Research article

*p*-coumaric acid, an active ingredient of *Panax ginseng*, ameliorates atopic dermatitis-like skin lesions through inhibition of thymic stromal lymphopoietin in mice

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Background: Atopic dermatitis (AD) is associated with chronic skin inflammatory reactions. *p*-coumaric acid (*p*CA) is an active ingredient of *Panax ginseng* Meyer (Araliaceae).

Methods: Here, we estimated an anti-AD effect of *p*CA on activated mast cells, activated splenocytes, and a mouse model of AD. Cytokines levels were measured by ELISA and protein activation was analyzed by Western blotting. 2,4-dinitrofluorobenzene (DNFB) was used to induce AD-like skin lesions.

Results: The treatment with *p*CA suppressed the productions and mRNA expressions of thymic stromal lymphopoietin (TSLP), TNF-α, IL-6, and IL-1β in HMC-1 cells. *p*CA downregulated the expressions of RIP2 and caspase-1, phosphorylated-(p)p38/pJNK/pERK, and pIkB in the supernatant of stimulated splenocytes. Comparing to DNFB-sensitized control group, *p*CA-treated group alleviated pathological changes of skin lesions. *p*CA decreased the proteins and mRNA expressions levels of TSLP, IL-6, and IL-4 in the skin lesions. Caspase-1 activation was also downregulated by *p*CA treatment in the AD-like lesions. The serum levels of histamine, IgE, TSLP, TNF-α, IL-6, and IL-4 were suppressed following treatment with *p*CA.

Conclusion: This study suggests that *p*CA has the potential to improve AD by suppressing TSLP as well as inflammatory cytokines via blocking of caspase-1/NF-κB signal cascade.

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1. Introduction

Atopic dermatitis (AD) is a recurrent and chronic inflammatory skin disorder characterized by debilitating itch, signs of anxiety and depression, impaired quality of life, sleep deprivation, and decreased productivity [1]. AD has become a worldwide public health problem with increasing prevalence in most countries [2].

In atopic disorders including AD and asthma, cytokine thymic stromal lymphopoietin (TSLP) has a fundamental role. A severe AD development was resulted from overexpressed TSLP in the skin of mice [3]. On the contrary, impairment of AD-like skin lesions was resulted from knockout of TSLP in the skin of mice [4]. Epithelial cells, keratinocytes as well as mast cells are closely involved in atopic diseases [5]. Mast cell activation was elevated in atopic disorder models, suggesting a role of mast cells in atopic disorder [6–8].

Caspases are involved in apoptosis, whereas caspase-1 which regulates inflammatory responses, is an inflammatory caspase and *p*CA is the most commonly appearing isomer in nature [13]. *p*CA is an active ingredient of fruit, leaves, and roots of *Panax ginseng* Meyer (Araliaceae) [14,15].

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P. ginseng exerts various activities such as anticancer, antioxidant, or antiinflammatory properties [15,16]. P. ginseng and ginsenosides have known to be effective in treating various skin diseases, including AD [18]. However, the regulatory mechanisms of pCA in AD have not been fully described. Thus, we explored whether pCA can ameliorate AD by means of in vitro and in vivo models.

2. Materials and methods

2.1. pCA and dexamethasone (DEX)

pCA (purity > 98%, Fluka™, Mexico City, Mexico) was prepared according to studies of Kim et al [19] and Cha et al [13]. DEX was solved considering study of Chen et al [20].

2.2. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

An MTT (Sigma Chemical Co.) assay was performed to estimate cytotoxicity, as described previously [21].

2.3. Intracellular calcium assessment

The intracellular calcium levels were examined using a fluorospectrometer (excitation 360nm, emission 450nm, Thermo Fisher Scientific, Waltham, MS, USA) for 100 s, as previously described [22].

2.4. Measurement of cytokines and IgE levels

The values of TSLP, TNF-α, IL-6, IL-1β, IL-4, IFN-γ, and IgE were measured by means of ELISA method (Pharmingen, Sandiego, CA, USA; R & D system Inc., Minneapolis, MN, USA), as previously described [23].

2.5. Quantification of gene expression

Polymerase chain reaction (PCR) was conducted considering the study of Han et al [24].

2.6. Western blotting

Western blotting was conducted considering the study of Moon et al [25].
2.7. Caspase-1 activity assay

The caspase-1 activities in the lysates and lesions homogenates were determined by means of a kit from R & D system, as described previously [26].

