Ursolic acid downregulates thymic stromal lymphopoietin through the blockade of intracellular calcium/caspase-1/NF-κB signaling cascade in HMC-1 cells

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Abstract. Thymic stromal lymphopoietin (TSLP) plays an important role in allergic disorders, including atopic dermatitis and asthma. Ursolic acid (UA) has various pharmacological properties, such as antioxidant, anti-inflammatory and anticancer. However, the effect of UA on TSLP regulation has not been fully elucidated. The aim of the present study was to analyze how UA regulates the production of TSLP in the human mast cell line HMC-1. Enzyme-linked immunosorbent assay, quantitative polymerase chain reaction analysis, western blotting, caspase-1 assay and fluorescent measurements of intracellular calcium levels were conducted to analyze the regulatory effects of UA. The results revealed that UA inhibited TSLP production and mRNA expression. In addition, UA reduced the activation of nuclear factor-κB and degradation of IκBα. Caspase-1 activity was increased by exposure to phorbol myristate acetate plus calcium ionophore, whereas it was reduced by UA. Finally, UA treatment prevented an increase in intracellular calcium levels. These results indicated that UA may be a useful agent for the treatment and/or prevention of atopic and inflammatory diseases, and its effects are likely mediated by TSLP downregulation.

Introduction

Atopic dermatitis (AD) is a recurrent and chronic inflammatory skin disorder that affects children as well as adults (1,2). The prevalence of AD is ~1-3% in adults and 25% in children of industrialized countries (3), and it has increased in the urban areas of such countries over the past decade (4). As a result of the increasing prevalence of AD, the burden of AD-associated medical costs has increased in industrialized countries (5). In addition, the recurrent eczema lesions, itching, lack of sleep and restricted diet may compromise the quality of life of AD patients (5).

Thymic stromal lymphopoietin (TSLP) is considered as a pivotal factor in the pathogenesis of allergic diseases, such as AD and asthma. In patients with AD, TSLP gene expression increased by epicutaneous house dust mite injections (6). The protein and mRNA expression levels of TSLP in skin lesions of AD patients are higher compared with those in healthy controls (7). In addition to epithelial cells and keratinocytes, mast cells also play an important role in atopic diseases (8). The increases in the population and activation of mast cells in AD models reported by several researchers (9-11) indicate the significance of mast cells in AD. HMC-1 is a human mast cell line (12). The effects of UA on the HMC-1 cell line were examined, as similar levels of TSLP are produced by HMC-1 and bone marrow-derived mast cells (13).

In general, protease caspases play critical roles in apoptosis, whereas caspase-1 is implicated in inflammatory responses (14-16). Deficiency of caspase-1 ameliorates dextran sulfate sodium-induced intestinal inflammation (17). In addition, TSLP expression and production were found to be mediated by caspase-1 and nuclear factor (NF)-κB signaling in HMC-1 cells in a previous study (18). Additionally, caspase-1 inhibitor treatment may decrease NF-κB activation, suggesting that caspase-1 acts as an upstream regulator of NF-κB (18).

Ursolic acid (UA; Fig. 1), a pentacyclic triterpenoid found in holy basil and apple peels (19), has various pharmacological properties, such as antioxidant, anti-inflammatory and anticancer (20,21). Recently, Gan et al (22) reported that UA ameliorates CCl4-induced liver fibrosis. However, the regulatory effect of UA on TSLP production by mast cells has not been fully elucidated. The aim of the present study was to

Key words: thymic stromal lymphopoietin, ursolic acid, nuclear factor-κB, caspase-1, intracellular calcium
investigate the effects of UA on the HMC-1 human mast cell line and determine whether UA can regulate TSLP production in mast cells.

