Pectolinarin Inhibited LPS-stimulated Inflammation in Microglial BV 2 Cells via NF-κB Signaling Pathway

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Research Article

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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 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 (1% DMSO), followed by LPS. ELISA, RT-PCR, NO 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 TNF-α and IL-6 in mRNA and protein levels induced by LPS. Moreover, the production of NO, iNOS and COX-2 were suppressed by Pec in LPS-stimulated microglial BV_2 cells. In addition, Pec inhibited LPS-induced inflammation via NF-κB signaling pathway, as evidenced by reduction of the phosphorylation of IKK, the degradation of IκBα and the nuclear translocation of p65.

Conclusions: Taken together, Pec exhibited anti-inflammatory effects in LPS-stimulated microglial BV_2 cells via NF-κB signaling pathway, which might provide therapeutic potential for neuro-inflammation and neurodegenerative diseases.

1. Introduction

Neuro-inflammation is a defense mechanism to multiple exogenous stimuli and pathogens in the central nervous system (CNS) [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 [5, 6], which play a vital role in the innate immune response and represent the first line of defense against invading pathogens and pro-inflammatory reactions [7, 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 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), cyclooxygenase-2 (COX-2) and nitric oxide synthase (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

(LBP) and glycosylphosphatidylinositol-anchored protein CD14, interacts with Toll-like receptor 4 (TLR4) and activates downstream signaling pathway. Activated TLR4 activates the NF-κB signal pathway [11], which is the most frequently used to investigate the mechanism of inflammatory responses in microglia. In response to external stimulation, IκB family members can be phosphorylated and degraded, 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]. Pec is a glycosylated flavone which is first isolated from *Linaria vulgaris* [18]. Moreover, Pec has widely reported due to its presence in many medicinal plants, such as *Cirsium* [19]. According to recent studies, Pec has turned out to be good biological activities, including anti-tumor, antioxidant [20], antiviral [21, 22], anti-inflammatory [23, 24] and anti-cancer effects. As for anti-inflammatory effects, a previous study showed that Pec inhibited the acid-induced writhing in mice in a dose-dependent manner [25]. 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.

2. Materials And Methods

2.1. Materials

Pectolinarin was purchased from Shanghai Yuanye Bio-Technology Co.,Ltd (Shanghai, China). LPS was obtained from Sigma (St. Louis, MO, USA). Dulbecco' modified Eagle's medium (DMEM) was obtained from Hyclone (Shanghai, China). Fetal bovine serum (FBS) and penicillin-streptomycin (P/S) were supplied from Gibco (Gaithersburg, MD, USA). PTGS2, iNOS, β-actin, P65, p-P65, IKKβ, p-IKKα/β, Lamin B1 antibodies and relative secondary antibodies were obtained from Cell Signaling Technology (Boston, MA, USA).

2.2. Cell culture and treatment

Murine microglial cell line BV_2 was provided by the National Infrastructure of Cell Line Resource (Wuhan University, China). BV_2 cells were cultured in DMEM supplemented with 10% FBS (v/v) and 1% P/S (v/v) 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 2 h prior to the addition of LPS (1μg/mL).

2.3. Cell viability

Cell Counting Kit (CCK8, MedChemExpress, 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 CCK-8 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 formula:

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

2.4. NO assay

Cells were firstly treated with Pec for 2 h and then stimulated by LPS (1μg/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.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The BV_2 cells were stimulated by LPS (1μg/mL) with or without Pec for 24 h, the cell supernatant was collected and centrifuged at 1000 r/min for 5 min. Then, the supernatant was diluted with the sample dilution buffer at appropriate ratio. The levels of TNF-α and IL-6 were examined by using ELISA Kit (Neobioscience Technology Co., Ltd., China) according to the manufacturer's instruction.

2.6. Real-time quantitative PCR (RT-PCR)

Total RNA of the cells was extracted by using Trizol (Life Technologies, 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 were shown in Table 1.

