the pretreatment of Pec. Activation of microglial cells also induces oxidative stress mediators such as iNOS, ROS, and COX-2. The iNOS is a major source of NO generation, which has neurotoxicity against complexes I and II in the respiratory chain and generates various deleterious reactive molecules [36]. NO generation is reduced with the decreasing of iNOS expression. In the present study, we demonstrated that Pec inhibited LPS-stimulated the production of ROS and reduced the expression of COX-2 and iNOS. These findings indicated that Pec inhibited inflammatory cytokines expression and oxidative stress reaction in LPS-stimulated microglial BV_2 cells, suggesting its potential role in the treatment of neurodegenerative diseases such as PD and AD.

A number of signaling pathways have been reported to be involved in neuro-inflammatory responses [37–39]. The NF-κB family of transcription factors is specially considered to play an important role in regulating the production of pro-inflammatory cytokines [40]. It is reported that NF-κB signaling pathway could regulate the production of TNF-α, IL-6, and IL-1β in LPS- or TNF-α-induced microglial cells. Non-activated NF-κB binds to inhibitors of the IκBα protein family and stays in the cytoplasm. With the LPS stimulation, NF-κB signaling could be activated with the IκBα kinase (IKK) activation, and the activated IKK would phosphorylate IκBα. Then, the IκBα dissociated, and the enhanced phosphorylation or degradation resulted in the downstream target p65 phosphorylation and translocation into the nucleus, which is associated with the secretion of inflammatory cytokines, such as TNF-α, IL-6, and IL-1β [41, 42]. In the present study, we found that LPS could enhance IKK and p65 phosphorylation and IκBα degradation. However, with the pretreatment of Pec, these effects could be blocked, indicating that Pec inhibits the inflammatory responses in LPS-stimulated microglial cells via NF-κB signaling pathway.

Conclusions

In conclusion, the present study demonstrated the neuro-protective effects of Pec on inhibiting the expression of pro-inflammatory cytokines and inflammatory mediators in LPS-stimulated microglial cells via NF-κB signaling pathway. As a natural flavonoid, Pec might provide a potential therapy for preventing and relieving the progression of neuro-inflammatory diseases.

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