Phillygenin inhibits the inflammation and apoptosis of pulmonary epithelial cells by activating PPARγ signaling via downregulation of MMP8

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Abstract. Acute lung injury (ALI) is often responsible for the high morbidity of critically ill patients. The present study aimed to investigate whether phillygenin (PHI) can inhibit inflammation and apoptosis of pulmonary epithelial cells by activating peroxisome proliferator-activated receptor γ (PPARγ) signaling. The in vitro model of ALI was established using lipopolysaccharide (LPS) and PHI was used to treat the LPS-induced cells. Cell viability was assessed using the MTT assay and the concentration levels of the inflammatory factors were detected by ELISA. Western blotting and reverse transcription-quantitative PCR were conducted to measure the expression levels of the inflammation- and apoptosis-associated proteins. The MMP8-overexpression plasmid was transfected into LPS-induced cells, which were treated with PHI treatment and the expression levels of PPARγ were detected via western blotting. PHI treatment suppressed the induction of inflammation and apoptosis of LPS-induced BEAS-2B cells. Furthermore, the expression levels of MMP8 in BEAS-2B cells induced by LPS were decreased following PHI treatment. Following transfection of the MMP8 overexpression plasmid into the LPS-induced BEAS-2B cells and subsequent treatment of these cells with PHI, the expression levels of PPARγ were decreased. In conclusion, it was shown that PHI inhibited the inflammation and apoptosis of pulmonary epithelial cells by activating PPARγ signaling via downregulating MMP8. These data may provide valuable information for future studies exploring the therapeutic effects of PHI for ALI.

Introduction

The lungs are one of the most vulnerable organs in the human body, and alveolar macrophages are the major inflammatory cells involved in the maintenance of the lung’s defense against foreign pathogens (1). Phagocytosis caused by these macrophages and the accompanied release of cytokines constitute the host cellular defense (2). Acute lung injury (ALI) is responsible for the high morbidity of critically ill patients (3). By damaging the alveolar epithelium, ALI can trigger the inflammatory response in the defensive system of the lung (4). Despite the complex mechanism that contributes to the occurrence of ALI, the damage to the epithelial cells can speed up the development of this disease (5,6). To date, specific pharmacological drugs have not been used in clinical practice for the treatment of ALI (7). Therefore, the severity of ALI and the potential targets or drugs that can treat this disease should be examined.

Phillygenin (PHI; 4-[(3S,3aR,6R,6aR)-6(3,4-dimethoxyphenyl)-1,3,3a,4,6,6a-hexahydrofurano[3,4-c]furan-3-yl]-2-methoxyphenol) (Fig. 1A) is a lignan component extracted from Forsythiae Fructus (8), and it has been reported to exert anti-inflammatory effects (9). The Toll-like receptor (TLR)4/MyD88/NF-κB signaling pathway, which can be activated by PHI, can inhibit lipopolysaccharide (LPS)-induced inflammation and further alleviate liver fibrosis (8). Furthermore, PHI has been reported to have significant antitumor effects in human esophageal and pancreatic cancer types (10,11). A search of the SwissTargetPrediction website suggested that PHI can bind to and regulate the expression of MMP8. Previous studies have indicated that MMP8 and MMP9 expression levels are elevated in pediatric patients with ALI (11,12). Decreased MMP8 activity and aberrantly increased MMP9 activity were noted in patients with ALI with prolonged disease progression (13). MMP8 serves an important role in inflammation and degradation of tight junction proteins (14). Increased expression of MMP8 is associated with the early inflammation stage of spinal cord injury (SCI) and addition of a specific MMP8 inhibitor can markedly alleviate the inflammatory response and cellular damage of SCI (14). The
use of MMP8 inhibitors on astrocytes can suppress the levels of inducible nitric oxide synthase, TNF-α, IL-1β, IL-6 and TLR2, and is accompanied by the activation of peroxisome proliferator-activated receptor γ (PPARγ) and the inhibition of NF-κB activity, which is induced by lipoteichoic acid (15). The notable therapeutic potential of curcumin in the treatment of neonatal ALI is mediated by activating PPARγ/heme oxygenase-1 signaling in an animal model (16). This finding provided evidence that activation of PPARγ may be a novel therapeutic strategy for the treatment of ALI. Therefore, the present study aimed to investigate whether PHI can inhibit the induction of inflammation and apoptosis of pulmonary epithelial cells by activating PPARγ signaling.