Table 1
Primer sequences

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

2.7. 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 followed by centrifuge at 13000 rpm for 10 min at 4°C. Nuclear proteins of BV_2 cells were extracted with the nuclear/cytoplasmic protein extraction kit (Beyotime biotechnology, Shanghai, China). The supernatant was collected, and concentrations were measured by Bradford assay (Biorad, CA, USA). For Western blot, proteins were separated by electrophoresis on 10–15% SDS-PAGE

and transferred to a polyvinylidene fluoride (PVDF) 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). Grayscale of each band was performed using Image J software (NIH, USA).

2.8. Statistical analysis

The experiment data were presented as Mean ± SD. The statistical significance was analyzed by one-way analysis of variance by GraphPad Prism 6.0. Differences were considered significant at the 95% confidence level (p□0.05). All experiments were performed at least three times.

3. Results

3.1. Effects of Pec on cell survival

Before determining the effects of Pec on anti-inflammation, we first examined the cytotoxicity of Pec (Fig. 1A) on BV_2 microglial cells. The effect of Pec on BV_2 cell viability was evaluated by CCK-8 assay. BV_2 cells were treated with vehicle (DMSO) or Pec (0.1, 1, 10, 50, 100 μM) for 24 h. As shown in Fig. 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.

3.2. 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 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) for 24 h, TNF-α and IL-6 expression was measured by RT-PCR. As shown in Fig. 2C and D, 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 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 (Fig. 2A and B).

3.3. Pec inhibited the expression of NO and COX-2 induced by LPS in BV_2 cells

In addition to pro-inflammatory cytokines release, many inflammatory mediators were also involved in inflammatory process in BV_2 cells, such as NO, and inflammatory enzymes iNOS, COX-2. To examine the effects of Pec on NO production of LPS-stimulated BV_2 cells, the supernatant was tested by Griess regents. As shown in Fig. 3C, LPS significantly augmented NO production, while Pec treatment decreased the expression of NO in BV_2 cells. Then, we investigated the expression of iNOS and COX-2, Pec

significantly reduced the LPS-stimulated increase of iNOS (Fig. 3A and B) and COX-2 (Fig. 4) compared with LPS treatment. These data indicated that Pec inhibited the accumulation of NO by regulating the iNOS and COX-2 expression, and it might be a potential inhibitor of microglial activation.

3.4. 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 Fig. 5A-C, 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 total protein of p65, LPS stimulation increased phosphorylation of p65. Pretreatment with Pec dramatically decreased the level of phosphorylated p65 (Fig. 6A and C). 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 (Fig. 6A and B). These results suggested that Pec inhibited inflammatory response in LPS-stimulated BV_2 cells via NF-κB signaling pathway.

4. Discussion

In recent years, several monomers have been indicated for their potential neuroprotective effects in various neurodegenerative diseases [26–29]. 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 [25]. However, the potential mechanism is not clear. In this study, we investigated the anti-inflammatory effects of Pec against LPS-stimulated neuro-inflammation via NF-κB signaling pathway in microglial BV_2 cells.

Accumulating evidence suggests that over-activated microglial cells were the symbol of neuro-inflammation [30]. It is reported that microglial cells can be over activated by LPS and release a variety of inflammatory cytokines [31]. 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 [32]. 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 induce inflammatory mediators such as COX-2 and iNOS. The iNOS is a major source of NO generation, which has neurotoxicity against complex I and II in the respiratory chain and generates various deleterious reactive molecules [33]. NO generation is reduced with the decreasing of iNOS expression. Our results showed that Pec inhibited the expression of COX-2 and iNOS in LPS-stimulated microglial cells. These findings suggest that Pec could inhibit the expressions of inflammatory cytokines and mediators in LPS-stimulated microglial cells.

A number of signaling pathways have been reported to involve in neuro-inflammatory responses [34–37]. The NF-κB family of transcription factors is specially considered to play an important role in regulating the production of pro-inflammatory cytokines [38]. 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 bounds to the inhibitor of IκBα family protein and is stayed in cytosol. 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 [39, 40]. 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.