Materials and methods

Materials. UA, phorbol myristate acetate (PMA), calcium ionophore A23187, avidin-peroxidase and dimethyl sulfoxide were obtained from Sigma-Aldrich; Merck KGaA. TMB substrate and tumor necrosis factor (TNF)-α antibodies were purchased from Pharmingen. Power SYBR®-Green PCR master mix was purchased from Applied Biosystems; Thermo Fisher Scientific, Inc. TSLP antibodies and caspase-1 assay kit were obtained from R&D Systems, Inc, Finally, IKKβ, PARP, GAPDH, IkBα and NF-κB p65 antibodies were obtained from Santa Cruz Biotechnology, Inc.

Cell culture. HMC-1 cells were cultured in IMDM with heat-inactivated fetal bovine serum (10%), streptomycin (100 µg/ml) and penicillin (100 U/ml) at 37°C with 5% CO2.

MTT assay. MTT assay was performed to measure cytotoxicity, as described previously (23). HMC-1 cells (3x10⁵) were incubated with UA (0.002-0.2 µg/ml) in 24-well plates, which were subsequently incubated with MTT solution (5 mg/ml) for 4 h. To dissolve the MTT formazan, 1 ml of dimethyl sulfoxide was added and 200 μl of supernatant were removed and transferred to a 96-well microplate. Finally, each well was read at 540 nm.

Cytokine assay. HMC-1 cells (3x10⁵) were pretreated with UA (0.002-0.2 µg/ml) for 1 h prior to stimulation with 0.05 µM PMA plus 1 µM calcium ionophore (PMAcI), and then incubated for 7 h. TSLP and TNF-α levels were assessed in the culture supernatants using ELISA, as described previously (24).

Quantitative polymerase chain reaction (qPCR) analysis. HMC-1 cells (1x10⁵) were pretreated with UA (0.002-0.2 µg/ml) for 1 h prior to PMACI stimulation, and then incubated for 5 h. qPCR was carried out using the Power SYBR-Green PCR master mix. mRNA detection was performed with the ABI StepOne real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) as described previously (25). PCR analysis was conducted using the following primers: TSLP, forward 5′-ccc AGG cTA TTc GGA AAc TcA G-3′ and reverse 5′-ccc AGG cTA TTc GGA AAc TcA G-3′; GAPDH, forward 5′-CCGACAGTCGAGCCGCATCTTTTT-3′ and reverse 5′-ACC AACGCCGTTGACTCCGACCTT-3′.

Caspase-1 assay. HMC-1 cells (5x10⁶) were pretreated with UA (0.002-0.2 µg/ml) for 1 h prior to PMACI stimulation, and then incubated for 1 h. Caspase-1 activation was evaluated using a caspase-1 assay kit, as described previously (26).

Nuclear and cytoplasmic extracts. HMC-1 cells (5x10⁶) were pretreated with UA (0.002-0.2 µg/ml) for 1 h prior to PMACI stimulation, and then incubated for 2 h. Isolation of nuclear and cytoplasmic proteins was carried out as described previously (27). In brief, the cells were washed in ice-cold phosphate-buffered saline (PBS) and centrifuged at 15,000 x g for 1 min. The cells were resuspended in 40 μl of a cold hypotonic buffer (10 mM HEPES/KOH, 2 mM MgCl₂, 0.1 mM EDTA, 10 mM KCI, 1 mM DTT, and 0.5 mM PMSF, pH 7.9). Next, the cells were swollen on ice for 15 min, lysed gently with 2.5 μl 10% Nonidet P-40 and centrifuged at 15,000 x g for 3 min at 4°C. The supernatant was then collected and used as the cytoplasmic extract. The pellets of nuclei were gently resuspended in 40 μl cold saline buffer (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT and 0.5 mM PMSF, pH 7.9) and placed on ice for 20 min. After centrifugation at 15,000 x g for 15 min at 4°C, the aliquots of supernatant containing the nuclear proteins were frozen in liquid nitrogen and stored at -70°C until analysis. Finally, the bicinechonic acid protein assay (Sigma-Aldrich; Merck KGaA) was used to measure the protein concentrations.

