Based on Proteomics Reveals the Anti-inflammatory Mechanism of Phillygenin by Inhibition NF- κ B Pathway

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Research

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Abstract

Background: Inflammation is a common pathological phenomenon when homeostasis is seriously disturbed. Phillygenin (PHI) is a lignin component isolated from *Forsythiae Fructus*, which showed a good anti-inflammation effect. However, the mechanisms of PHI on anti-inflammation have not yet been systematically elucidated.

Methods: In the study, the lipopolysaccharide (LPS)-induced RAW264.7 cell inflammation model was established to investigate mechanisms of PHI on inflammation. The effect of PHI on LPS-induced IL-1 β and PGE2 inflammatory factors was detected by ELISA, and the mRNA expression of IL-1 β , IL-6 and TNF- α was detected by RT-qPCR. Proteomics studies the signaling pathways that may be affected by PHI. Molecular docking technology was used to study the possible targets of PHI on NF- κ B pathway. Western blot was performed to detect progressive changes in protein expression.

Results: The research showed that PHI significantly inhibited the robust increase of IL-1 β and PGE2, and lowered the transcriptional level of inflammatory genes including IL-6, IL-1 β and PGE2 in LPS-stimulated RAW264.7 cells. Proteomics results indicated that PHI was involved in the regulation of multiple signaling pathways. Molecular docking results indicated that PHI has an affinity for most proteins in NF- κ B pathway. Western blot analysis showed that PHI inhibited LPS-induced NF- κ B pathway activation.

Conclusion: PHI inhibits the activation of NF- κ B pathway, thereby inhibiting the expression of related inflammatory genes and the release of cytokines, thus showing remarkable anti-inflammatory effect.

1. Background

Inflammation is one of the most common clinical symptoms. It is a defense response of body to ensure removal of harmful stimuli and repair of damaged tissues[1]. When human immune cells are affected by external stimuli, some small molecule soluble proteins or peptides that can transmit information between cells and have specific immunoregulatory functions are secreted by body itself and participate in or cause inflammatory reactions. These substances are called inflammatory factors, such as interleukin (IL)-1 β , IL-6, tumor necrosis factor (TNF)- α , nitric oxide (NO) and prostaglandin E2 (PGE2)[2]. Nowadays, many studies have reported that inflammatory response can also cause some autoimmune diseases or cancer, and is closely related to many chronic diseases such as arthritis, osteoporosis[3], asthma[4], Alzheimer's disease[5], cardiovascular disease[6], cancer[7] and obesity[8] and so on .

Forsythia Fructus ("Lianqiao" in Chinese) is a kind of heat clearing and detoxifying traditional Chinese medicine and is the fruit of *Forsythia suspensa* (Thunb.) Vahl. [9]. It is often used in modern medicine to treat acute respiratory infections, skin purulent infections, acute nephritis, hepatitis, meningitis and other diseases [10]. *Forsythia* has more than 150 main chemical components, including lignans, phenylethanoid glycosides, pentacyclic triterpenoids and flavonoids and so on. In recent years, the anti-inflammatory activity and mechanism of many active ingredients of *Forsythia Fructus*, such as phillyrin, have been reported [11–14]. However, another active ingredient in *Forsythia Fructus* is phillygenin (PHI)

(4-[(3S,3aR,6R,6aR)-6-(3,4-dimethoxyphenyl)-1,3,3a,4,6,6a-hexahydrofuro[3,4-c]furan-3-yl]-2-methoxyphenol) that is often overlooked (Fig. 1). PHI is a glycoside of phillyrin, which is one of the important lignans in *Forsythia Fructus* and is regarded as the fingerprint component of *Forsythia Fructus* [15]. Studies have shown that phillyrin was poorly water-soluble and the oral absorption effect is not satisfactory [16]. In addition, it was found that phillyrin was rapidly metabolized to PHI by human intestinal bacteria [17]. Another study suspected that PHI might have the involvement of phillyrin in its anti-inflammatory effects [18]. Pre-study in our group, PHI dose-dependently inhibited the production expression of NO, TNF- α and IL-6 in LPS-stimulated RAW264.7 macrophages, and PHI also inhibited inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein expression and gene transcription [19]. Although some reports have shown that PHI has made some progress in its anti-inflammatory activity in inflammatory diseases, its exact anti-inflammatory mechanism has not been clearly characterized. Proteomics as one of the most promising methods and technologies to predict disease biomarkers can reflect the signal transduction pathway and reflect the mechanism of action of drugs [20]. The combination of proteomics and pharmacology has been proved to be a means to study the mechanism of drug action.