5. 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.

Abbreviations

Pec, pectoinarin; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor α; IL-6, interleukin 6; NO, nitric oxide; iNOS, inducible Nitric Oxide synthase; COX-2, cyclooxygenase 2; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; IKK, inhibitor of nuclear factor kappa-B kinase; IκBα, inhibitor of NF-κB; CNS, central nervous system; PD, Parkinson’s disease; AD, Alzheimer’s disease; LBP, LPS-binding protein ; TLR4, Toll-like receptor 4; RT-PCR, Real-time quantitative PCR; IL-1β, interleukin-1β; DMEM, Dulbecco’ modified Eagle’s medium; FBS, Fetal bovine serum; PTGS2, prostaglandin-endoperoxide synthase 2; ELISA, enzyme-linked immunosorbent assay; PVDF, polyvinylidene fluoride.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data used during this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that there are no conflicts of interest.

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Authors’ contributions

B.F.C. and H.J.Y.: experimental design, manuscript wring and editing; X.F.: experimentation, data analysis and manuscript writing; S.Q.J. and H.H.Y.: data collection and analysis; J.J.L., T.M. and J.Z.: collection and/or assembly of data; L.W. and Y.D.Z.: data analysis and interpretation.

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Figures

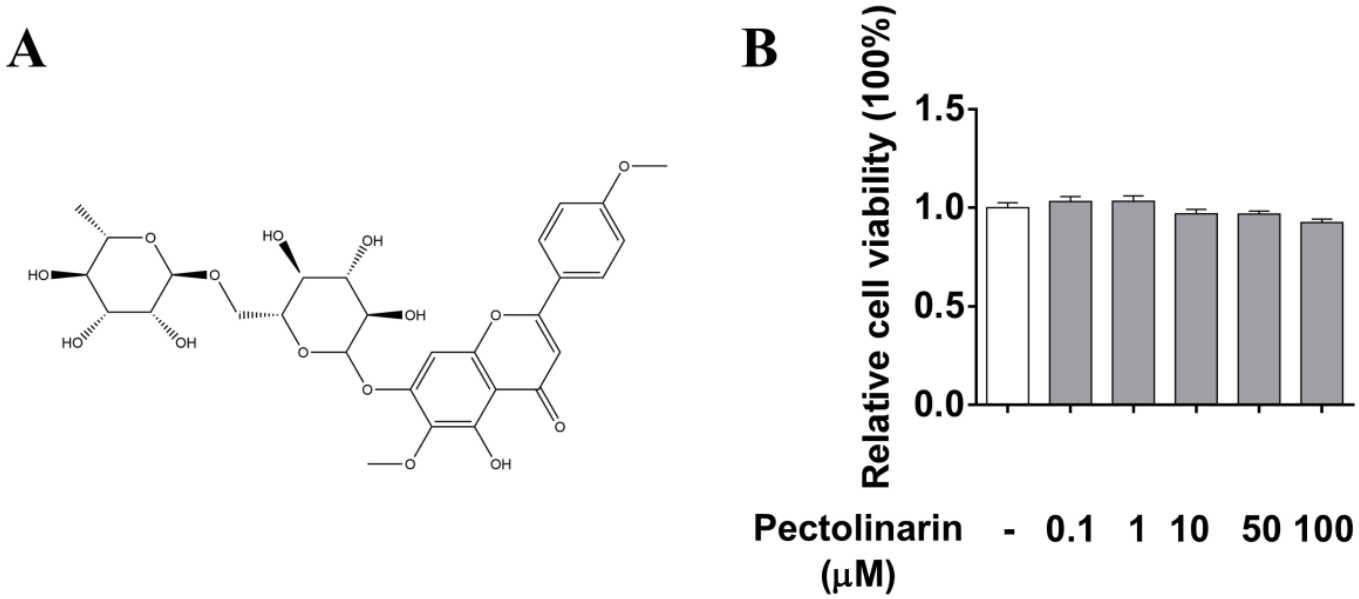

Figure 1

Effects of Pec on cell survival. (A) Chemical structure of Pec. (B) BV2 cells were stimulated with different concentrations of Pec for 24h and the cell viability was determined by CCK8 assay. All data were

presented as means ± SD of three times.

