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Research Article

Physalin B Suppresses Inflammatory Response to Lipopolysaccharide in RAW264.7 Cells by Inhibiting NF-κB Signaling

Yanjun Yang,[1] **Lang Yi**,[2] **Qing Wang**,[2] **Bingbing Xie**,[2] **Congwei Sha**,[3] **and Yan Dong**[2]

[1]*Department of Pharmacy, Guangdong Food and Drug Vocational College, Tianhe District, Guangzhou 510520, China*
[2]*Department of Immunology, Institute of Clinical Pharmacology, Guangzhou University of Chinese Medicine, Baiyun District, Guangzhou 510405, China*
[3]*Guangdong Provincial Institute of Biological Products and Materia Medica, Baiyun District, Guangzhou 510440, China*

Correspondence should be addressed to Yanjun Yang; ncyyj@163.com and Yan Dong; 1462523594@qq.com

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Physalin B from *Physalis angulata* L. (*Solanaceae*) is a naturally occurring secosteroid with multiple biological activities. But its anti-inflammatory activity and mechanism remain unclear. Physalin B effects on RAW264.7 macrophages stimulated by lipopolysaccharide (LPS) were observed in this study. The expression and secretion of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) induced by LPS were significantly inhibited by physalin B. Meanwhile, the NF-κB nuclear translocation induced by LPS was inhibited by physalin B. The anti-inflammatory effects of physalin B could not be inhibited by mifepristone (RU486), the blocker of glucocorticoid receptor. In conclusion, physalin B can suppress inflammatory response to LPS in macrophages by inhibiting the production of inflammatory cytokines via NF-κB signaling.

1. Introduction

Inflammation is a fundamental pathological phenomenon and a complex biological response participating in the development of diverse diseases [1–4]. Inflammation is usually mediated by eicosanoids and cytokines released by injured or infected cells, especially activated immunocytes. TNF-α and IL-6 are key inflammatory cytokines in macrophages activated by LPS [5, 6]. Although helpful to combat against infection, vigorous inflammatory cytokines may lead to edema, cellular metabolic stress, and tissue necrosis. NF-κB, an immediate early transcriptional activator, plays a central role in inflammatory response by binding with the promoters to induce transcription of proinflammatory genes, such as TNF-α and IFN-γ [7]. NF-κB signaling is well known to be involved in various diseases, including inflammation and cancers, and thus has attracted attention as a drug target [8].

Physalin B, a naturally occurring secosteroid isolated from the stems and aerial parts of *Physalis angulata* L. (*Solanaceae*) (Figure 1), possesses a unique 13,14-seco-16,24-cycloergostane skeleton, an H-ring with a C_{14}–O–C_{27} bond and a cage-shaped structure, with a highly oxygenated, complex structure similar to glucocorticoid. In addition to the intriguing structure, there is considerable interest in the biological activities of physalin B. In the experiments *in vivo*, the physalin B anti-inflammatory effect appeared to be mostly due to the activation of glucocorticoid receptors, which represented novel therapeutic options for the treatment of inflammatory diseases [9]. Physalin B was thought to have the potential to be an effective chemotherapeutic lead compound for the treatment of malignant melanoma [10]. It showed strong cytotoxicity against multiple tumor cell lines [11] and antimitotic activity for the first cleavage [12] and inhibited the growth of several human leukemia cells [13]. Physalin B was considered responsible for the antimicrobial activity, at the concentration of 200 μg/ml, and physalin B exhibited about 85% of the inhibitory activity observed with the mixture of physalins (pool) containing physalins B, D, F, and G, at the

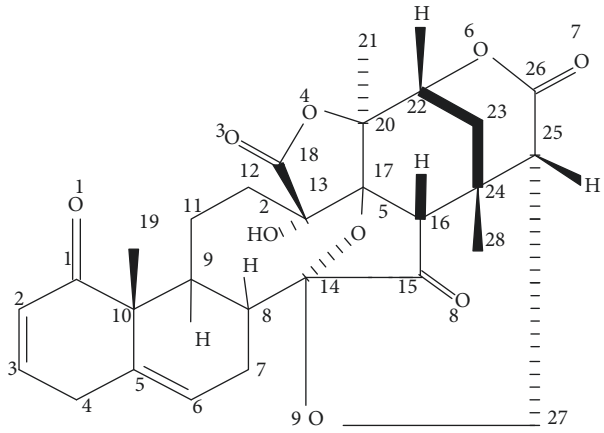

Figure 1: The structure of physalin B.