Western blot analysis. HMC-1 cells (5x10⁵) were pretreated with UA (0.002-0.2 µg/ml) for 1 h prior to PMACI stimulation. Proteins of obtained lysates were separated and transferred to nitrocellulose paper, as described previously (28). In brief, the cell lysates were prepared in a sample buffer containing sodium dodecyl sulfate (SDS). The samples were heated at 95°C for 5 min and briefly cooled on ice. Following centrifugation at 15,000 x g for 5 min, the proteins in the cell lysates were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. The membrane was blocked with 5% skimmed milk in PBS-Tween-20 for 1 h at room temperature and then incubated with primary antibodies (1:500 dilution for all primary antibodies; NF-κB; cat. no. sc-8008; IkBα; cat. no. sc-847; IKKβ; cat. no. sc-7607; PARP; sc-8007; GAPDH; cat. no. sc-32233; all purchased from Santa Cruz Biotechnology, Inc.) overnight at 4°C and secondary antibodies (mouse anti-rabbit IgG-HRP: 1:5,000; goat anti-mouse IgG-HRP: 1:5,000; cat. no. sc-205; goat anti-rabbit IgG-HRP, 1:5,000; cat. no. sc-205; all purchased from Santa Cruz Biotechnology, Inc.) antibodies for 1 h at room temperature. Finally, the protein bands were visualized by an enhanced chemiluminescence solution (Amersham; GE Healthcare) following the manufacturer’s instructions. All protein expression levels were quantitated using ImageJ software (National Institutes of Health).

Fluorescent measurements of the intracellular calcium level. HMC-1 cells (1x10⁵) were pretreated with Fura-2/AM for 30 min. After washing twice with medium containing the extracellular calcium chelator EGTA (0.5 mM), the cell suspension (1x10⁵) was seeded into a 96-well plate and pretreated with UA (0.002-0.2 µg/ml) for 20 min. Next, the cells were stimulated with PMACI for 5 min. Fluorescence was measured at 440 nm (excitation, 360 nm) in a spectrofluorometer (29).

Statistical analysis. IBM SPSS 23.0 (IBM Corp.) was used to statistically analyze the results. Statistical analyses included performing independent t-tests and analysis of variance with Tukey’s post hoc test. The differences were considered statistically significant at P<0.05, and the results are presented as mean ± standard error of the mean.

Results

Effect of UA on the production of TSLP. To evaluate the effect of UA on the production of TSLP in HMC-1 cells, HMC-1 cells
were exposed to PMACI for 7 h. The levels of TSLP were evaluated with ELISA. Exposure to PMACI elevated the production of TSLP in HMC-1 cells (Fig. 2A); however, the elevated TSLP production was significantly lowered by UA (0.02 and 0.2 µg/ml; Fig. 2A). The TSLP production levels at concentrations of 0.002, 0.02 and 0.2 µg/ml were 0.127±0.006, 0.111±0.003 and 0.101±0.004, respectively. The levels of TSLP production in the blank and control groups were 0.079±0.002 and 0.135±0.008, respectively. Treatment with UA (0.2 µg/ml) reduced the TSLP production up to 61.442±6.947%. However, UA alone did not notably change the level of TSLP production from that in the blank (PBS-treated cells) group (data not shown). When HMC-1 cells were treated with UA at concentrations of 0.002-0.2 µg/ml, cell viability did not change (Fig. 2C). Higher concentrations of UA (2 and 20 µg/ml) did not achieve further TSLP inhibition (Fig. 2A). In addition, prolonged UA pretreatment did not achieve further TSLP inhibition (Fig. 2D). A 1-h pretreatment with UA inhibited TSLP production by up to ~60%, whereas a 24-h UA pretreatment produced a ~40% TSLP inhibition (Fig. 2D). This may be due to spontaneously released TSLP. Furthermore, when UA was added 1 h after PMACI stimulation, it did not significantly inhibit TSLP production (Fig. 2E).