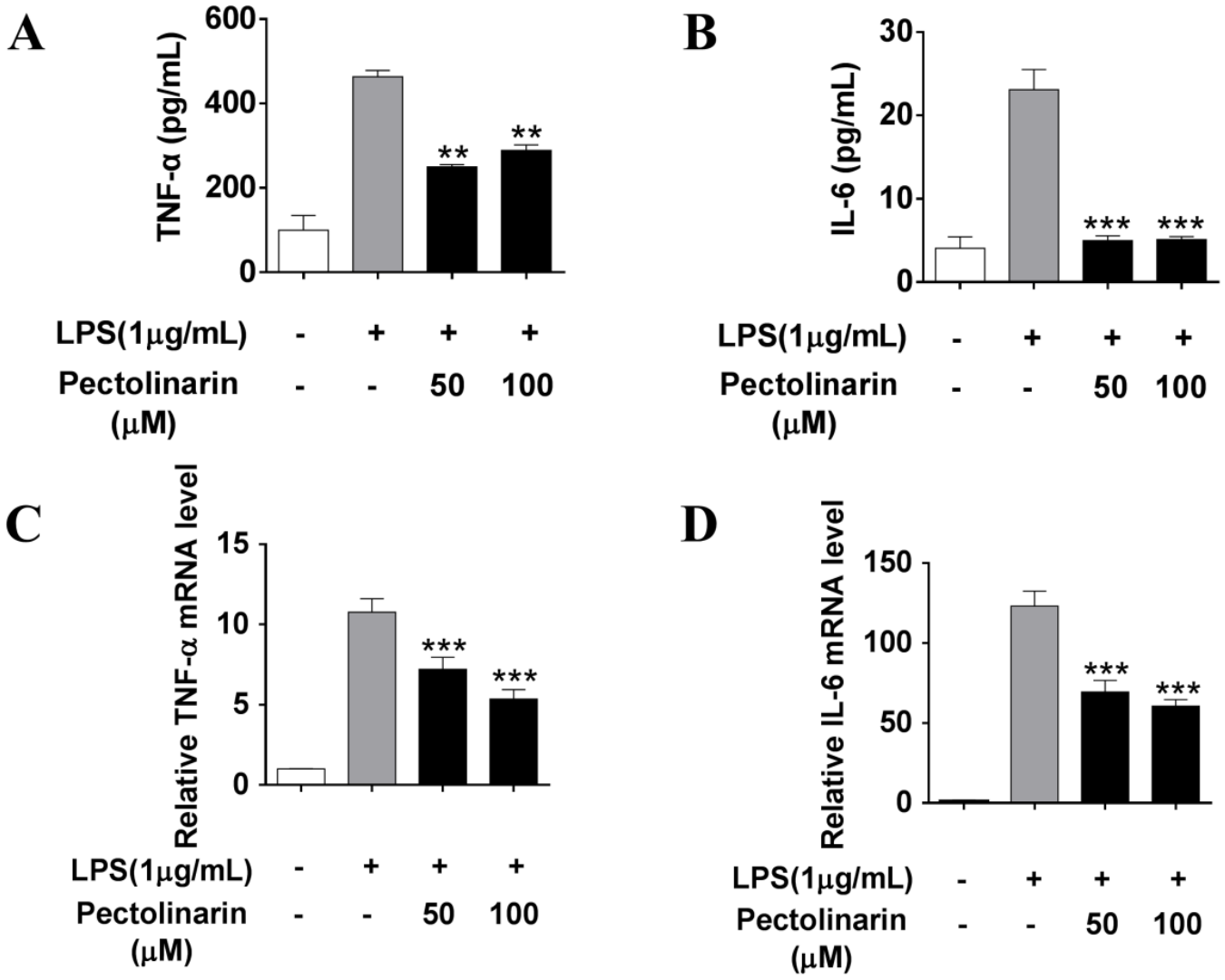

Figure 2

Effects of Pec on the production of pro-inflammatory cytokines (TNF-α and IL-6) in LPS-induced BV2 cells. Cells were pretreated with Pec of different concentrations for 1h, then treated with 1μg/mL LPS for 24h. (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<0.05, **p<0.01 and ***p<0.001 vs. LPS-treated group.