2.8. Nuclear and cytoplasmic extract preparation

Harvested HMC-1 cells were used to prepare cytoplasmic and nuclear extracts, as previously described [21].

2.9. AD-like skin lesions induction

All treatment and procedure complied with internationally accepted principles for laboratory animal care according to the United States guidelines (NIH publication no. 85-23, revised in 1985). General treatment and process of an AD mouse model are shown in supplementary Fig. 1B. AD-like skin lesions were induced, as described previously [24]. Ethics for animal study (KHUASP (SE)-18-022) was approved by the animal care committee of Kyung Hee University.

2.10. Histological analysis

The mast cells, inflammatory cells, and epidermal thickness in de-paraffinized and re-hydrated tissue slides were visualized by means of hematoxylin and eosin (H&E) or toluidine blue staining, as previously described [4].

2.11. Histamine assay

Serum histamine was evaluated by o-phthaldialdehyde (Sigma Chemical Co.) fluorospectrometric procedure, as described previously [27].

2.12. Statistical analysis

Each value was showed as mean ± standard error of mean (SEM). A SPSS statistical program (version 25, IBM) was used. Independent t-test was used to compare the medians of two groups (B vs (PMA plus A23187; PMACI), vehicle vs control). ANOVA with Tukey post hoc test was employed to compare differences among PMACI/control group vs pCA or DEX-treated groups. P values lower than 0.05 were considered to be significant.
The therapeutic potential on intracellular calcium level in pm (50 of inactivated group, B (0.5, 5, and 50 BAPTA-AM-treated group in HMC-1 cells. The treatment with phore (PMACI)-stimulation showed a marked increase in intracellular calcium level. However, treatment with pCA led to a significant downregulation in intracellular calcium levels (Fig. 1B). The therapeutic potential on intracellular calcium level in pCA (50 μg/ml)-treated group was comparable with calcium chelator BAPTA-AM-treated group in HMC-1 cells. The treatment with pCA (5 and 50 μg/ml), alike DEX, reduced the productions and mRNA expressions of TSLP, IL-6, IL-1β, and tumor necrosis factor (TNF)-α, in HMC-1 cells (p < 0.05; Fig. 1A and B). pCA alone did not produce a significant change on these levels (Fig. 1).

3. Results

3.1. pCA treatment alleviated inflammatory cytokines levels in HMC-1 cells

In general, inflammatory cytokines released from mast cells play a critical role during induction of AD [28]. Thus, we investigated a regulatory effect of pCA on inflammatory cytokines levels in activated HMC-1 cells. First, we found that 0.5, 5, and 50 μg/ml of pCA and 10mM of DEX did not show cytotoxicities in activated HMC-1 cells (Fig. 1A). The protein kinase C activator plus calcium ionophore (PMACI)-stimulation showed a marked increase in intracellular calcium level. However, treatment with pCA led to a significant downregulation in intracellular calcium levels (Fig. 1B). The therapeutic potential on intracellular calcium level in pCA (50 μg/ml)-treated group was comparable with calcium chelator BAPTA-AM-treated group in HMC-1 cells. The treatment with pCA (5 and 50 μg/ml), alike DEX, reduced the productions and mRNA expressions of TSLP, IL-6, IL-1β, and tumor necrosis factor (TNF)-α, in HMC-1 cells (p < 0.05; Fig. 1C and D). pCA alone did not produce a significant change on these levels (Fig. 1).

3.2. pCA treatment reduced RIP2 and caspase-1 expressions in activated HMC-1 cells

For investigation about the inhibitory mechanism of pCA on inflammatory cytokines, we measured the expression levels of receptor interacting protein 2 (RIP2) and caspase-1 in activated HMC-1 cells. As indicated in Fig. 2A and B, Western blotting showed that PMACI addition significantly upregulated the activation levels of RIP2 and caspase-1 (p < 0.05). 5 and 50 μg/ml of pCA treatment significantly suppressed the activation levels of RIP2 and caspase-1 in activated HMC-1 cells (p < 0.05; Fig. 2A and B). In addition, pCA (0.5, 5, and 50 μg/ml) decreased the activities of caspase-1 in activated HMC-1 cells (p < 0.05; Fig. 2C). pCA itself did not influence inactivated HMC-1 cells (Fig. 2A–C). The alleviated expressions of RIP2 and caspase-1 in pCA (50 μg/ml)-treated group were comparable with those of DEX-treated group in HMC-1 cells (Fig. 2A–C).

