Original Article
Platycodin D suppresses mast cell-mediated allergic inflammation

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Abstract: Mast cell-mediated allergic inflammation is involved in allergic rhinitis (AR). Platycodin D (PLD) is one of the main effective ingredients from the root of *Platycodon grandiflorum*. It is known to have anti-tumor, anti-inflammatory, and anti-allergic activities. However, the effects of PLD on AR have not yet been fully elucidated. In this study, we evaluated the anti-allergic and anti-inflammatory effects of PLD on phorbol-12-myristate 13-acetate plus calcium inophore (PMACI)-induced human mast cells. In this study, we found that PLD significantly inhibited the expression of TNF-α and IL-1β and histamine release in the PMACI-stimulated HMC-1 cells; it also inhibited the PMACI-induced caspase-1 activation in HMC-1 cells. Moreover, it inhibited the PMACI-induced IκB-α degradation and MAPK activation in HMC-1 cells. Taken together, our results showed that PLD inhibits PMACI-induced IL-1β expression and caspase-1 activity by suppressing the activation of NF-κB signaling pathway and MAPK signaling pathway in HMC-1 cells. Therefore, our data suggest the possible therapeutic application of PLD in the treatment of AR.

Keywords: Platycodin D (PLD), allergic rhinitis (AR), mast cells, inflammation

Introduction

Allergic rhinitis (AR) is an inflammatory disorder of the upper airway, affecting 5-22% of the general population, and its prevalence is increasing [1, 2]. It is characterized by the symptoms of sneezing, itchiness, rhinorrhea, and nasal congestion [3]. These symptoms are attributed to the release of histamine and other active substances by mast cells [4]. These substances act on the vasculature, smooth muscle, connective tissue, and mucous glands, resulting in the recruitment of activated immune and inflammatory cells to the site of inflammatory lesion, thereby amplifying and sustaining the inflammatory condition [5]. So, inhibition of mast cell function could provide benefit in AR and other inflammatory diseases.

Platycodin D (PLD) is one of the main effective ingredient from the root of *Platycodon grandiflorum*. It has been shown to possess anti-inflammation [6], anti-virus [7], heptoprotection [8], immunoregulation [9] and anti-tumor [10] properties. For example, PLD inhibited the production of nitric oxide and secretion of TNF-alpha in activated RAW 264.7 cells [11]. In

addition, it has anti-allergic effects. Wang *et al*. reported that PLD inhibited ovalbumin-induced increases in Raw and eosinophil count in airway; IL-4, IL-5, and IL-13 were recovered in bronchoalveolar lavage fluid [12]. However, the effects of PLD on AR have not yet been elucidated. In this study, we evaluated the anti-allergic and anti-inflammatory effects of PLD on phorbol-12-myristate 13-acetate plus calcium inophore (PMACI)-induced human mast cells. Our results showed that PLD significantly inhibited PMACI-induced pro-inflammatory cytokines expression and caspase-1 activity via modulation of the JNK- and ERK-MAPKs and NF-κB-dependent pathways in mast cells.

Materials and methods

Reagents and cell culture

PLD was purchased from Chengdu Herb purify Co. Ltd (20 mg, J-013-120321, HPLC ≥ 98%). The human mast cell line (HMC-1) was grown in Iscove's media (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and glutamine 2 mM in a 5% CO_2 incubator at 37°C. HMC-1 cells at passages

ranging from 4 to 8 were used throughout the study. HMC-1 cells (3×10^5 cells/ml) were treated with PLD (1, 10, or 50 µg/ml) for 1 h prior to stimulation with PMACI.

MTT assay

The HMC-1 cells (2×10^5 cells/ml) were cultured in microplate wells for 24 h following treatment with PLD (1, 10, or 50 µg/ml) and incubated with 20 µl of MTT solution (5 mg/ml) for an additional 4 h at 37°C in an atmosphere of 5% CO_2 and 95% air. Consecutively, 250 µl of DMSO were added to extract the MTT formazan and the absorbance of each well at 540 nm was read using an automatic microplate reader.