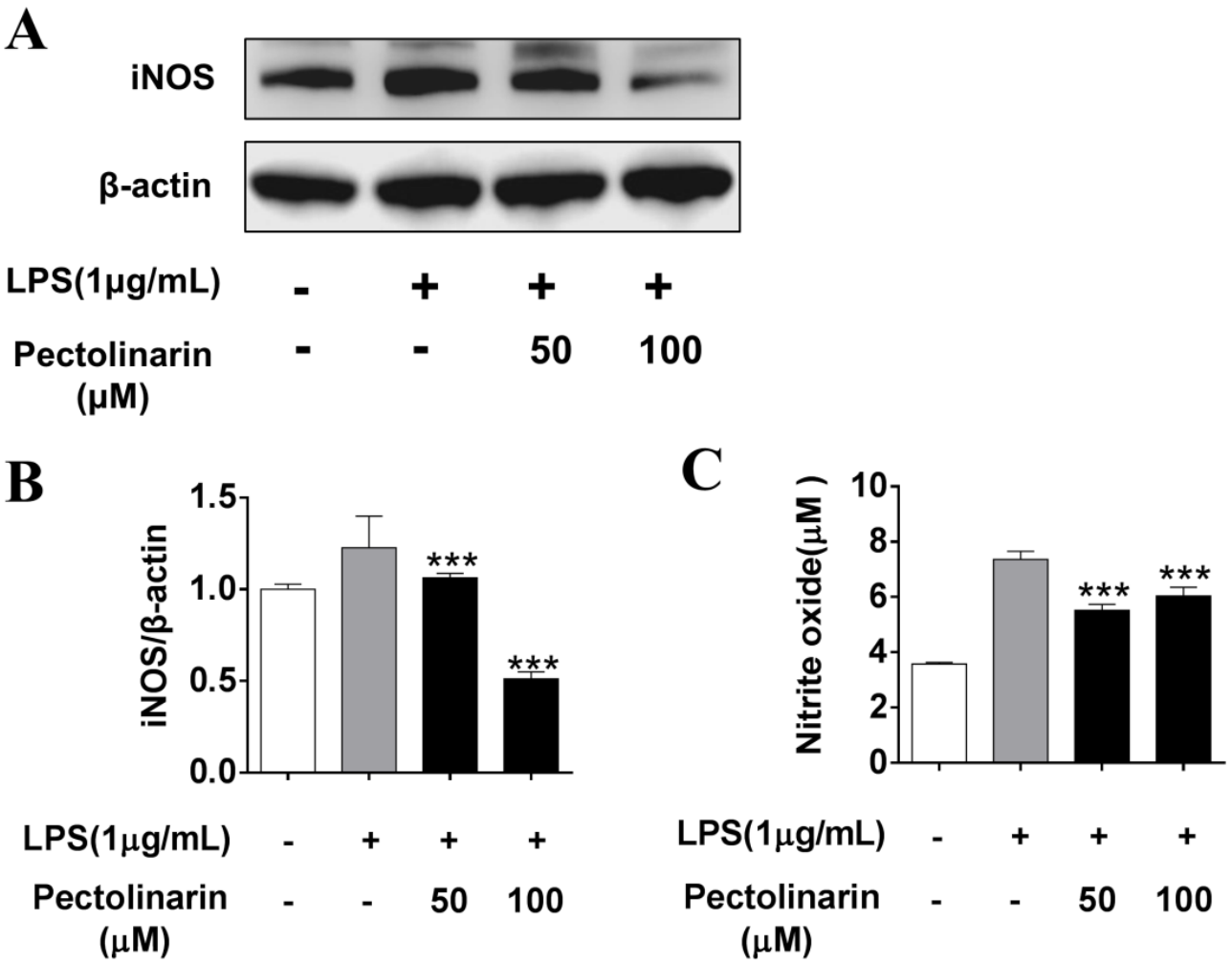

Figure 3

Effects of Pec on the production of NO and iNOS in LPS-induced BV2 cells. Cells were pretreated with different concentrations of Pec for 1h, then treated with 1μg/mL LPS for 24h. (A, B) The iNOS expression was determined by Western blot. β-actin 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<0.05, **p<0.01 and ***p<0.001 vs. LPS-treated group.

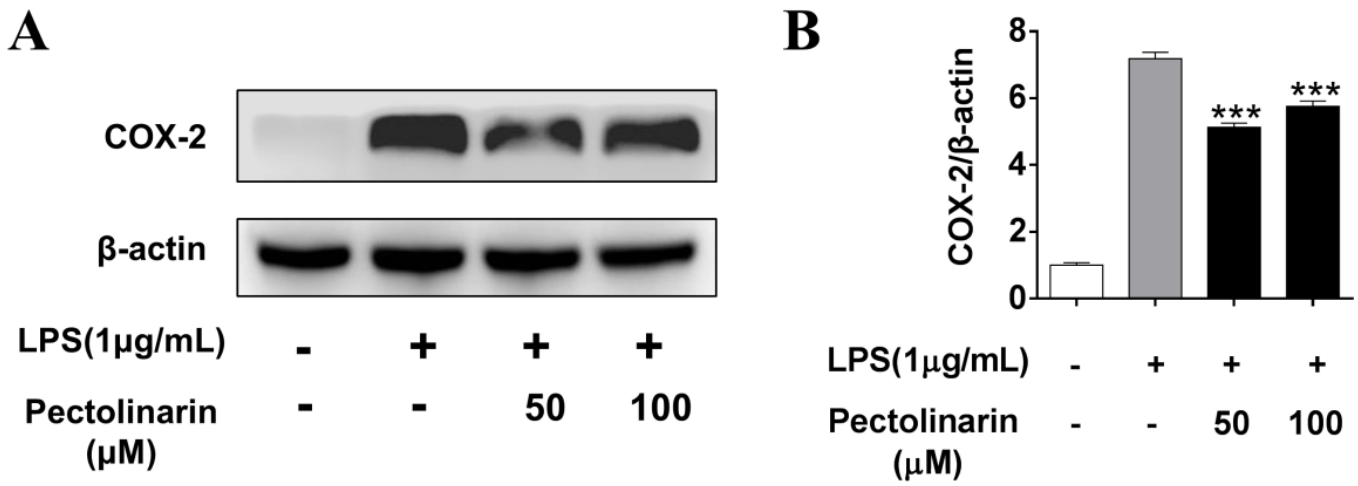

Figure 4

Effects of Pec on the production of COX-2 in LPS-induced BV2 cells. Cells were pretreated with different concentrations of Pec for 1h, then treated with 1μg/mL LPS for 24h. (A, B) The COX-2 expression was determined by Western blot. β-actin was used as an internal control. All data were presented as the mean ± SD of three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 vs. LPS-treated group.