same concentration [14]. Physalin B exhibited a minimum inhibitory concentration (MIC) value (128 μg/ml) against *Mycobacterium tuberculosis* H37Rv strain [15]. Thus, physalin B has the potential to regulate a broad range of biological events. However, the underlying mechanisms of physalin B remain largely unknown.

This study observed the anti-inflammatory underlying mechanism of physalin B in RAW264.7 macrophages stimulated by LPS. The roles of the nuclear factor kappa B (NF-κB) and the glucocorticoid receptor in physalin B anti-inflammatory effect were analyzed. The study might provide evidence for physalin B as the lead structure of anti-inflammatory drugs.

2. Materials and Methods

2.1. Plant Material. Physalin B was isolated from *Physalis angulata* (*Physalis angulata* L.). The herb was collected from Puning in Guangdong Province by Mr. Wen-Biao Chen and identified by Dr. Qing-Qian Zeng. The specimens (GICMM number 5) were stored in the Guangdong Institute of Chinese Materia Medica.

2.2. Chemicals and Reagents. The isolated compound was analyzed by HR mass spectrometry, and its purity was found to be 100% for small-molecule single-crystal X-ray diffraction analysis. LPS (from *Escherichia coli* 0111 : B4) was purchased from Sigma (St. Louis, USA). The monoclonal antibodies IκB (4814S), NF-κB p65 (6956S), and β-actin (2118S) were purchased from Cell Signaling Technology (CST, USA). Mouse TNF-α (E09483-1643) and IL-6 (E09362-1643) ELISA detection kits were purchased from eBioscience (California, USA). TRIzol (15596026) was purchased from Invitrogen. Reverse Transcription Kit (135600) and SYBR Green Quantitative PCR Kit (262000) were purchased from Toyobo (Osaka, Japan). The ECL chemiluminescence detection kit was purchased from Thermo (Waltham, MA, USA).

2.3. Isolation and Purification of Physalin B. The air-dried and milled whole plants of *P. angulata* (8 kg) were extracted with ethanol (85%) three times (3 × 30 L) under immersion, for 1 week each. After filtration and evaporation of the solvent under reduced pressure, the combined crude ethanolic extract (1067 g) was mashed and then dissolved successively with petroleum ether, EtoAc, and *n*-BuOH to afford dried petroleum ether-soluble (101 g), EtoAc-soluble (49 g), and *n*-BuOH-soluble (48 g) fractions, respectively. Accordingly, the EtoAc-soluble extract was subjected to medium-pressure column chromatography over silica gel (LC60A 40–63 micron) and eluted using a step gradient of a petroleum ether and EtoAc solvent system (100 : 0, 100 : 1, 80 : 1, 50 : 1, 25 : 1, 10 : 1, 5 : 1, 3 : 1, 2 : 1, 1 : 1) at a flow rate of 50 ml·min^{-1}, pressure 20 bar, to obtain ten fractions (F1–F10) based on the TLC profile. Each fraction was concentrated in vacuo. Further purification of subfraction F7 (petroleum ether and EtoAc solvent system: 5 : 1) by repeated column chromatography over silica gel (LC60A 40–63 micron) with petroleum ether-EtoAc (100 : 0 to 1 : 1) followed by thin-layer chromatography gave transparent crystals, physalin B, suitable for X-ray diffraction analysis (80.0 mg).