Histamine determination

Determination of histamine contents was examined as previously described [13]. In brief, HMC-1 (1×10^6 cells/ml) were pre-incubated with PLD 30 min prior to incubation with (PMACI) (1 µM), and the histamine contents were measured by the o-phthaldialdehyde spectrofluorometric procedure. The fluorescence intensity was measured at an emission wavelength of 438 nm and an excitation wavelength of 353 nm using a spectrofluorometer (Shimadzu).

Quantitative real-time PCR

Total RNA was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and 5 mg RNA of each sample was reverse transcribed using SuperScript RT kit (Invitrogen, Carlsbad, CA, USA). Subsequently, real-time PCR was performed with SYBR Premix Ex Taq (TaKaRa Biotechnology) using an ABI Prism 7900 instrument (Life Technologies, Carlsbad, CA). The following primers were used: TNF-α, 5'-ATGGGC-TCCCTCTCATCAGT-3' (sense), 5'-GCTTGGTGG-TTTGCTACGAC-3' (antisense); IL-1β, 5'-GGGGT-ACCTTAGGAAGAC ACAAATTG-3' (sense), 5'-CC-GGATCCATGGCACCTGTAC ATCA-3' (antisense); β-actin, 5'-CTTAGTTGCGTTACACCCTTTCTTG-3' (sense), 5'-CTGTCACCTTCACCGTTCCAGTTT-3' (antisense). The RT-PCR cycling parameters were performed as follows: denaturation at 95°C for 15 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s. For analysis, the expression of target genes was normalized by the gene β-actin. Based on the DDCt method,

relative amounts of mRNA were expressed as $2^{-\Delta\Delta Ct}$.

Western blot

Total proteins were extracted from HMC-1 cells, and the concentrations of proteins were assayed by BCA protein assay kit. The equal amounts (30 mg) of proteins were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel, and transferred to a polyvinylidene difluoride membrane. The membranes were blocked by 2.5% nonfat milk for 1 h at 37°C, then incubated with primary antibodies against TNF-α (1:1500), IL-1β (1:1500), IκB-α (1:1500), p38 (1:1000), phospho-p38 (1:1000), ERK (1:1000), phospho-ERK (1:1000), JNK (1:1000), phospho-JNK (1:1000) and GAPDH (1:2000) at 4°C overnight. The blot was then incubated with appropriate horseradish peroxidase conjugated secondary antibody (Abcam, Cambridge, MA). Immunoreactive bands were identified using enhanced chemiluminescence, according to the manufacturer's instructions, and quantified by densitometry.

Caspase-1 activity assay

HMC-1 cells (3×10^6) were treated with PLD (1, 10, or 50 µg/ml) for 1 h before stimulation with PMACI incubated for 2 h. The enzymatic activity of caspase-1 was measured according to the manufacturer's specification using a caspase assay kit (R&D Systems). Catalytic activity of caspase-1 from cell lysate was measured by proteolytic cleavage of WEHD-pNA for 4 h at 37°C. The plates were read at 405 nm.

Statistical analysis

Results were expressed as mean ± SD. All statistical analyses were carried out using SPSS 16.0. Differences between treatment conditions were assessed for statistical significance using one-way ANOVA, followed by the LSD or Dunnett's t test method. P values < 0.05 were considered statistically significant.

Results

Effects of PLD on HMC-1 cells viability

We first examined the effects of PLD on HMC-1 cells viability using the MTT assay. As shown in **Figure 1**, compared with untreated HMC-1 cells, treatment with PLD did not obviously affect cell viability.
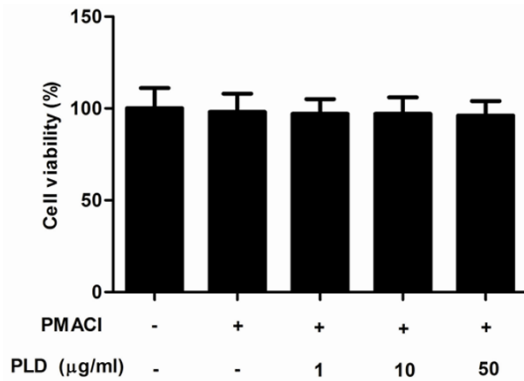
Figure 1. Effects of PLD on HMC-1 cells viability. Cells were pretreated with PLD for 30 min prior to PMACI (1 μM) stimulation for 2 h. Cell viability was evaluated by an MTT assay. All experiments were repeated at least three times. The values shown represent the mean ± SD.
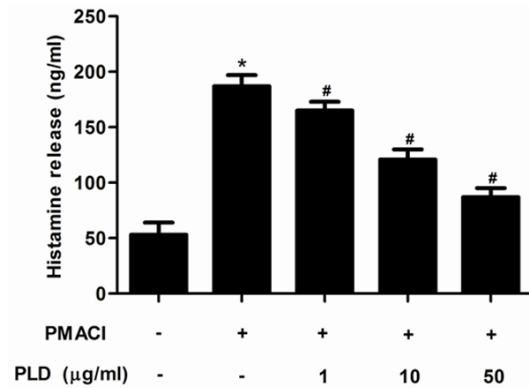