LPS-induced macrophage inflammatory response is a common inflammation model. The NF- κ B signaling pathway is the most important downstream pathway in the signaling pathway mediated by LPS [21]. NF- κ B consists of two subunits belonging to the Rel family composed of homologous or heterodimers. In resting cells, the NF- κ B dimer binds to I κ B protein to cover the nuclear localization signal of NF- κ B, so that the complex formed by NF- κ B and I κ B stays in the cytoplasm. When the cell receives external stimulation, I κ B α / β is phosphorylated and ubiquitinated and then dissociate with NF-dimer which make the nuclear localization signal of NF- κ B exposed. So NF- κ B enters cell nucleus and exerts transcriptional regulatory effects which result in the gene transcription of iNOS, COX-2, IL-6, IL-1 β and TNF- α and so on [22–24].

In this study, we conducted a comprehensive study based on quantitative proteomics and pharmacological experiments to clarify the anti-inflammatory mechanism of PHI. Our results showed that PHI inhibited the production of various inflammatory factors by inhibiting the activation of NF- κ B signaling pathway. These results provided new ideas for the signal pathway of PHI to inhibit the inflammatory response, and also supplied the basis for the subsequent development and research of PHI.

2. Materials And Methods

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies [25].

2.1 Materials

PHI and dexamethasone (DEX) (purity above 99%) were obtained from Mansite-Biotechnology Co. Ltd (Sichuan, China). RAW264.7 cells were obtained from Obio Technology Corp (Changsha, China). Lipopolysaccharide was obtained from Sigma-Aldrich (St. Louis, MO, United States). High glucose

Dulbecco's Modified Eagle Medium and Fetal Bovine Serum were obtained from Gibco (Australia). IL-1 β Elisa kits and PGE2 Elisa kits were obtained from Elabscience Biotechnology Co., Ltd (Wuhan, Hubei, China). Trizol reagent was obtained from Ambion Life Technologies (CA, USA). 5X All-In-One MasterMix and EvaGreen 2XqPCR MasterMix-No Dye were obtained from Applied Biological Materials (abm) Inc (Canada). RIPA lysis buffer, PMSF and BCA Protein Assay Kit were obtained from MultiSciences(Lianke)Biotech Co., Ltd (Hangzhou, Zhejiang, China). iTRAQ kit was obtained from AB Sciex. Rabbit anti-p-p65, mouse anti-p65, rabbit anti-IKK β , rabbit anti-IkBa, rabbit anti-IkBa, Goat anti-Mouse IgG-HRP and Goat anti-Rabbit IgG-HRP were obtained from Multi Sciences (LIANKE) Biotech Co., Ltd. (Hangzhou, Zhejiang, China).

2.2 Cell Culture

Mouse macrophage cell line RAW264.7 was cultured in DMEM complete medium containing antibiotics (100 U / mL penicillin, 100 U / mL streptomycin) and 10% heat-inactivated fetal bovine serum (FBS) at 37 ° C, 5% CO₂ conditions in a humidified incubator.

2.3 Cell Viability Assay

To evaluate the effect of PHI on the viability of RAW264.7 cells. RAW264.7 cells were seeded at a density of 1×10^5 cells / mL on a 96-well cell culture plate, and DMEM solutions containing different concentrations of PHI or DEX (0, 1, 5, 10, 25, 50, 150 and 200 μ g/mL) were added for 24 hours, respectively. MTT solution was added to each well to incubate for additional 4 hours. And then carefully removed the supernatant and added 150 μ L of dimethyl sulfoxide (DMSO) solution to each well. After the crystals in the culture plate were completely dissolved, the absorbance was measured at 570 nm through a microplate reader and calculated the cell proliferation rate.

2.4 ELISA for IL-1 β and PGE2

RAW 264.7 cells were plated at a density of 1×10^5 cells/mL in 24-well plates and divided into six groups. The control group was only treated with DMEM. LPS group was treated with 10 ng/mL LPS. In LPS + PHI group, DMEM solution containing LPS (10 ng / mL) and PHI (5, 50, 100 μ g / mL) was added; In the positive control group, DMEM containing LPS (10 ng / mL) and DEX (50 μ g / mL) was added. Cells were incubated in the cell incubator for 24 h. Cell culture supernatants were used to determinate the concentrations of IL-1 β and PGE2 according to the instructions of the ELISA kit and measured each OD value at 450 nm and then calculated the cytokine release level based on the OD value.