Physalin B, colorless prismatic crystals (petroleum ether-EtoAc), mp253-254°C. HR-EI: m/z 510.1883($C_{28}H_{30}O_9$, calcd. for 510.1884), ^{1}H-NMR (400 MHz, acetone-d6)δ: 6.92 (^{1}H, m, H-3), 6.50(^{1}H, s, 13-OH), 5.86(^{1}H, dd, J = 10.4, 2 Hz, H-2), 5.61 (^{1}H, brd, J = 6 Hz, H-6), 4.56(^{1}H, brs, H-22), 4.40 (^{1}H, dd, J = 13.6, 4.6 Hz, H-27), 3.76(^{1}H, d, J = 13.6, H-27), 1.88(^{3}H, s, CH_3-21), 1.31(^{3}H, s, CH_3-28), 1.19 (^{3}H, s, CH_3-19).^{13}C-NMR (100 MHz, acetone-d6)δ: 205.3(C-1), 127.7 (C-2), 147.3(C-3), 33.3(C-4), 136.2(C-5), 124.7(C-6), 25.4 (C-7), 41.1(C-8), 34.4(C-9), 53.6(C-10), 25.2(C-11), 26.3 (C-12), 80.0(C-13), 107.8(C-14), 209.7(C-15), 56.1(C-16), 81.9(C-17), 172.7(C-18), 17.6(C-19), 81.5(C-20), 22.0(C-21), 77.6(C-22), 32.8(C-23), 31.6(C-24), 50.9(C-26), 167.5(C-26), 61.7(C-27), 25.6(C-28).

2.4. Cell Culture. The culture of RAW264.7 cells was obtained the way we had established in our lab [16].

2.5. Cell Viability. RAW264.7 cells (1 × 10^5/ml) were seeded in a 96-well plate and incubated at 37°C overnight. Cells were stimulated with various concentrations of physalin B for 24 h, and PBS was used as vehicle control. At the end of the incubation, 10 μl of MTT (5 mg/ml in PBS) solution was added and incubated for an additional 2 h. After DMSO solubilized the formazan crystals, it was measured using an enzyme-linked immunosorbent assay (Molecular Devices, Sunnyvale, CA) at 595 nm. The relative cell viability was calculated and compared with the absorbance of the untreated control group.

2.6. Detection of TNF-α and IL-6 Release. RAW264.7 cells were treated with positive drug (1 μM dexamethasone) or different concentrations of physalin B (20, 10, and 5 μM) for 2 h and then stimulated with 1 μg/ml LPS for 8 h. PBS was used as the control. After the incubation, the culture supernatant was collected. Concentration of inflammatory cytokines (TNF-α and IL-6) was analyzed by ELISA.

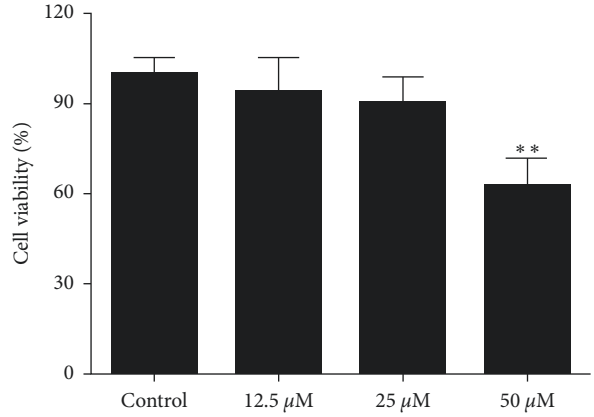

Figure 2: Effects of physalin B on cell viability of RAW264.7 macrophages. Each value indicated the mean ± SEM and was representative of results obtained from three independent experiments. $^{**}P < 0.01$ versus control.