Figure 2. Effects of PLD on PMACI-induced the expression of TNF-α and IL-1β. Cells were pretreated with PLD for 30 min prior to PMACI (1 μM) stimulation for 2 h. A. The mRNA levels were measured by RT-PCR; B. The protein levels were measured by Western blot. All experiments were repeated at least three times. The values shown represent the mean ± SD, the symbol * indicates differences from the control group at $P < 0.05$; the symbol # indicates differences from PMACI group at $P < 0.05$.
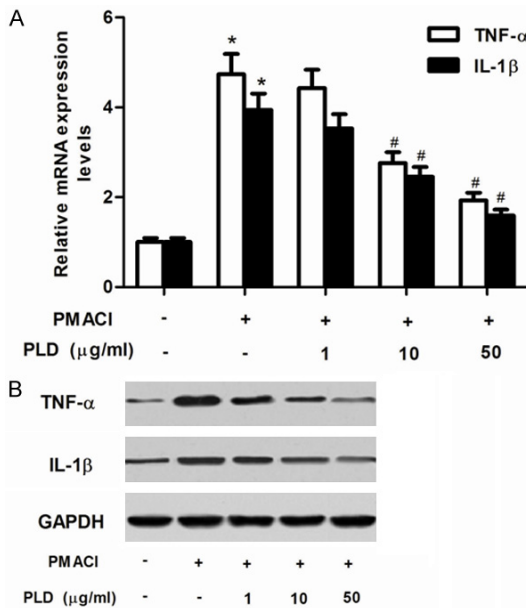
Effects of PLD on PMACI-induced the expression of TNF-α and IL-1β

It is well known that TNF-α and IL-1β play critical roles in the progress of AR, therefore, we inves-

Figure 3. Effects of PLD on PMACI-induced histamine release in HMC-1 cells. Cells were pretreated with PLD for 30 min prior to PMACI (1 μM) stimulation for 2 h. Histamine levels were measured using the histamine assay kit. PLD inhibited PMACI-induced histamine release from HMC-1 in a dose-dependent manner. All experiments were repeated at least three times. The values shown represent the mean ± SD, the symbol * indicates differences from the control group at $P < 0.05$; the symbol # indicates differences from PMACI group at $P < 0.05$.

tigated the effects of PLD on PMACI-induced the expression of TNF-α and IL-1β in HMC-1 cells. As shown in **Figure 2A**, PMACI significantly induced the mRNA expression levels of TNF-α and IL-1β. However, treatment of HMC-1 cells in the presence of PLD significantly inhibited PMACI-induced the mRNA expression of TNF-α and IL-1β. The protein levels of TNF-α and IL-1β were also significantly inhibited by the treatment of PLD (**Figure 2B**).

Effects of PLD on PMACI-induced histamine release in HMC-1 cells

Then, we investigated the effect of PLD on histamine release in PMACI-induced HMC-1 cells. As shown in **Figure 3**, PMACI significantly induced histamine release. Whereas, PLD inhibited PMACI-induced histamine release from HMC-1 in a dose-dependent manner.

