3,4,5-Trihydroxycinnamic acid suppresses phorbol-12-myristate-13-acetate and A23187-induced mast cell activation in RBL-2H3 cells

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Received November 26, 2022; Accepted February 10, 2023

DOI: 10.3892/etm.2023.11926

Abstract. Previously, anti-inflammatory properties of 3,4,5-Trihydroxycinnamic acid (THC) has been reported in lipopolysaccharide (LPS)-induced RAW264.7 murine macrophage cells and in an LPS-induced sepsis BALB/c mice animal model. However, the effect of THC on the anti-allergic effect in mast cells has not been elucidated. The current study aimed to demonstrate the anti-allergic properties of THC and its underlying mechanism. Rat basophilic leukemia (RBL-2H3) cells were treated with phorbol-12-myristate-13-acetate (PMA) and A23187, a calcium ionophore, to be activated. The anti-allergic effect of THC was determined by measuring cytokine and histamine release. Western blotting was conducted to determine mitogen-activated protein kinases (MAPKs) activation and nuclear factor-κB (NF-κB) translocation. THC significantly suppressed PMA/A23187-induced tumor necrosis factor α secretion and THC also significantly attenuated degranulation, releasing β-hexosaminidase and histamine in concentration-dependent manners. Furthermore, THC significantly attenuated PMA/A23187-induced cyclooxygenase 2 expression and nuclear translocation of NF-κB. THC significantly suppressed PMA/A23187-induced increased phosphorylation of p38 mitogen-activated protein kinase, phosphorylated (p-)extracellular signal-regulated kinase 1/2 and p-c-Jun N-terminal kinase in RBL-2H3 cells. Overall, the results demonstrated that THC exhibited anti-allergic action by significantly attenuating degranulation of mast cells through the inhibition of MAPKs/NF-κB signaling pathway in RBL-2H3 cells.

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Abbreviations: THC, 3,4,5-trihydroxycinnamic acid; PMA, phorbol-12-myristate-13-acetate; TNF-α, tumor necrosis factor α; COX-2, cyclooxygenase 2; NF-κB, nuclear factor-κB; MAPKs, mitogen-activated protein kinases; ERK1/2, extracellular signal-regulated kinase 1/2; JNK, c-Jun N-terminal kinase; p38, p38 mitogen-activated protein kinase

Key words: 3,4,5-trihydroxycinnamic acid, phorbol-12-myristate-13-acetate, COX-2, NF-κB, RBL-2H3 cells, MAPKs

Introduction

Mast cells, located in the skin, lungs and mucosal surfaces, are crucial effector cells of both innate and adaptive immune defense against bacterial infection and various toxins (1,2). Activated mast cells release a broad range of immune mediators such as histamine, hexosaminidase, cytokines, lipid compounds and vasoactive amines to defend host (3). In addition to their positive effects of mast cells in host defense, abnormally activated mast cells are also reported to have detrimental effect in various allergic conditions including bronchial asthma, eczema, hay fever and food allergies (4,5). In allergic diseases, the excessive release of various mediators such as TNF-α from abnormally activated mast cells can initiate immediate hypersensitivity response associated with allergy. Therefore, pharmacological intervention of the proliferation and migration of mast cells could be a valuable approach for the attenuation of allergic conditions (6).

MAPKs have been reported to play a crucial role in the cytokine production of activated mast cells and subsequent proliferation and differentiation of mast cells (7). MAPKs consist of extracellular signal-regulated kinase1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (p38) (8). Phosphorylation of ERK has been reported to regulate various mast cell responses such as proliferation, migration and differentiation in allergic conditions (9). JNK has been also reported to be involved in an inflammatory response of activated mast cells (10). Activated JNK results in the expression of transcription factor AP1, which subsequently leads to the expression of many inflammatory mediators (10). P38 has been reported to be involved in the production of pro-inflammatory cytokines in activated mast

cells by increasing nuclear translocation of pro-inflammatory transcription factor, NF-κB (11).