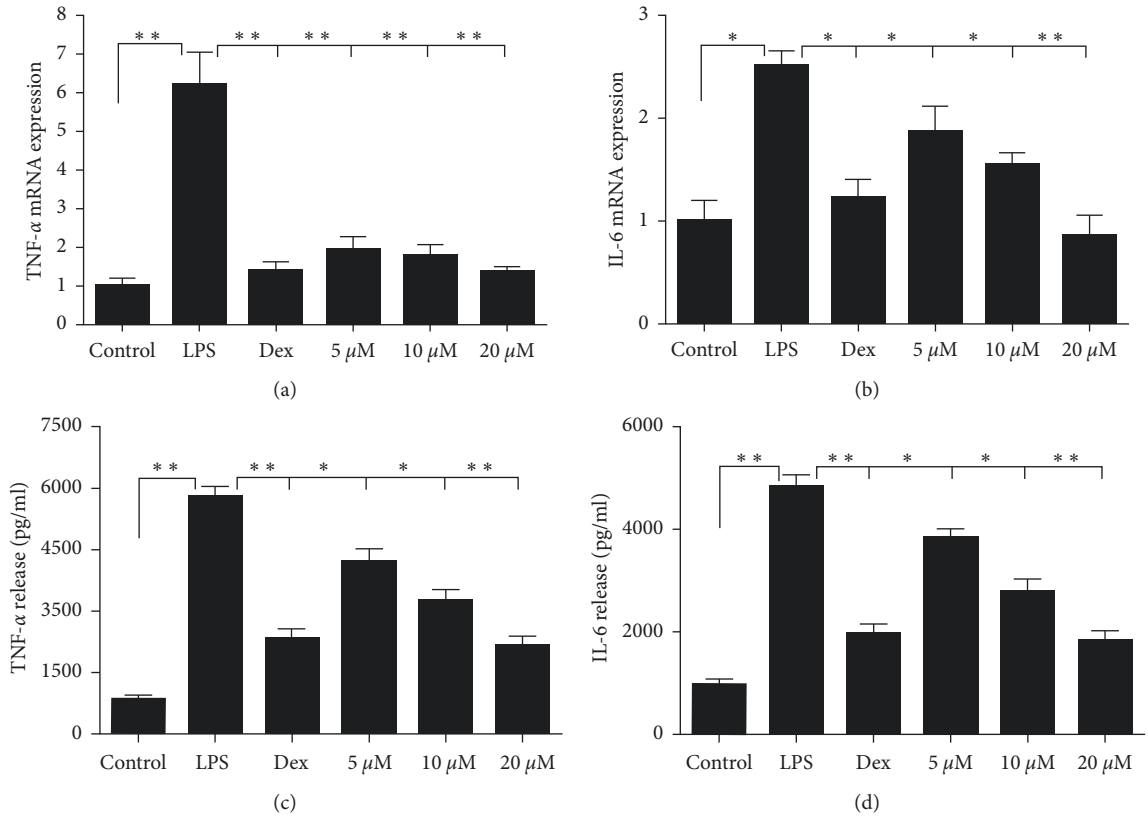

Figure 3: Effects of physalin B on LPS-induced TNF-α and IL-6 expression. RAW264.7 cells were treated with different concentrations of physalin B (5, 10, and 20 μM) for 2 h and then stimulated with 1 μg/ml LPS for 8 h. (a, b) Total cellular RNA was extracted and reverse transcribed. The relative mRNA levels of TNF-α and IL-6 were detected by real-time PCR as described in Materials and Methods. GAPDH was used as a loading control. (c, d) The TNF-α and IL-6 protein levels were detected by ELISA. Each value indicated the mean ± SEM and was representative of results obtained from three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

2.7. Real-Time PCR for TNF-α and IL-6 Assay. RNA was extracted from RAW264.7 cells by TRIzol method. cDNA synthesis and the quantitative PCR were obtained as described in the previous study of our lab [17].

2.8. Western Blotting for IκBα and NF-κB p65 Protein Assay. For Western blotting assay, we used KeyGEN Nuclear and Cytoplasmic Protein Extraction Kit to extract NF-κB p65 and IκBα protein and BCA protein assay kit to measure the protein concentrations. Protein samples (50 μg) were fractionated by 10% SDS-PAGE and transferred onto PVDF membranes (Bio-Rad). Nonspecific reactivity was blocked by 5% BSA for 2 h at room temperature, followed by primary antibodies for anti-mouse NF-κB p65, IκBα, GAPDH diluted to 1 : 1000 and then by goat anti-mouse HRP-conjugated secondary antibody at 1 : 15000. The specific proteins were