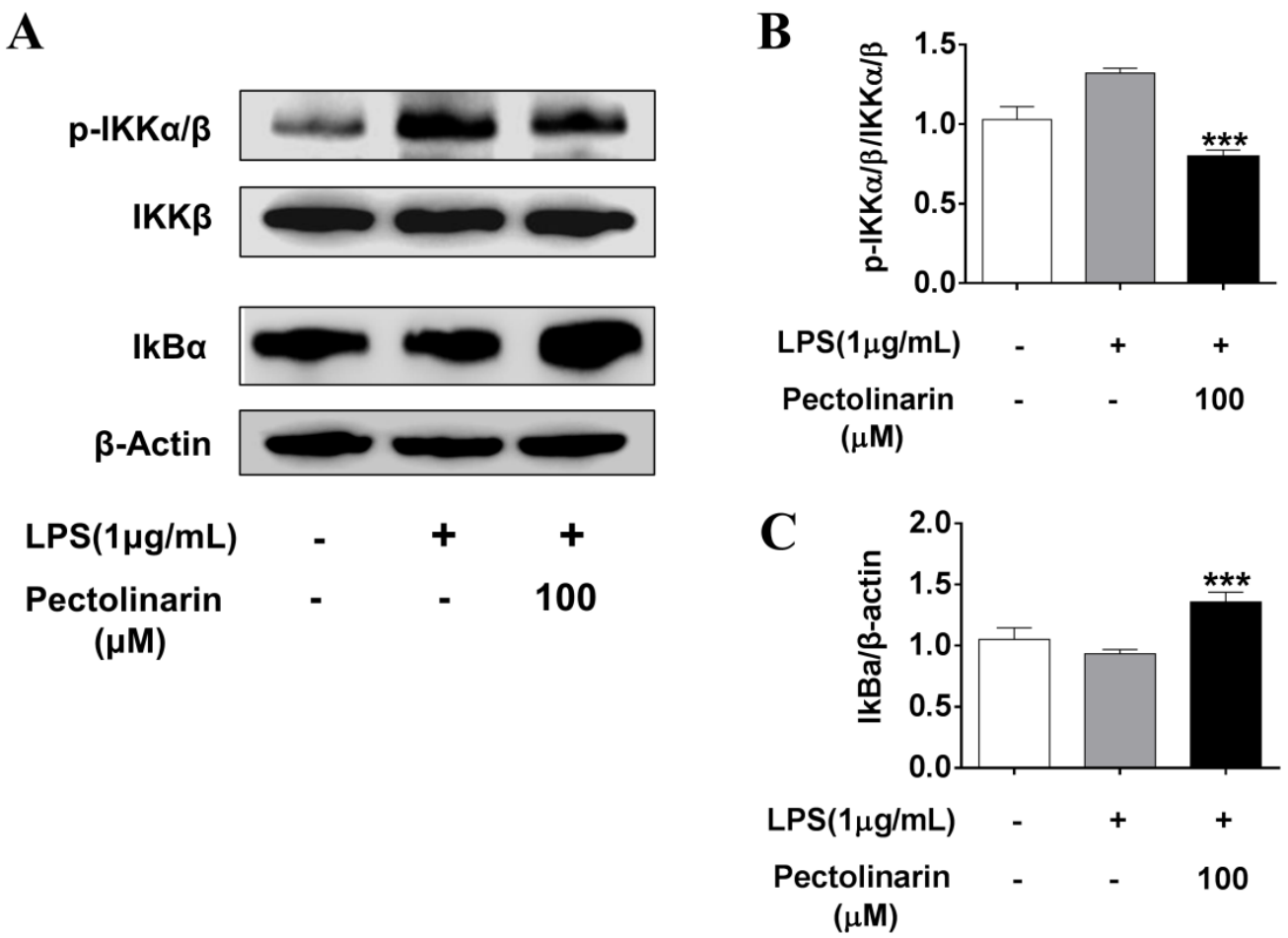

Figure 5

Effects of Pec on NF-κB signaling pathway in LPS-induced BV2 cells. Cells were pretreated with different concentrations of Pec for 1h, then treated with 1μg/mL LPS for 24h. (A-C) IKKβ, IKKα/β phosphorylation, IκBα and β-actin expression were determined by Western blot. The non-phosphorylated form of each targeted protein was used as loading control, β-actin was used as an internal control. All data were presented as the mean ± SD of three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 vs. LPS-treated group.