Effects of PLD on caspase-1 activation in HMC-1 cells

Caspase-1 plays a critical role in several important inflammatory diseases, so, in order to investigate the regulatory mechanisms of PLD on inflammatory cytokine expression, we detected the effects of PLD on caspase-1 activation in HMC-1 cells. As shown in **Figure 4**, PMACI significantly induced caspase-1 activity,
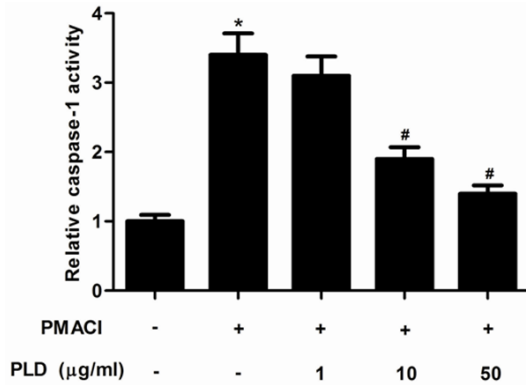
Figure 4. Effects of PLD on caspase-1 activation in HMC-1 cells. Cells were pretreated with PLD for 30 min prior to PMACI (1 μM) stimulation for 2 h. The enzymatic activity of caspase-1 was confirmed by colorimetric assay. All experiments were repeated at least three times. The values shown represent the mean ± SD, the symbol * indicates differences from the control group at $P < 0.05$; the symbol # indicates differences from PMACI group at $P < 0.05$.

Figure 5. Effects of PLD on the activation of NF-κB in HMC-1. Cells were pretreated with PLD for 30 min prior to PMACI (1 μM) stimulation for 2 h. Equal amounts (30 μg protein per lane) of total proteins were separated by SDS-PAGE and analyzed by immunoblotting with anti-IκBα and anti-GAPDH antibodies. All experiments were repeated at least three times. The values shown represent the mean ± SD, the symbol * indicates differences from the control group at $P < 0.05$; the symbol # indicates differences from PMACI group at $P < 0.05$.
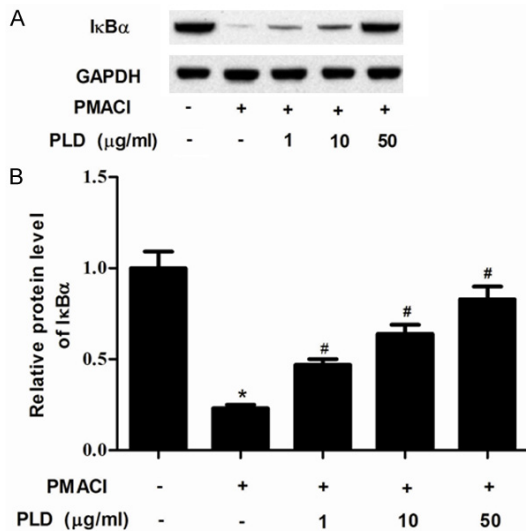
whereas, the treatment of PLD inhibited the PMACI-induced caspase-1 activation in HMC-1 cells.

Effects of PLD on PMACI-induced NF-κB activation in HMC-1 cells

NF-κB is an important transcriptional regulator of inflammatory cytokines and plays a crucial role in immune and inflammatory responses. We postulated that PLD mediates its effects at least partly through the suppression of NF-κB activation. As shown in **Figure 5**, PMACI stimulation resulted in IκB-α degradation, whereas, PLD inhibited the PMACI-induced IκB-α degradation in HMC-1 cells.

Effects of PLD on PMACI-induced MAPK activation in HMC-1 cells

Activation of MAPK signaling pathway has also been reported to participate in inflammatory responses. Therefore, we investigated the effects of PLD on activation of MAPK signaling pathway. As shown in **Figure 6**, PMACI significantly increased the protein expression levels of p-p38, p-ERK and p-JNK, however, PLD could decreased the protein expression levels of p-p38, p-ERK and p-JNK induced by PMACI.

Discussion

The main findings of our study are: 1st) PLD significantly inhibited the expression of TNF-α and IL-1β in the PMACI-stimulated HMC-1 cells; 2nd) PLD inhibited PMACI-induced histamine release from HMC-1 in a dose-dependent manner; 3rd) PLD inhibited the PMACI-induced caspase-1 activation in HMC-1 cells; 4th) PLD inhibited the PMACI-induced IκB-α degradation in HMC-1 cells; 5th) PLD could decreased the protein expression levels of p-p38, p-ERK and p-JNK induced by PMACI.