3.3. pCA treatment attenuated MAPKs and pIkKβ/pIkBα/NF-κB expressions in activated HMC-1 cells

We further evaluated whether pCA would down-regulate MAPKs, p38/c-Jun N-terminal kinase (JNK)/extracellular signal-regulated kinase (ERK) and pIkKβ/pIkBα/NF-κB signaling pathways which play a pivotal role in inflammatory diseases [29]. As shown in Fig. 2D and E, PMACI addition significantly upregulated the phosphorylation levels of p38, JNK, and ERK compared to those of inactivated group, B (p < 0.05). The treatment with pCA (50 μg/ml), alike DEX-treated group, significantly decreased the expression levels increased by PMACI (p < 0.05; Fig. 2D and E). Also, PMACI addition upregulated the phosphorylated-(p)IkKβ, pIkBα, and NF-κB expression levels (p < 0.05; Fig. 2F and G). pCA (50 μg/ml), alike DEX-treated group, reduced the expression levels (p < 0.05; Fig. 2F and G). pCA alone did not produce a significant change on these levels (Fig. 2D–G).

3.4. pCA treatment decreased inflammatory cytokines in activated splenocytes

Activation with anti-CD3 and anti-CD28 antibodies results in the activation of primary splenic cells and the production of inflammatory cytokines [30,31]. Hence, to identify an inhibitory effect of pCA treatment on splenic T cells-derived inflammatory cytokines, we estimated TSLP, TNF-α, IL-6, and IFN-γ levels on supernatant in anti-CD3 and anti-CD28 antibodies-stimulated splenocytes. Table 1 showed that the productions of TSLP, TNF-α, IL-6, and IFN-γ upregulated by anti-CD3 and anti-CD28 antibodies were inhibited by 50 μg/ml of pCA (p < 0.05). These inhibitory effects of pCA were comparable to DEX group.

3.5. pCA treatment alleviated AD-like symptoms from skin lesions

We figured out that pCA treatment ameliorated the levels of inflammatory cytokines in vitro. Hence, we tried to verify whether pCA regulates pathological symptoms of AD-like skin lesions in a 2,4-dinitrofluorobenzene (DNFB)-induced AD mouse model. Fig. 3A showed that pCA treatment markedly alleviated the striking hemorrhage, excretion, and erosion compared to control group in DNFB-induced AD-like skin lesions. pCA treatment reduced the epidermal thickening as well as accumulation of inflammatory cells (middle panel, p < 0.05) and mast cells (lower panel, p < 0.05) in the lesions (Fig. 3B and C). In addition, pCA treatment significantly inhibited the scratching behavior (p < 0.05; Fig. 3D). Treatment with DEX, as observed in the pCA-treated group, alleviated the pathological symptoms of AD-like skin lesions (Fig. 3).

3.6. pCA treatment inhibited inflammatory cytokine levels and caspase-1 activation in the skin lesions

Next, we investigated whether pCA can reduce TSLP, IL-6, and IL-4 levels in the skin lesions. TSLP, IL-6, and IL-4 protein levels were suppressed by pCA (p < 0.05; Fig. 4A). Also, pCA suppressed the TSLP, IL-6, and IL-4 mRNA expressions in the skin lesions (p < 0.05; Fig. 4B and C). DEX decreased the TSLP, IL-6, and IL-4 proteins and mRNA expressions (p < 0.05; Fig. 4A–C). Treatment with pCA reduced inflammatory cytokine levels through blocking of caspase-1 signal cascade in HMC-1 cells. Hence, we finally investigated whether pCA can modulate protein expression and activity of caspase-1 in the skin lesions. DNFB sensitization enhanced the protein levels of caspase-1, whereas pCA or DEX significantly
reduced the protein expressions of caspase-1 in the skin lesions \((p < 0.05; \text{Fig. } 4\text{D and }E)\).

CA or DEX significantly alleviated activities of caspase-1 in the skin lesions \((p < 0.05; \text{Fig. } 4\text{F})\).