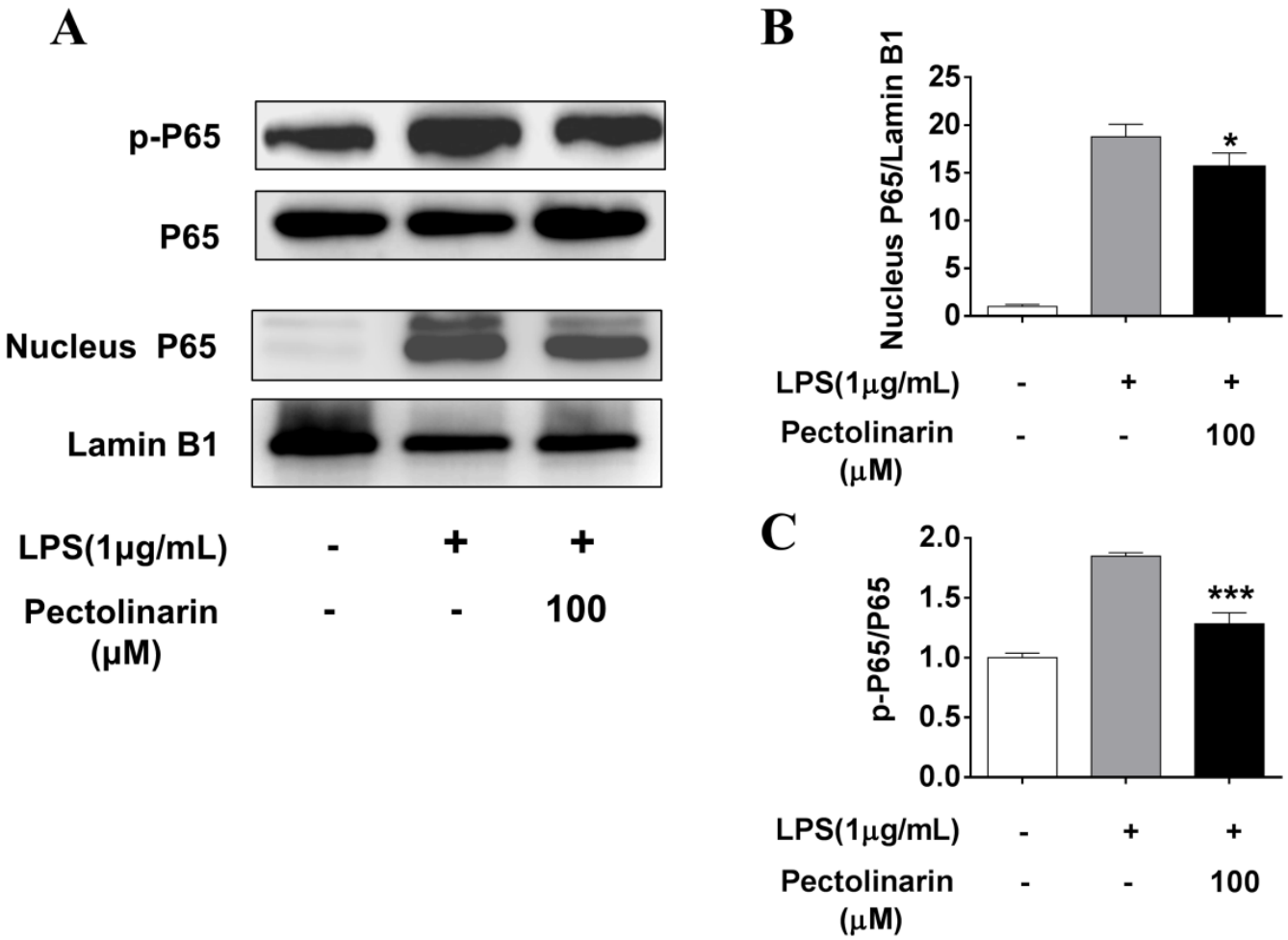

Figure 6

Effects of Pec on NF-κB signaling pathway in LPS-induced BV2 cells. Cells were pretreated with different concentrations of Pec for 1h, then treated with 1μg/mL LPS for 24h. (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, **p<0.01 and ***p<0.001 vs. LPS-treated group.

Supplementary Files

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- supplementaryinformation.pdf