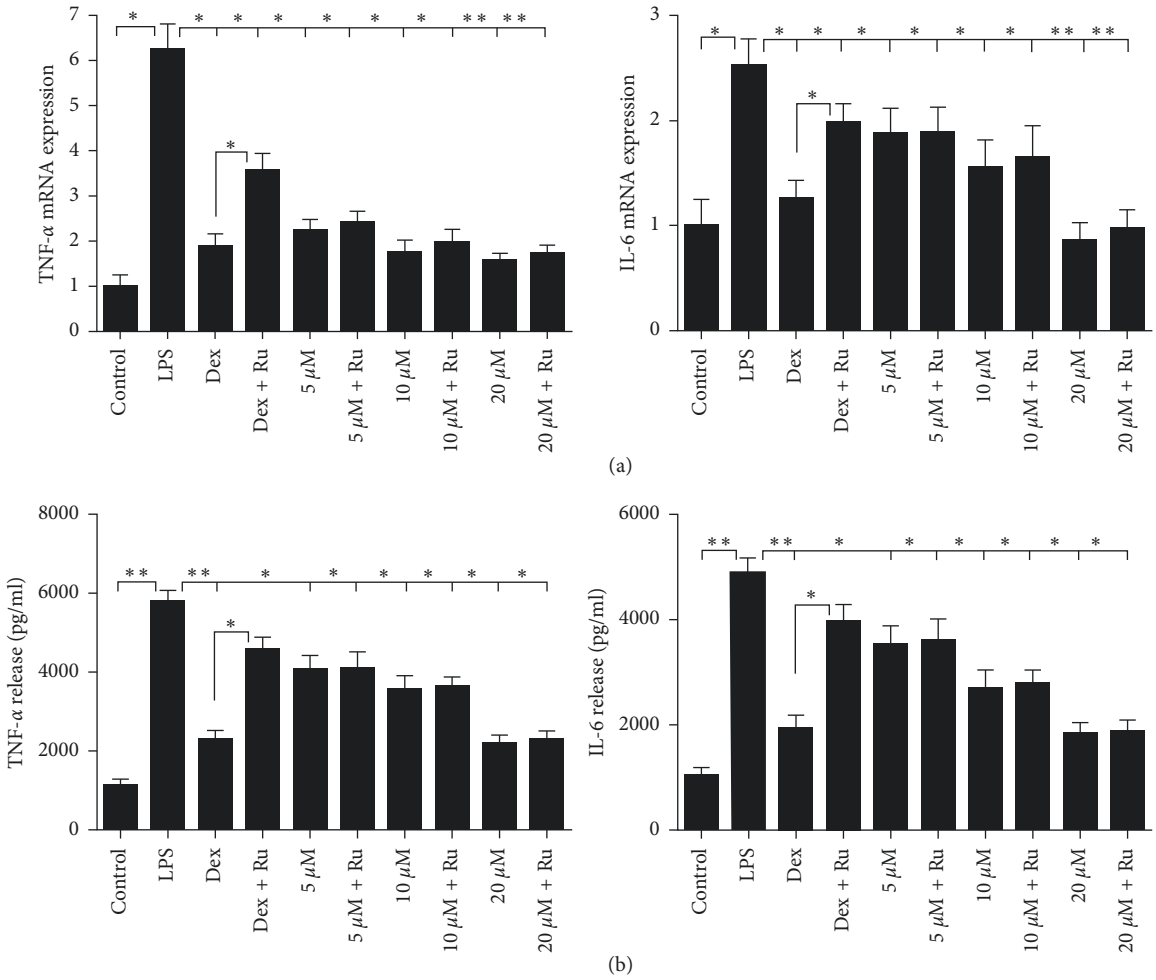

Figure 4: Effects of GR antagonist on the inhibition of TNF-α and IL-6 by physalin B. RAW264.7 cells were treated with different concentrations of physalin B (5, 10, and 20 μM) or Dex (1 μM) for 2 h with/without 1 h pretreatment of RU486 (10 μM) and then stimulated with 1 μg/ml LPS for 8 h. (a, b) Total cellular RNA was extracted and reverse transcribed. The relative mRNA levels of TNF-α and IL-6 were detected by real-time PCR as described in Materials and Methods. GAPDH was used as a loading control. (c, d) The TNF-α and IL-6 protein levels were detected by ELISA. Each value indicated the mean ± SEM and was representative of results obtained from three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

detected by exposing membranes to Kodak X-Omat films, and densitometric analysis was performed by using the Quantity One to scan the signals.