3.7. pCA treatment decreased the serum histamine, IgE, and inflammatory cytokines in AD-like mouse model

Histamine and IgE induces inflammatory cell infiltration and scratching behaviors, and elevates inflammatory cytokines levels in AD [32]. Thus, we estimated the histamine, IgE, TSLP, IL-6, IL-4, and TNF-α, levels in the serum of DNFB-sensitized mice. Table 2 showed that pCA or DEX reduced the histamine, IgE, TSLP, IL-6, IL-4, and TNF-α levels increased by DNFB-sensitization \((p < 0.05)\).

4. Discussion

In general, protein kinase C (PKC) activation and intracellular calcium upregulation result from mast cell activation by binding with antigens [33]. To reenact this circumstance, we used PMA for PKC activation and calcium ionophore for intracellular calcium
elevation in HMC-1 cells. Our preliminary experiment showed that exposure to PMACI increased the mRNA expression and production of TSLP in HMC-1 cells [12]. Deficiency of TSLP ameliorated skin inflammation in a murine AD model [34]. Intradermal injection of recombinant TSLP increased scratching behavior in a murine AD model [35]. TSLP expression was elevated in lesional skin from AD patients [36]. Our results presented that the protein and mRNA expression levels of TSLP in mast cells, sponcocyes, and serum were downregulated by pCA treatment (Figs. 1C and D, 4A–4C; Tables 1 and 2). Hence, we can assume that pCA might be beneficial to treat atopic and inflammatory disorders. Also, cytokine TNF-α, IL-1β, and IL-4 increased in AD patients [37]. IL-6 levels increased in skin lesions of AD patients [38]. Higher levels of IFN-γ showed in AD patients in comparison with control subjects [39]. In the present study, pCA improved the levels of TNF-α, IL-1β, IL-6, IL-4, and IFN–γ, validating a potential of pCA in AD.

Calcium chelator BAPTA-AM reduces RIP2 expression in HMC-1 cells, suggesting that calcium is an upstream regulator of RIP2 [40]. RIP2 induces caspase-1 activation by promoting its oligomerization [41]. Treatment with pCA prevented the increase of intracellular calcium levels, activation of RIP2 and caspase-1, phosphorylation of p38, JNK, and ERK [46]. Caspase-1 increases p38 phosphorylation blocking of calcium/RIP2/caspase-1 signaling in HMC-1 cells. Thus, the results of the present study showed that phosphorylation of p38, JNK, and ERK, activation of NF-κB. We showed that pCA downregulated intracellular calcium levels, activation of RIP2 and caspase-1, phosphorylation of p38, JNK, and ERK, activation of NF-κB, as well as phosphorylation of IKKβ and IκBα in HMC-1 cells (supplementary fig. 2). Additionally, pCA decreased production of TSLP, TNF-α, IL-6, IL-4, and IFN–γ in the supernatant of stimulated splenic cells. Treatment with pCA alleviated AD-like skin lesions and decreased the protein and mRNA levels of TSLP, IL-6, and IL-4, as well as caspase-1 activation in the skin lesions. Histamine, IgE, TSLP, TNF-α increased in AD patients [51]. Oral administration of pCA decreased an infiltration of mast cell into skin lesions (Fig. 3B and C). Serum histamine levels in AD patients were elevated compared with healthy controls [52]. Anti-histamine therapy results in an improvement of AD symptoms, with a remarkable amelioration in pruritus [52]. Administration of pCA produced a decrease in serum histamine levels and scratching behaviors (Table 2; Fig. 3D). Therefore, we presuppose that pCA may be useful to reduce histamine in AD.

Finally, epidermal thickening and inflammatory cells infiltration are histological features of AD in human [50]. AD-like skin lesions, epidermal thickening, and inflammatory cells infiltration were reduced by pCA administration (Fig. 3). Choi and colleagues reported that an infiltration of mast cell into skin lesions is elevated in AD, presenting a role of mast cells in AD [51]. Oral administration of pCA decreased an infiltration of mast cell into skin lesions (Fig. 3B and C). Serum histamine levels in AD patients were elevated compared with healthy controls [52]. Anti-histamine therapy results in an improvement of AD symptoms, with a remarkable amelioration in pruritus [52]. Administration of pCA produced a decrease in serum histamine levels and scratching behaviors (Table 2; Fig. 3D). Therefore, we presuppose that pCA may be useful to reduce histamine in AD.

### Conflicts of interest

The authors have no conflicts of interest to declare.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at [https://doi.org/10.1016/j.jggr.2020.06.004](https://doi.org/10.1016/j.jggr.2020.06.004)

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