Effect of UA on mRNA expression of TSLP. To determine the regulatory effect of UA on the TSLP mRNA expression, a variety of concentrations (0.002-0.2 µg/ml) of UA were added as pretreatment for 1 h prior to the exposure of HMC-1 cells to PMACI. Exposure to PMACI elevated TSLP mRNA expression, whereas the elevated TSLP mRNA expression was lowered by UA treatment (Fig. 2B). The TSLP mRNA expression values at concentrations of 0.002, 0.02 and 0.2 µg/ml were 22.667±2.333, 17.333±1.333 and 15.667±1.453, respectively. The relative expression levels of TSLP mRNA in the blank and control groups were 1.800±0.640 and 25.333±1.856, respectively.

Effects of UA on activation of NF-κB and degradation of IkBα. To investigate whether the regulatory effect of UA is mediated by NF-κB/IkBα signaling, the activation of NF-κB p65 and degradation of IkBα were assessed by western blot analysis. Exposure to PMACI elevated NF-κB activation in the nuclear extract, whereas the elevated NF-κB activation was lowered by UA treatment (Fig. 3). The relative intensity values for NF-κB activation in the blank, control and UA groups (0.002, 0.02 and 0.2 µg/ml) were 0.408±0.025, 0.629±0.039, 0.533±0.034, 0.455±0.028 and 0.424±0.053, respectively. However, UA treatment did not produce a significant change in cytoplasmic NF-κB protein levels (Fig. 3). Proteolytic degradation of IkBα results in activation of NF-κB (30,31); thus, we investigated whether the regulatory effect of UA is due to IkBα degradation. Exposure to PMACI elevated IkBα degradation in the cytoplasmic extract; however, the elevated IkBα degradation was lowered by UA treatment (Fig. 3). The relative intensity values of IkBα in the blank, control and UA groups were 0.438±0.019, 0.288±0.010, 0.304±0.016, 0.363±0.006 and 0.384±0.012, respectively. Phosphorylation and degradation of IkBα is due to IkB kinase (IKK) complex activation, and the IKK complex consists of three core subunits (IKKα, IKKβ and IKKγ), among which IKKβ is predominant (32); thus, we investigated whether IkBα degradation by UA is due to IKKβ. Exposure to PMACI elevated the IKKβ protein levels; however, the elevated IKKβ protein levels were reduced by UA treatment (Fig. 3). The relative intensity values of IKKβ in the blank, control and UA groups were 0.223±0.019, 0.298±0.004, 0.287±0.003, 0.253±0.006 and 0.299±0.007, respectively.

Effect of UA on the activation of caspase-1. The level of caspase-1 activation was evaluated with a caspase-1 assay kit to examine whether the effect of UA was mediated through caspase-1 activation. Exposure to PMACI increased the levels of caspase-1 activation, whereas the elevated caspase-1 activation was lowered by UA treatment (Fig. 4). The levels of caspase-1 activation in the blank, control and UA groups were 0.281±0.005, 0.335±0.007, 0.330±0.009, 0.307±0.004 and 0.299±0.007, respectively.

Effect of UA on calcium level. An increase in the intracellular calcium levels has been reported to enhance caspase-1 activation (33). Thus, the regulatory effect of UA on intracellular calcium levels was examined in HMC-1 cells. Exposure to PMACI increased intracellular calcium levels; however, this increase was prevented by UA treatment (Fig. 5).

Effect of UA on pro-inflammatory cytokine levels in HMC-1 cells. The pro-inflammatory cytokine tumor necrosis factor (TNF)-α is overexpressed in AD (34). To substantiate the presence of UA effects in AD, the levels of TNF-α were measured in HMC-1 cells. Exposure to PMACI increased TNF-α production in HMC-1 cells (Fig. 6); however, this increase in TNF-α production was markedly reduced by treatment with 0.2 µg/ml UA (Fig. 6).

Discussion

In the present study, UA was shown to suppress the production and mRNA expression of TSLP in HMC-1 cells. In addition, UA reduced NF-κB activation, IkBα degradation and caspase-1 activity in HMC-1 cells. Finally, it was demonstrated that UA downregulated intracellular calcium levels in HMC-1 cells.