Materials and methods

Sample collection. The clinical study protocol was approved by Gaolangang Hospital of Zhuhai People's Hospital (approval no. 2020-012; Zhuhai, China) between 2019/09 and 2020/02. Signed informed consent forms were obtained from the child's family for the collection and use of the specimens. Briefly, patients who were admitted to the pediatric intensive care unit of Gaolangang Hospital of Zhuhai People's Hospital were enrolled. Inclusion criteria were pediatric patients [age, 2-10 years; 28 (46.7%) females and 32 (53.3%) males] who were intubated and mechanically ventilated with a ratio of partial pressure of arterial oxygen (PaO₂) to the fraction of inspired oxygen (FiO₂) of ≤300 (adjusted to 253 in Salt Lake City due to altitude), bilateral pulmonary infiltrates and no clinical evidence of left atrial hypertension. Patients were excluded if they were <2 years of age; had respiratory failure from cardiac disease; had hypoxemia without bilateral infiltrates; had received a bone marrow or lung transplant; were supported on extracorporeal membrane oxygenation; had a non-pulmonary condition that could be exacerbated by the prone position; had participated in other clinical trials within the preceding 30 days; or if there was a decision to limit life support. Blood samples were collected within the first 24 h of diagnosis. The serum samples of pediatric patients with ALI (n=30) and normal subjects (n=30) were collected for further analysis following submission of the informed consent form.

Cell lines and treatment. The pulmonary epithelial cell line (BEAS-2B) was obtained from the American Type Culture Collection. The cells were maintained in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) containing 10% FBS (Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO₂. For LPS induction, 1 µg plasmids were transfected into BEAS-2B cells using Lipofectamine³ 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Following incubation for 6 h, the medium was replaced with RPMI-1640 medium with 10% FBS (Thermo Fisher Scientific, Inc.). After 48 h of incubation, the cells were collected and the transfection efficiency was analyzed by reverse transcription-quantitative PCR (RT-qPCR).

MTT assay. Cells were seeded in 96-well plates at a density of 3,000/well and were treated by different concentrations of PHI for 24 h. A total of 10 µl MTT solution was added to the medium and the cells were cultured for an additional 4 h. Dimethyl sulfoxide was added into each well to dissolve the formazan particles. The absorbance was measured at 450 nm using a microplate reader (Molecular Devices, LLC).

TUNEL assay. BEAS-2B cells treated with PHI (12.5, 25 or 50 µg/ml; 24 h; 37˚C) and LPS were fixed with 4% paraformaldehyde at 25˚C for 15 min. Then, a TUNEL kit (Roche Applied Science) was used to detect apoptotic cells according to the manufacturer's protocol. Subsequently, cell nuclei were counterstained with 0.2 µg/ml DAPI at room temperature for 15 min and mounted with glycerol gelatin (Sigma-Aldrich; Merck KGaA). An Olympus fluorescence microscope (magnification, x200) was used to acquire the images in ≥3 randomly selected fields of view.

ELISA. BEAS-2B cells were plated in 96-well plates (8x10⁴ cells/ml) and incubated with LPS in the presence or absence of PHI for 24 h. The concentration levels of IL-1β, IL-6 and TNF-α in the cell supernatant were measured by ELISA kits according to the manufacturer's recommendations. The following ELISA kits were purchased from Beyotime Institute of Biotechnology, IL-1β ELISA kit (cat. no. PA012), IL-6 ELISA kit (cat. no. PA130) and TNF-α ELISA kit (cat. no. PT518). The absorbance at 450 nm was detected by a microplate reader.