2.5 RNA Extraction and RT-qPCR Analysis

The mRNA expression of TNF- α , IL-6, IL-1 β was measured by RT-qPCR. After treatment, supernatant was discarded and cells were washed twice with PBS. Total RNA was extracted by Trizol Reagent. The experiment was performed according to the procedure of the amplification kit, and GAPDH was used as an internal reference. The formula of $2^{-\Delta\Delta C_t}$ was used to calculate the expression changes of target genes. The gene primer sequences were shown in Table 1.

Table 1
Primers used for RT-qPCR in this study.

Gene	Forward (5'-3')	Reverse (5'-3')
GAPDH	GGCCTTCCGTGTTCTACC	TGCCTGCTTCACCACCTTC
TNF- α	ATGAGAAGTTCCCAAATGGC	CTCCACTTGGTGGTTTGCTA
IL-6	AGTCCGGAGAGGAGACTTCA	ATTTCACGATTTCCAGAG
IL-1 β	TGTGAAATGCCACCTTTTGA	CCTCTTCGACACCGTCGATG

2.6 Proteomic analysis

Sample Preparation

The experiment was divided into control group, LPS group (10 ng / mL LPS), LPS + PHI group (10 ng / mL LPS + 100ug / mL PHI). After raw264.7 cells were cultured for 24 hours with LPS in the presence or absence of PHI, the total protein was isolated using ice-cold RIPA lysate. The protein concentration was determined by BCA method. The extracted proteins were digested with trypsin and labeled according to the instructions of the iTRAQ kit.

Mass Spectrometry Detection

The extracted samples were detected by Q Exactive™ Plus Combined Quadrupole Orbitrap™ Mass Spectrometer. A 75 μ m id $\times$ 12 cm C18 column was used for the experiment. The composition of mobile phase A was 0.1% FA + 2% acetonitrile + 98% water; the composition of mobile phase B was 0.1% FA + 98% acetonitrile + 2% water; the liquid flow rate was 300 nL / min. The liquid phase gradient was set as follows: 5–8% B for 8 min, 8–22% B for 50 min, 22–32% B for 12 min, 32–90% B for 1 min, 90% B for 7 min and 90 – 5% B for 2 min.

The ion source voltage was set to 2 kV, the automatic gain control (AGC) was set to 3e6, the scanning range was 300–1400 m / z, and the resolution was 70,000. The strongest ion peaks with 2 charges were selected for further high energy fragmentation, collision capacity is set to 27%, and dynamic exclusion time is set to 18 s.

Data Analysis

The files were searched using MaxQuant (version 1.5.2.8). The filling of data gaps, normalization, and difference screening ($P < 0.05\%$) were all performed using the standard software set of perseus.

2.7 Molecular docking

Docking study was performed by the Discovery Studio 3.5 software. The proteins in NF-κB signaling pathway (P65, IκBα, IKKβ, GSK-3β, CBP, JNK, NIK, P38, TAK1, CBC13, RSK1, NAK) were selected as the research objects. The three-dimensional structure of all protein and the corresponding known ligand structure were obtained from the Protein Data Bank (<https://www.rcsb.org>). Table 2 showed information on target proteins and corresponding ligands of NF-κB pathway. The downloaded proteins and ligands structures were imported into Discovery Studio 3.5 software and performed molecular docking optimization firstly, and then molecular docking work was implemented to get consistency score. If the consistency score of PHI with the target protein was no less than that of the ligand with the target protein, the protein was considered to have a good affinity with PHI.

Table 2
Target protein of NF-κB pathway and corresponding ligands.