Pro-inflammatory cytokines, particularly TNF-α and IL-1β, are known to play an important role in triggering and sustaining allergic inflammation in mast cells [14-16]. TNF-α promotes inflammation, granuloma formation and tissue fibrosis and is an initiator of cytokine related inflammatory responses by stimulating cytokine production. It is also responsible for eosinophil survival through partial inhibition of apoptosis, thereby contributes to chronic inflammation [17]. IL-1β is also produced from mast cells and its accumulation is associated with the progress of AR [18]. In this study, we observed that PLD significantly inhibited the expression of TNF-α and IL-1β in the
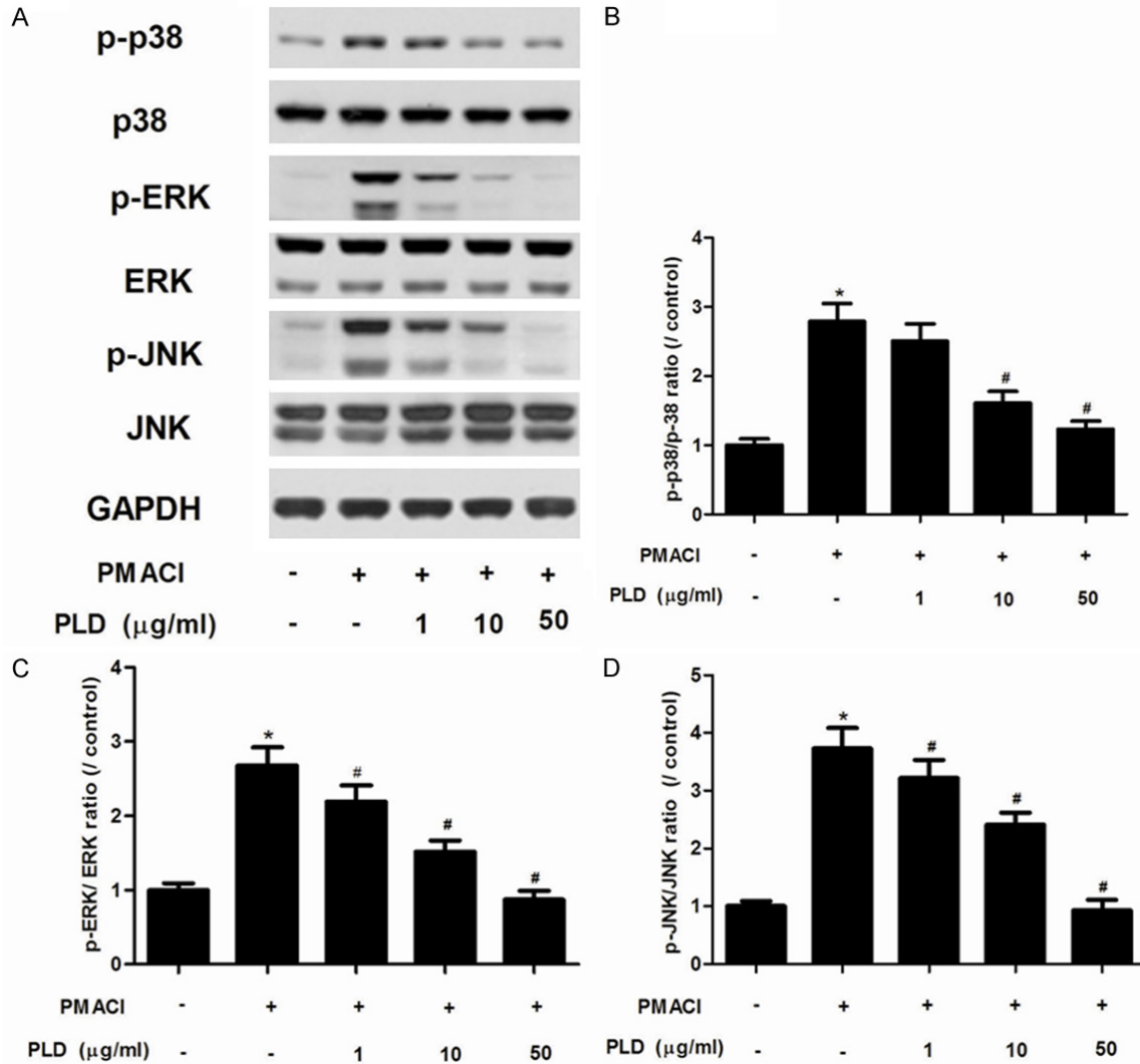
Figure 6. Effect of PLD on phosphorylation of MAPKs in HMC-1. Cells were pretreated with PLD for 30 min prior to PMACI (1 µM) stimulation for 30 min. (A) Equal amounts (30 µg protein per lane) of total proteins were separated by SDS-PAGE and analyzed by immunoblotting with anti-p-p38, anti-p38, anti-p-ERK, anti-ERK, anti-p-JNK and anti-JNK antibodies. Quantification of (B) p-p38/p38, (C) p-ERK/ERK and (D) p-JNK/JNK. All experiments were repeated at least three times. The values shown represent the mean ± SD, the symbol * indicates differences from the control group at $P < 0.05$; the symbol # indicates differences from PMACI group at $P < 0.05$.