2.9. Statistical Analysis. Results were showed as mean ± SEM. One-way analysis of variance (ANOVA) and Tukey multiple comparison tests were used to analyze the data. $P < 0.05$ was considered statistically significant. All analyses were performed using SPSS 13.0 for Windows.

3. Results and Discussion

3.1. Cell Viability. In order to determine the working concentration of physalin B, RAW264.7 cells were treated with 12.5, 25, and 50 μM physalin B. We found that physalin B at 50 μM can markedly reduced the cell viability of RAW264.7 cells compared with control ($P < 0.01$), but others did not show any significant difference (Figure 2).

3.2. Effect of Physalin B on the mRNA and Protein Levels of TNF-α and IL-6. In order to evaluate the effect of physalin B on LPS stimulation, ELISA was used for determining the levels of inflammatory cytokines IL-6 and TNF-α. LPS (1 μg/ml) significantly increased the mRNA and protein levels of TNF-α and IL-6. However, dexamethasone (1 μM) significantly inhibited the mRNA and protein levels of TNF-α and IL-6 ($P < 0.01$). Physalin B decreased TNF-α and IL-6 mRNA and protein levels significantly at 5, 10, and 20 μM in a concentration-dependent manner ($P < 0.05$ or $P < 0.01$) (Figure 3).

3.3. The Effect of GR Antagonist on the Inhibition of TNF-α and IL-6 Expression by Physalin B. To analyze whether the anti-inflammatory effect of physalin B relied on the glucocorticoid receptor (GR), we chose the GR selective antagonist mifepristone (RU486). Our study showed that RU486 did not inhibit the effect of physalin B on the levels of TNF-α and IL-6

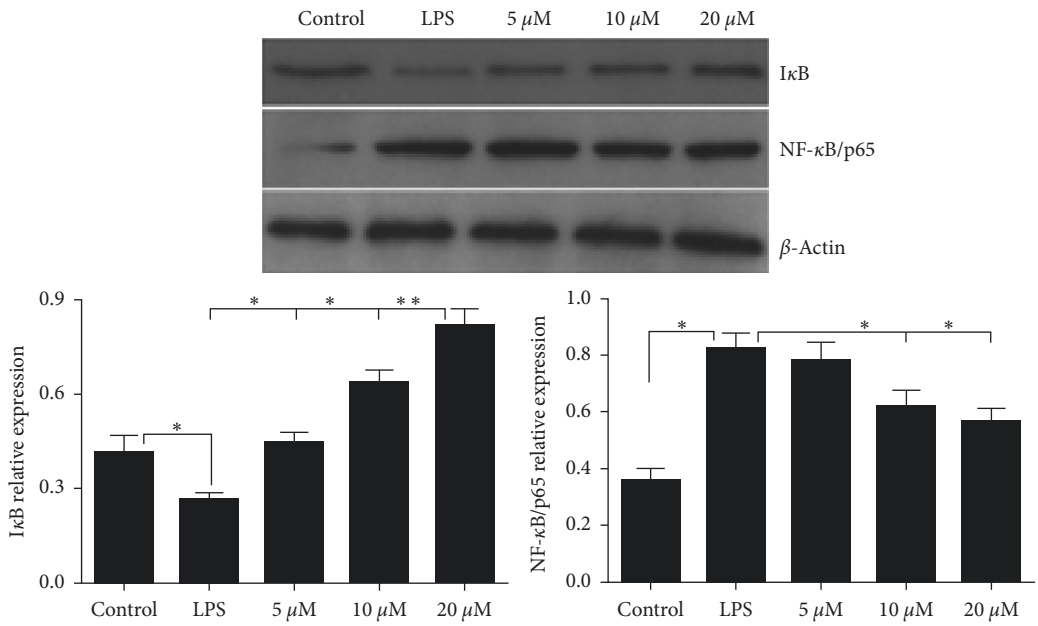

Figure 5: The effect of physalin B on LPS-induced NF-κB activation in RAW264.7 cells. RAW264.7 cells were treated with different concentrations of physalin B (5, 10, and 20 μM) for 2 h and then stimulated with 1 μg/ml LPS for 30 min. Nuclear extract was prepared, and the translocation of NF-κB subunits to nucleus was analyzed by Western blot analysis using specific antibodies. Cytoplasmic extract was also prepared, and IκB expression was analyzed. Three independent experiments were performed. $^{*}P < 0.05$.