Targets	PDB Number	Ligands
P65	1LEI	DTV
IκBα	4KBA	1QM
IKKβ	4KIK	KSA
GSK-3β	1GNG	7YG
CBP	1JSP	1KX
JNK	2NO3	859
NIK	4IDV	13V
P38	1A9U	SB2
TAK1	4L3P	1UH
CBC13	3HCT	GLZ
RSK1	2Z7Q	ACP
NAK	3K03	LIK

2.8 Western Blot Analysis

After treatment, cells were washed twice with PBS. 380 μL RIPA lysate buffer supplemented with PMSF, protein phosphatase inhibitor and protease inhibitor was added on cells to lyse for 10 min on ice. The lysates were collected with a cell scraper and the cells were broken by ultrasonic cell crusher. Then the supernatant was obtained through centrifugation at -4 °C,12000 rpm condition and the protein concentration was measured with BCA protein assay reagent according to the manufacturer's instructions. Samples were separated by 10% SDS-PAGE electrophoresis and then electrotransferred to a polyvinylidene fluoride (PVDF) membrane in ice bath. Then the PVDF membrane was blocked in 5% skim milk at room temperature for 2 hours. After blocking for 2 h, the membrane was incubated with primary

antibodies diluted 1000 times through 5% BSA solution overnight at 4 °C in a shaking incubator. Finally, the primary antibodies were recycled and the membrane was incubated with the horseradish peroxidase-labeled secondary antibody (antibody : 5% skim milk = 1: 5 000) at 37 °C for 1 hour. After washing, the protein bands were detected and visualized with the chemiluminescence kit using a chemiluminescence detection system.

2.9 Statistical Analysis

Data were presented as means $\pm$ SD and SPSS 25.0 was used to statistical analyses. Comparison between groups was performed by one-way analysis of variance (ANOVA). All the experiments were repeated at least three times. The results were considered statistically significant if p value < 0.05 .

3. Results

3.1 Effects of PHI and DEX on the proliferation of RAW264.7 cells

MTT method was used to investigate the changes of cell proliferation activity of RAW264.7 cells treated with different concentrations of PHI and DEX. As shown in Fig. 2, compared with normal control, PHI had no significant inhibitory effect on cell proliferation at concentrations up to 100 $\mu\text{g/mL}$, and DEX had no significant inhibitory effect on cell proliferation at a concentration of 0 to 50 $\mu\text{g/mL}$. Therefore, in the subsequent experiments, low, medium and high concentrations (5, 50, 100 $\mu\text{g/mL}$) of PHI that did not significantly inhibit cell proliferation were selected for further research, and DEX (50 $\mu\text{g/mL}$) was used as a positive control.

3.2 Effects of PHI on levels of IL-1 β and PGE2

The contents of inflammatory factors IL-1 β and PGE2 in the supernatant of cell culture were determined by ELISA. When LPS acted on RAW264.7 cells, the contents of IL-1 β and PGE2 in cell supernatant were increased significantly compared with the normal control. The contents of IL-1 β and PGE2 in cell supernatant of LPS + PHI groups at different concentrations were significantly lower than those in LPS groups and were dose-dependent. The contents of IL-1 β and PGE2 in the supernatant of the DEX group were significantly lower than those in the LPS group. There was no significant difference in IL-1 β and PGE2 content between 50 $\mu\text{g/mL}$ PHI group and 50 $\mu\text{g/mL}$ DEX group. The results were shown in Fig. 3.

3.3 Effect of PHI on LPS-induced inflammatory factor mRNA expression in RAW264.7 cells

In order to further explore the effect of PHI on LPS-induced inflammatory factor gene expression, RT-qPCR was used to detect the levels of IL-6, IL-1 β and TNF- α . As shown in Fig. 4, compared with the normal control, LPS induced significantly expression of IL-6, IL-1 β and TNF- α mRNA. Compared with the LPS group, IL-6, IL-1 β and TNF- α mRNA expression was down-regulated in a concentration-dependent manner by treating PHI (5, 50, 100 $\mu\text{g/mL}$). Compared with dexamethasone, IL-1 β was no significant difference in

mRNA expression between PHI and dexamethasone at the same concentration of 50 µg/mL. These results indicated that PHI suppressed the inflammatory response by down-regulating the expression of IL-6, IL-1 β and TNF- α mRNA in LPS-induced RAW2644.7 cells inflammation and showed a strong anti-inflammatory effect.

3.4 Effect of PHI on LPS-induced RAW264.7 cell proteome changes

The proteomic profiles were analyzed by Q ExactiveTM Plus Combined Quadrupole OrbitrapTM Mass Spectrometer to research differences of protein expression in LPS-induced Raw264.7 cells inflammation after PHI treatment. The results showed that a total of 4318 proteins were identified and contained quantitative information in this study. By comparing the expression levels of total proteins, the differentially expressed proteins in the three groups were screened. The fold change was more than 1.3 times as the change standard of significant up-regulation and less than 1.3 times as the change standard of significant down-regulation. Based on this criteria, a total of 292 differentially expressed proteins were screened between normal control and LPS group. The results showed that compared with the control group, 171 proteins were up-regulated and 121 proteins were down-regulated in LPS group. These 292 proteins were then screened between PHI treatment group and LPS group. The results showed that 62 (36.3%) up-regulated proteins and 30 (24.8%) down-regulated proteins in LPS group compared with LPS + PHI were significantly reversed by PHI treatment. The results were shown in Fig. 5.