PMACI-stimulated HMC-1 cells, exhibiting a dose-independent manner. Therefore, we suggest that PLD exerts anti-allergic effects via down-regulation of the expression of TNF-α and IL-1β.

Histamine, a main content of granules in mast cells, exerts affects chronic inflammation and regulates several essential events of immune response such as immune cell maturation, polarization, and lymphocyte responsiveness [19]. In this study, we observed that PLD inhibited PMACI-induced histamine release from HMC-1 in a dose-dependent manner.

Caspase-1 plays a crucial role in the regulation of cytokines. Several studies demonstrated that caspase-1 activity was significantly increased in an animal model of AR, as compared with normal mice. It has been reported that caspase-1 is found predominantly in the cytoplasm of cells, where it proteolytically converts pro-IL-1β into the mature form involved in inflammation [20]. In this study, we observed that PLD inhibited the PMACI-induced caspase-1 activation in HMC-1 cells. These results suggest that the anti-allergic effects of PLD are also associated with the inhibition of caspase-1 activation.

To gain insight into the mechanism of mast cell inhibition by PLD, we investigated its effect on NF-κB activation in mast cells. Before cell stimulation, NF-κB subunits are sequestered in the cytoplasm by its inhibitory proteins (IκB). Upon stimulation, IκB-α is phosphorylated by its kinase, which leading to nuclear localization of NF-κB and inducing the transcriptional activation of target genes [21]. Previous studies showed that NF-κB plays a critical role in chronic inflammatory diseases, including AR [22, 23]. Wang et al. reported that NF-κB p65 may upregulate the transcription and secretion of TNF-α and IL-6, resulting in the overexpression of MUC5AC in allergic inflammatory processes [24]. In this study, we found that PLD suppressed PMACI-induced IκBα degradation in HMC-1 cells. These results suggest that PLD inhibits PMACI-induced the expression of TNF-α and IL-1β and caspase-1 activation by suppressing the activation of NF-κB signaling pathway.

The MAPK cascade is an important signaling pathway in inflammatory responses [25, 26]. The induction of inflammatory cytokine genes requires activation of all three types of MAPKs. A growing body of evidence indicates that activation of ERK, and JNK by environmental stimuli play a significant role in the cytokine expression in mast cells [14, 27, 28]. In this study, we found that PLD suppressed the protein expression levels of p-p38, p-ERK and p-JNK induced by PMACI in HMC-1 cells. These results suggest that PLD inhibits PMACI-induced the expression of TNF-α and IL-1β and caspase-1 activity by suppressing the activation of MAPK signaling pathway.

In conclusion, our results showed that PLD inhibits PMACI-induced the expression of TNF-α and IL-1β and caspase-1 activity by suppressing the activation of NF-κB signaling pathway and MAPK signaling pathway in HMC-1 cells. Therefore, our data suggest the possible therapeutic application of PLD in the treatment of AR.

Disclosure of conflict of interest

None.

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