When mast cells are activated, there are increases in the activation of protein kinase C (PKC) and intracellular calcium levels (35). To replicate this condition in the present study, PMA
was used to activate PKC, and calcium ionophore to increase the levels of intracellular calcium. Exposure to PMACI was reported to increase the production and mRNA expression of TSLP in HMC-1 cells (18), and high levels of TSLP...
have been detected in the skin lesions of AD patients (36). Moreover, it has been suggested that TSLP enhances skin inflammatory responses in a murine AD model (37), whereas dexamethasone, an anti-inflammatory drug, inhibits expression of TSLP in a murine model of AD (38). The results of this study revealed that the production and mRNA expression of TSLP were reduced by UA treatment in HMc-1 cells (Fig. 2). Therefore, UA appears to be helpful in the treatment of atopic and inflammatory disorders.

Lee and Ziegler (39) suggested that TSLP expression is mediated by NF-κB. Our previous report clarified that TSLP production and mRNA expression were regulated via NF-κB signaling in mast cells (18). The present study demonstrated that NF-κB activation and IκBα degradation were lowered by UA treatment (Fig. 3). Furthermore, Shen et al (40) reported that NF-κB is a critical transcription factor for the production of TSLP. Thus, it may be hypothesized that UA reduces TSLP levels via blockade of NF-κB signaling in HMc-1 cells.

Pro-inflammatory stimuli generally activate caspase-1 (41). Several reports have demonstrated that caspase-1 activation results from pro-inflammatory stimulation, such as exposure to PMACI (42,43). In the present study, caspase-1 activation was found to be lowered by UA treatment (Fig. 4). Thus, it may be hypothesized that UA suppresses the production and mRNA expression of TSLP by blocking caspase-1 activation in HMc-1 cells.
modulation of intracellular calcium levels (45). Tang et al (45) reported that UA inhibits mitochondrial calcium release. In the present study, UA treatment prevented an increase in intracellular calcium, suggesting that UA contributes to the prevention of ER and mitochondrial calcium release (Fig. 5). An increase in intracellular calcium levels promotes the activation of caspase-1 (33), whereas caspase-1 activation in HMC-1 cells may be reduced by treatment with the calcium chelator BAPTA-AM (46). The results of the present study revealed that an increase in intracellular calcium may be prevented by UA treatment (Fig. 5). Thus, UA appears to decrease TSLP levels via blockade of calcium/caspase-1/NF-κB signaling in HMC-1 cells (Fig. 7). When UA was added following PMACI stimulation, it did not produce a significant change in TSLP inhibition (Fig. 2E). Thus, UA may exert a preventive effect rather than a therapeutic effect on AD. Finally, UA significantly attenuated the effects of PMACI; however, the levels of TNF-α, calcium fluorescence, TSLP/GAPDH and NF-κB/GAPDH did not return to those observed in non-PMACI-treated cells. High-fat diet exacerbated AD-like skin lesions in NC/Nga mice and increased TSLP levels in skin lesions, whereas pro-inflammatory cytokines, such as interleukin (IL)-4, IL-13, interferon-γ and TNF-α did not exhibit significant changes (47). To confirm the role of TSLP in AD-like skin lesions, when Moon et al (47) prepared TSLP knockout NC/Nga mice, TSLP deficiency markedly decreased AD-like skin lesions. Although the present conditions differ from those in the previous report (47), TSLP inhibition by UA may contribute significantly to the amelioration of the symptoms of allergic and atopic disorders.

In conclusion, the present study demonstrated that UA inhibits the production and mRNA expression of TSLP in HMC-1 cells. Moreover, UA decreased the activation of NF-κB, degradation of IκBα and activation of caspase-1. Furthermore, UA downregulated the levels of intracellular calcium. Therefore, these results suggest that UA, through its ability to downregulate TSLP, may be a valuable agent for the treatment and/or prevention of atopic and inflammatory diseases.

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Availability of data and materials

The data generated and analyzed during the present study are available from the corresponding authors on reasonable request.

Authors' contributions

PDM and NRH wrote the manuscript and conducted all experiments. JSL analyzed the data. HMK and HJJ designed the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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