Gene Ontology (GO) is an important bioinformatics analysis method and tool for expressing various properties of genes and gene products. GO annotations are divided into three first-level categories including Biological Process (BP), Cellular Component (CC), and Molecular Function (MF), which explain the biological role of proteins from different perspectives. We conducted statistics on the distribution of the selected differential proteins in the GO secondary annotations. As shown in Fig. 6, according to the analysis, the cellular composition of these proteins was mainly cytoplasm (51 proteins), cytosol (16 proteins) and nucleoli (10 proteins). In biological processes, these proteins were mainly involved in the cell's response to interferon- β (8 proteins), innate immune response (14 proteins), response to viruses (8 proteins) and so on. The molecular functions mainly included nucleotide binding (22 proteins), RNA binding (13 proteins) and protein binding (34 proteins).

3.5 Protein pathway enrichment analysis

In order to visualize the pathways with significant enrichment of differentially expressed proteins by PHI treatment, Cytocape software was used to examine the enrichment of 92 PHI-regulated differential proteins in the KEGG pathway database, REACTOME pathway database and Wiki pathway database. Figure 7 depicted a global pathway network enriched for GO terms at $P < 0.05$. The results showed that PHI mainly affected three pathways, including RIG-I-like receptor signaling pathway, NF-kappa B signaling pathway, and Chagas disease (American trypanosomiasis).

3.6 Molecular docking

Molecular docking experiments were performed to further predict if PHI exerted an anti-inflammatory effect through NF- κ B signaling pathway. Figure 8 showed the consistency score results of docking of the target protein with PHI or ligand molecules in NF- κ B pathway. The results showed that the consistency scores of PHI docking with P65, I κ B α , IKK β , GSK-3 β , CBP, NIK, P38, CBP13, RSK1 were higher than the consistency score of these proteins with their corresponding ligands, while consistency scores of PHI docking with JNK, TAK1 and NAK were lower than that of with their corresponding ligands.

3.7 Effect of PHI on LPS-induced NF- κ B pathway in RAW264.7 cells

Proteomics results and molecular docking indicated that PHI might be able to regulate NF- κ B signaling pathway. To confirm this result, we investigated the effect of PHI on the expression of key proteins in NF- κ B signaling pathway induced by LPS through western blot analysis. The results showed that the expression of IKK β protein increased significantly after LPS stimulation, and PHI could significantly reverse the increase. After LPS stimulation, the levels of P65 and I κ B α phosphorylation were up-regulated significantly, while the levels of P65 and I κ B α phosphorylation were down-regulated significantly by PHI treatment. Between 50 μ g / mL PHI and 50 μ g / mL DEX, there was no significant difference in the inhibitory effects on the expression of IKK β , P-I κ B α and P-P65. These results showed that PHI inhibited the activation of LPS-induced NF- κ B signaling pathway by inhibiting the expression of IKK β and the phosphorylation of P65 and I κ B α . The results were shown in Fig. 9.

4. Discussion

Macrophages are immune effector cells and play a very important role in body's immune system. They are also central cells that initiate the production of inflammatory mediators in body [26–28]. LPS is the main product of Gram-negative bacteria. It can stimulate the activation of immune cells such as macrophages and cause systemic inflammation. The LPS model is a classic inflammation model [29, 30]. Therefore, in this study, we selected LPS-stimulated RAW264.7 cells as a model for inflammation research. PGE2 is an important inflammatory mediator involved in the inflammatory process and can mediate arterial dilatation and increase microvascular permeability. IL-1 β is a marker of body's early inflammation. It can also induce the production of inflammatory factors such as IL-6 and IL-8 while mediating the inflammatory response. As a multifunctional cytokine, IL-6 has two-way functions of anti-inflammatory and pro-inflammatory. Its effect is related to the content in the tissue, and excessive production of IL-6 will cause a series of inflammatory damage. TNF- α is a classic inflammatory indicator, which has the coordination and regulation effect on IL-1 β , IL-6, etc., and is at the center of the inflammatory cascade. In our study, we found that PHI significantly reduced the IL-1 β and PGE2 production and inhibited IL-6, IL-1 β and TNF- α mRNA expression. At the same time, our study found that the inhibitory effect of medium concentration PHI on the IL-1 β and PGE2 production and IL-6, IL-1 β and TNF- α mRNA expression was very similar to that of the positive control drug DEX, and the inhibitory effect of high concentration PHI was stronger than DEX, suggesting that PHI has great development value in future study.