RT-qPCR. Total RNA in the cells and the serum of pediatric patients with ALI was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocol. Subsequently, the extracts were dissolved and the final RNA purity was measured by the Nucleic Acid/Protein Analyzer (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA was synthesized using 5X All-In-One RT Master Mix (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocols. RT-qPCR reactions were performed using SYBR-Green Master Mix (Invitrogen; Thermo Fisher Scientific, Inc.). The reaction conditions were as follows: Initial denaturation at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 30 sec. The mRNA levels of the target genes were normalized to the levels of the GAPDH gene. The 2⁻ΔΔCq (17) method was used for analysis of the data. The sequence of primer pairs were as follows: MMP8 forward (F), 5'-AACAGGCATGTGACCTGTTT-3' and reverse (R), 5'-AAACAGGTGATACCAATGAGCCTT-3'; and GAPDH F, 5'-CTGAGTACGCTGGAGTCTC-3' and R, 5'-TGATGATCTTCGAGGCTTGGTC-3'.
Western blot analysis. BEAS-2B cells were lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology) and the protein content was estimated using a BCA assay. The samples (30 µg) were subsequently loaded on SDS-PAGE gels (10, 12 or 15%), which were prepared previously. The gels were then transferred to nitrocellulose membranes. The membranes were first blocked using fat-free milk (5%) in TBS for 2 h at room temperature and then the membranes were incubated at 4˚C for 24 h with primary antibodies against the following: Cox-2 (1:1,000; cat. no. sc-376861), Bcl-2 (1:1,000; cat. no. sc-7382), Bax (1:1,000; cat. no. sc-7480), caspase3 (1:1,000; cat. no. sc-7272), caspase9 (1:1,000; cat. no. sc-56076), MMP8 (1:1,000; cat. no. sc-137044), PPARγ (1:1,000; cat. no. sc-7273), GAPDH (1:2,000; cat. no. sc-47724; all purchased from Santa Cruz Biotechnology, Inc.), iNOS (1:1,000; cat. no. 13120), cleaved (c)-caspase3 (1:1,000; cat. no. 9661) and c-caspase9 (1:1,000; cat. no. 9509; all purchased from Cell Signaling Technology, Inc.). Following which, the membranes were incubated with HRP-conjugated secondary antibody (1:1,000; cat. nos. 7074 and 7076; Cell Signaling Technology, Inc.) for 50 min at 25˚C. TBS with Tween-20 (0.2%) was used to wash the membranes to remove non-specific binding of the antibodies and the ECL luminescence agent (Santa Cruz Biotechnology, Inc.) was added. The images were captured in a Bio-Rad chemiluminescence imager (Bio-Rad Laboratories, Inc.). The expression levels of the proteins were analyzed using ImageJ software version 1.4 (National Institutes of Health) and normalized to those of the control.

Statistical analysis. Data analysis was performed with SPSS version 23.0 (IBM Corp.) and GraphPad (version 5.0; GraphPad Software, Inc.). All experiments were repeated three times and the data are expressed as the mean ± standard deviation. Two group comparisons were performed using an unpaired Student's t-test was used for the comparison between two group of samples and statistical differences between groups were analyzed using one-way ANOVA followed by a Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

PHI treatment suppresses the inflammatory response in LPS-induced BEAS-2B cells. To determine the effects of PHI on
pulmonary epithelial cells, BEAS-2B cell viability was detected. Treatment of BEAS-2B cells with PHI (12.5, 25 and 50 µg/ml) indicated no apparent difference in cell viability, while significant differences were noted at 100 µg/ml PHI treatment (Fig. 1B). The cell viability of BEAS-2B cells treated with LPS was markedly decreased. This effect was partially reversed by PHI treatment at 12.5, 25 and 50 µg/ml (Fig. 1C). Therefore, the following concentrations of PHI were selected for subsequent studies: 12.5, 25 and 50 µg/ml. ELISA and western blot analysis indicated that the levels of inflammatory cytokines and inflammation-associated proteins were elevated following LPS induction, which could be gradually reversed by PHI treatment at increasing concentrations (Fig. 1D-G). Therefore, these results indicated that PHI treatment suppressed the inflammatory response in LPS-induced BEAS-2B cells.