mRNA and protein ($P > 0.05$) in contrast to dexamethasone (Figure 4).

3.4. The Effects of Physalin B on IκB and NF-κB/p65 Protein Level. Stimulation with LPS caused IκB decreasing in cytoplasm and NF-κB p65 increasing in nucleus. However, when the cells were pretreated with physalin B, the LPS effect on IκB and NF-κB p65 was reversed. Our data revealed that, in the cells cotreated with LPS and physalin B, the LPS-induced p65 was suppressed. These results demonstrated that physalin B could inhibit NF-κB activation.

Physalins share a unique 13,14-seco-16,24-cycloergostane skeleton, with a highly oxygenated and complex structure. Type B physalins, such as physalin B, have an H-ring with a C_{14}–O–C_{27} bond and a cage-shaped structure. The AB-ring of physalins that is commonly found in plant steroids was suggested to be involved in biological activities. For instance, Ma and coworkers suggested that the A-ring of physalin A could form a covalent bond with cysteine residues of IKKβ [18]. In contrast, little attention was paid to the contribution of the cage-shaped right-sided structure in Type B physalins (e.g., physalin B). Masaki et al. [19] hypothesized that the unique partial structure would play an important role in the biological activity.

Also, physalin B has a similar glucocorticoid structure. Glucocorticoids have anti-inflammatory activities with many adverse effects, such as osteoporosis, metabolic diseases, high blood pressure, and so on.

From our results, physalin B significantly inhibited the mRNA expression and secretion of TNF-α and IL-6 in macrophages induced by LPS at the concentrations without obvious cytotoxicity (Figures 2 and 3). In addition, the results showed that RU486 inhibited the anti-inflammatory effects of dexamethasone, but not physalin B in RAW264.7 cells (Figure 4). It suggests that physalin B does not require GR for the anti-inflammatory activity *in vitro*, which is different from Vieira's research [9]. Physalin B, although with secosteroidal chemical structure, did not act through the glucocorticoid receptor in macrophages, which means its structure group different from glucocorticoid is the active core.

NF-κB, an immediate early transcriptional activator, participates in inflammatory responses and acute phase through increasing the expression of immediate early inflammatory genes by binding with the promoters, including TNF-α, IFN-γ, NOS II, ICAM, and so on [20]. IκBα is the main regulator of NF-κB, and NF-κB combined with IκBα is inactivated [21]. When IκBα is degraded in cytoplasm, NF-κB can translocate to the nucleus and transcriptional activation is activated. From the results (Figure 5), the decreased IκBα protein level in cytoplasm and the increased NF-κB p65 protein level in nucleoprotein induced by LPS were reversed by physalin B. These results provide evidence that physalin B exerts anti-inflammatory effect by inhibiting NF-κB activation.

4. Conclusions

Physalin B, a naturally occurring secosteroid from *Physalis angulata* L., can inhibit inflammatory response in LPS-induced macrophages by inhibiting NF-κB activation *in vitro*. Its anti-inflammatory effect is independent on the glucocorticoid receptor. Our study suggests that physalin B could be a potential new therapeutic agent against inflammation.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that none of the authors has any kind of conflicts of interest related to the present work.

Authors' Contributions

Lang Yi, Qing Wang, and Bingbing Xie performed the cell-based assay experiments. Yanjun Yang and Congwei Sha performed the isolation and purification of physalin B. Yanjun Yang drafted the manuscript. Yanjun Yang and Yan Dong supervised and coordinated the study and revised the manuscript.

Acknowledgments

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