Nowadays, the proteomics technology has become an important support for the rapid development of modern biotechnology. Proteomics essentially refers to studying the characteristics of proteins on a large scale and understanding key pathways through information network analysis [31]. In this work, a comprehensive approach combining proteomics and pharmacological experimental studies is used to research the anti-inflammatory effects of PHI and mechanism of action. Through GO analyses, we observed that the biological processes were quite active in the differentially expressed proteins, including cellular response to interferon-beta, innate immune response, response to virus. Functionally, they were mainly involved in nucleotide binding, RNA binding, and protein binding. Key pathways that might be affected by pathway enrichment analysis included RIG-I-like receptor signaling pathway, NF- κ B signaling pathway, and Chagas disease (American trypanosomiasis). RIG-I-like receptor was one of the recognition receptors of innate immunity. It activated and regulated the intrinsic immunity of cells by recognizing the characteristics of RNA and DNA that were usually absent in the host transcriptome and was a key antiviral pathway [32]. Chagas disease was an anthroponosis which resulted in cardiomyopathy, arrhythmia and so on, and American continent was the epicenter of the disease[33]. NF- κ B, a multi-directional nuclear transcription factor, regulated the expression of various cytokines, enzymes and other genes, and adjusted body's immune, inflammatory response and cell proliferation, as well as affected the expression of inflammatory mediators, adhesion molecules, etc., subsequently to regulate protein synthesis [34–36]. In the results of pathway enrichment, NF- κ B signaling pathway was closely related to the occurrence of inflammation. Therefore, subsequent experiments were carried out around NF- κ B signaling pathway.

Molecular docking study is an effective way to understand the interaction between ligands and proteins [37]. The purpose of ligand-protein docking is to explore the major binding modes when a ligand binds to a protein with a known three-dimensional structure [38]. Based on the fact that PHI has been confirmed to have a good anti-inflammatory effect and the results of proteomic analysis, molecular docking experiments were performed to further study the effect of PHI on the binding of each target protein of NF- κ B signaling pathway. The results showed that the consistency scores of PHI docking with P65, I κ B α , IKK β , GSK-3 β , CBP, NIK, P38, CBP13, RSK1 proteins were not lower than the consistency scores of these proteins with their corresponding ligands. These indicated that PHI had a good affinity for most proteins in NF- κ B pathway and anti-inflammatory effects might be derived from NF- κ B pathway. Of course, binding energies of proteins and molecules by the molecular docking alone is insufficient to judge PHI molecular mechanism. Therefore, comprehensive evaluation needs to be combined with biological experiments.

According to molecular docking and proteomics analysis, PHI might regulate NF- κ B signaling pathway. To further confirm there results, we performed western blot experiments. P65 is a very important protein in NF- κ B family and is a key signaling molecule in the inflammatory process. It is also a relatively well-known activated macrophage pathway protein that can secrete a large number of various inflammatory factors after activation [39–41].The results indicated that PHI inhibited the activation of LPS-induced NF- κ B signaling pathway by inhibiting the expression of IKK β and the phosphorylation of P65 and I κ B α . The results were consistent with molecular docking and proteomics analysis. At the same time, our study

found that the inhibitory effect of medium concentration PHI on LPS-activated IKK β and the phosphorylation levels of P65, I κ B α was very similar to that of the positive control drug DEX and the inhibitory effect of high concentration PHI was stronger than DEX which suggested that PHI had a great inhibition on LPS-activated NF- κ B pathway.