**Figure 2. PHI treatment suppresses the induction of apoptosis in BEAS-2B cells.** (A and B) Cell apoptosis of LPS-induced BEAS-2B cells was detected using a TUNEL assay (magnification, x200; scale bar, 100 µm). ***P<0.001 vs. control; ###P<0.001 vs. LPS group. (C) Western blotting was used for the analysis of the expression levels of the apoptosis-associated proteins. ***P<0.001 vs. control; #P<0.05, ##P<0.01, ###P<0.001 vs. LPS group. PHI, phillygenin; LPS, lipopolysaccharide; c-, cleaved.

**PHI treatment suppresses the induction of BEAS-2B cell apoptosis.** Induction of cell apoptosis plays a critical role in the development of ALI (18). The apoptotic effects of PHI on BEAS-2B cells were confirmed by TUNEL assay and western blot analysis. As shown in Fig. 2A and B, the induction of cell apoptosis was increased by LPS, while increasing doses of PHI alleviated the effect of LPS on BEAS-2B cell apoptosis. In addition, the expression levels of the pro-apoptotic proteins were increased by LPS, while they were decreased by PHI. The opposite finding was noted for the anti-apoptotic protein Bcl-2, which demonstrated decreased expression following treatment with LPS and increased expression by PHI in a dose-dependent manner (Fig. 2C). Taken together, the data revealed that PHI treatment suppressed the induction of BEAS-2B cell apoptosis.
PHI treatment decreases the expression levels of MMP8 in LPS-induced BEAS-2B cells. Serum samples of normal subjects and pediatric patients with ALI were collected and assessed by RT-qPCR and western blot analyses. As demonstrated in Fig. 3A and B, the expression levels of MMP8 were significantly increased in patients with ALI. Following the addition of increasing doses of PHI in LPS-induced BEAS-2B cells, western blot analysis demonstrated that the expression levels of MMP8 were gradually decreased (Fig. 3C). Therefore, PHI treatment decreased the expression levels of MMP8 in LPS-induced BEAS-2B cells.

PHI treatment suppresses induction of inflammation in LPS-treated BEAS-2B cells by downregulating MMP8 expression. To directly assess the effects of MMP8 on LPS-induced BEAS-2B cells, which were treated with PHI, 50 µg/ml PHI was selected for subsequent experiments and MMP8-overexpression models were established. RT-qPCR and western blot analyses indicated significantly higher levels of MMP8 in the pcDNA3.1-MMP8 group compared with those of the control group (Fig. 4A and B). The expression levels of the inflammatory factors, which were inhibited by PHI in LPS-induced BEAS-2B cells, were significantly elevated following transfection of pcDNA3.1-MMP8 into the cells (Fig. 4C-E). Moreover, the inflammation-associated proteins exhibited decreased expression following PHI treatment in LPS-induced BEAS-2B cells, which could be partially reversed by MMP8 overexpression (Fig. 4F). These results indicated that PHI treatment suppressed the inflammation of LPS-induced BEAS-2B cells.

PHI treatment activates PPARγ signaling by downregulating MMP8 expression. The induction of apoptosis of LPS-treated BEAS-2B cells was detected by TUNEL staining. PHI decreased the number of apoptotic cells in LPS-induced BEAS-2B cells, while pcDNA3.1-MMP8 increased it, indicating that PHI treatment suppressed the induction of apoptosis in LPS-treated BEAS-2B cells (Fig. 5A and B). Moreover, the changes in the protein levels of the anti-apoptotic and pro-apoptotic proteins further demonstrated that MMP8 overexpression partially reduced the inhibitory effects of PHI on cell apoptosis (Fig. 5C). The results from the western blot analysis indicated that PPARγ expression was significantly decreased following LPS treatment of the cells, while additional PHI treatment rescued its expression (Fig. 5D). This change was partially reversed by MMP8 overexpression (Fig. 5D). Therefore, these results suggested that PHI treatment activated PPARγ signaling by downregulating MMP8 expression.