5. Conclusion

In summary, this study demonstrated the anti-inflammatory effect and mechanism of PHI on LPS-induced RAW264.7 cell inflammation. Firstly, we demonstrated that PHI inhibited the release of LPS-induced inflammatory factors and the transcription of related inflammatory genes. The signaling pathways that PHI might affect have been studied through proteomics and bioinformatics analysis. And then through molecular docking technology to study the affinity between PHI and NF- κ B inflammatory pathway target protein, it was found that PHI had a good affinity for NF- κ B pathway. Finally, western blot was used to further verify that PHI inhibited the activation of LPS-induced NF- κ B signaling pathway by inhibiting the expression of IKK β and phosphorylation of P65 and I κ B α . On the whole, PHI had shown its anti-inflammatory effect by inhibiting the activation of the NF- κ B pathway, thereby suppressing the expression of related inflammatory genes and the release of cytokines. The research deeply explored the effects, targets, and mechanism of PHI on inflammation to provide scientific references for the study of PHI in the future and improve the overall understanding of PHI.

List Of Abbreviations

PHI: Phillygenin; LPS: lipopolysaccharide; IL: interleukin; TNF- α : tumor necrosis factor α ; NO: nitric oxide; PGE2: prostaglandin E2; iNOS: inducible nitric oxide synthase; COX-2: cyclooxygenase-2; DEX: dexamethasone; FBS: fetal bovine serum; DMSO: dimethyl sulfoxide; PVDF: polyvinylidene fluoride; GO: Gene Ontology.

Declarations

Ethics approval and consent to participate

Not applicable

Consent to publish

Not applicable

Availability of data and materials

The datasets in this study are available to provide by corresponding authors on reasonable request.

Competing interests

The authors report no conflicts of interest.

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Authors' Contributions

M.Z.(Mengting Zhou), Y.T. (Yunqiu Tang) and Y.L. (Yunxia Li) designed the study and directed the study's optimization strategy. M.Z. and Y.T. summarized the Results and Discussion sections of the text. WB, qPCR and proteomics were performed by Y.T., M.L., and M.Z. Y.T., X.Z., L.L. and Y.D. conducted the biological experiments and analyzed the data. All the authors read and approved the final manuscript.

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Figures

Figure 1

Chemical structure of phillygenin.

Figure 2

Effect of Phillygenin and dexamethasone on proliferation of RAW264. 7 cells. Cell viability was determined by MTT assay. RAW264.7 Cells were treated with PHI or DEX. Compare with control, * P ≤ 0. 05, **P ≤ 0. 01.

Figure 3

Effect of PHI on LPS-induced release of inflammatory factors in raw264.7 cells. RAW264.7 cells were treated with various concentrations of PHI (5,50,100 ug/mL) or 50 ug/mL DEX in the presence of 10ng/mL LPS for 24 hours. Then the IL-1β and PGE2 level were determined by ELISA, respectively. Compare with control, ##P ≤ 0. 01≤Compare with LPS, **P ≤ 0. 01.

Figure 4

Effect of PHI on LPS-induced inflammatory factor mRNA expression in RAW264.7 cells. RAW264.7 cells were treated with various concentrations of PHI (5,50,100 ug/mL) or 50 ug/mL DEX in the presence of 10ng/mL LPS for 24 hours. RAW264.7 cells were treated for 24 hours. The relative mRNA expressions of

IL-6 (A), TNF-α (B) and IL-1β (C) were detected by RT-qPCR analysis. Compare with control, ##P ≤ 0. 01
Compare with LPS, **P ≤ 0. 01.

Figure 5

Venn diagram. Summary data for all differentially expressed proteins from 3 groups of samples are shown. The control group was added with DMEM solution; LPS group (10ng/mL LPS), LPS+PHI group (10ng/mL LPS + 100 ug/mL PHI). RAW264.7 cells were treated for 24 hours.

Figure 6

GO analysis of biological process (A), cellular components (B) and molecular functions (C) for selected differential proteins.

Figure 7

Visualization of pathways with significant enrichment of differentially expressed proteins by PHI treatment. Pathway enrichment analysis of differential proteins predicted that PHI might affect LPS-induced signaling pathways in RAW264.7 cells.

Figure 8

Consistency scores of target proteins docking PHI and corresponding ligands on NF-κB pathway.

Figure 9

PHI inhibited LPS-induced activation of NF-κB signaling pathway. The control group was added with DMEM solution; LPS group (10ng/mL LPS), LPS+PHI group (10ng/mL LPS + 5, 50, 100 ug/mL PHI); The positive control group (10ng / mL LPS + 50 ug/mL DEX). RAW264.7 cells were treated for 24 hours. The expression of IKKβ, IκBα and p65 proteins were detected by western blot. Compare with control, ^{##}P < 0.01. Compare with LPS, ^{**}P < 0.01.

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