Discussion

Forsythiae Fructus is used as a single herb or added in compound prescriptions in Asia (19). Dong et al (20) was the first to report the use of this type of dried fruit as liangqiao (Forsythia). It has been used for the treatment of infectious diseases, including acute nephritis, since it exhibits heat-clearing and detoxification effects (21). The Forsythia Fructus extract further exerts anti-inflammatory, antibacterial, antiviral and antioxidant effects (22,23). PHI is the extract from Forsythia Fructus, which demonstrates antitumor, antioxidant and hepatoprotective effects in vivo and in vitro (24,25). A previous study has observed the anti-inflammatory activity of PHI in mouse lymphocytes (9). Moreover, Forsythia Fructus could enhance the defense mechanism in rats with LPS-induced liver injury (26). Concurrently, the present study indicated the importance of
PHI in alleviating inflammation and reducing apoptosis in LPS-induced BEAS-2B cells.

The generation of ROS and the reduction in MMP expression levels have been reported to be important events in triggering apoptosis. PHI was previously found to increase ROS and decrease MMP levels in promyelocytic leukemia HL-60 cells, indicating its effects on the suppression of cell apoptosis (27). A previous study reported the effects of MMP8 deletion on the improvement of septic patient survival and the suppression of the inflammatory response in a murine model (28). MMP8 inhibitors have also been considered key regulators in mitigating myocardial injury (29). In the present study, MMP8 was found to be highly expressed in the serum of pediatric patients with ALI, whereas PHI treatment decreased the levels of MMP8 expression. Further experiments confirmed the regulatory role of PHI in the expression of MMP8, and it was concluded that PHI alleviated inflammation and reduced apoptosis in LPS-induced BEAS-2B cells by downregulating MMP8 expression levels.

PPARs are ligand-activated transcription factors that exert anti-inflammatory effects on certain diseases, including brain injury (30,31). Furthermore, PPARs also regulate the transcription of genes with critical roles in various cellular processes (32,33). These transcription factors belong to the superfamily of nuclear receptors and they are present in a variety of tissues for cellular homeostasis. As PPARs are involved in the development of numerous diseases, such as brain and peripheral inflammation and cancer, and they are considered to be potential molecular targets (30,34,35). Previously, it was shown that PPARγ plays an important role in the regulation of gene expression, such as that of lipoprotein(a), IL-1 and TNF-α (36,37). The activation of PPARγ can also regulate different cellular processes, including cell proliferation, metabolism and inflammation (38). In the present study, PPARγ signaling was inhibited by LPS induction in BEAS-2B cells, whereas PHI treatment led to an increase in the expression of PPARγ in LPS-induced BEAS-2B cells. However, MMP8 overexpression partially alleviated the effects of PHI on activating the PPARγ signaling pathway.
In conclusion, the present study demonstrated that PHI inhibited inflammation and apoptosis of pulmonary epithelial cells by activating PPARγ signaling via downregulation of MMP8 expression, which may guide future studies on PHI and provide a theoretical basis for the therapeutic potential of PHI. However, there are numerous limitations of the present study. After validating the effect of PHI on LPS-induced inflammation and apoptosis in lung epithelial cells in vitro, it would improve the results of the present study if experiments and comparison tests with positive control tests had been performed in animal models.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

PY and YL collaborated on the manuscript, including designing the research, collecting and analyzing the experiments and data, and writing the manuscript. PY and YL confirmed the authenticity of all the raw data. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

The clinical study protocol was approved by Gaolangang Hospital of Zhubai People’s Hospital (Zhuhai, China; approval no. 2020-012). Signed informed consent forms were obtained from the children’s guardians for the collection and use of the specimens.

Patient consent for participation

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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