Anti-inflammatory property of caffeic acid derivatives were reported in multiple studies (12-15). 3,4,5-Trihydroxycinnamic acid (THC), a derivative of caffeic acid, was originally reported in Rooibos tea (16) and has been demonstrated to possess a variety of pharmacological actions such as anti-inflammatory and neuroprotection actions (17-19). Previously, we reported that THC significantly suppressed LPS-induced expression of pro-inflammatory mediators through the suppression of NF-κB activation in BV2 microglial cells (20). We also demonstrated that THC suppressed LPS-induced inflammation via the upregulation of HO-1 through Nrf2 activation in RAW264.7 macrophage cells and increased the survival of animal in an LPS-induced endotoxemia mouse model (21,22). Caffeic acid phenethyl ester (CAPE), an ester derivative of hydroxycinnamic acid, was reported to inhibit cytokine-induced NF-κB signaling in macrophage cells (23) and plays an important role in the regulation of the host immune response (24). Recently, CAPE has been demonstrated to exert anti-allergic effects by inhibiting MAPK signaling and NF-κB activation abnormally activated HMC-1 human mast cells (6). Given that THC exerts wide range of anti-inflammatory actions and its derivative, CAPE possesses anti-allergic property, THC might also exhibit anti-allergic action in mast cells. Therefore, the objective of the current study was to examine the anti-allergic action of THC and its underlying mechanism in PMA/A23187-challenged RBL-2H3 mast cells in order to provide an useful therapeutic agent that could suppress various allergic conditions.

Materials and methods

Reagents and cell culture. Phorbol 12-myristate 13-acetate (PMA) and A23187 were purchased from Sigma-Aldrich; Merck KGaA). 3,4,5-Trihydroxycinnamic acid (THC) was purchased from AApin Chemicals Limited. Rat basophilic leukemia (RBL-2H3) cells were purchased from the Korea cell line bank (KCLB), KCLB cat #22256. RBL-2H3 cells were maintained in medium RPMI 1640 (RPMI 1640; Hyclon Laboratories) containing 10% heat-inactivated fetal bovine serum and 100 U/ml penicillin-streptomycin (Gibco) at 37˚C, 5% CO_2. Cells were incubated in the presence of the indicated concentrations of THC and then stimulated with 50 nM of PMA and 1 μM of A23187 for the indicated times.

TNF-α release assays. RBL-2H3 cells were incubated with THC (10~100 μM) for 1 h and then incubated in the presence or absence of PMA/A23187 for 30 min. TNF-α released into the medium of RBL-2H3 cultures was detected using enzyme-linked immunosorbent (ELISA) kits (R&D System) according to the manufacturer's instructions.

β-Hexosaminidase and histamine release assay. To examine the effect of THC on degranulation, the concentrations of β-hexosaminidase and histamine release were quantitatively measured. These enzymes are restricted within granules in mast cells and has been utilized as granule markers (25). RBL-2H3 cells were cultured in 12-well plates for 24 h. Then the supernatant was removed and the cells were further incubated with various concentrations of THC diluted in PIPES buffer for 1 h at 37˚C. After pretreatment, cells were washed twice with PIPES buffer, then stimulated with PMA/A23187 for 30 min at 37˚C. And 20 μl of supernatant was used to react with 80 μl of substrate buffer (2 mM 4-*p*-nitrophenyl-*N*-acetyl-β-$_D$-glucosaminide in 0.05 M sodium citrate buffer, pH 4.5) 30 min at 37˚C. The reaction was terminated with the addition of 200 μl of stop buffer (0.1 M $NaHCO_3$, pH 10). The absorbance was determined at 405 nm using microplate spectrophotometer (SpectraMax M5, Molecular Devices). The amount of histamine was detected by *o*-phthalaldehyde (OPT) spectroflurometric procedure. To 0.5 ml of media from each well, 0.1 ml of 1 M NaOH and 25 μl of OPT (1% (w/v) in methanol) were added. The supernatant was incubated for 4 min at room temperature. The reaction stopped by the addition of 50 μl of 3 M HCl. The absorbance was measured at excitation and emission wavelengths of 360 and 450 nm, respectively, using microplate spectrophotometer (SpectraMax M5, Molecular Devices).

Preparation of cytoplasmic and nuclear fractions. RBL-2H3 cells were treated with 10, 50, and 100 μM concentrations of THC for 1 h prior to PMA/A23187 treatment. Cells were washed with ice-cold PBS, and harvested, and centrifuged at 15,000 x g for 10 min at 4˚C. Cytoplasmic and nuclear extracts were prepared as described previously (26). Briefly, cells were resuspended in 40 μl of an ice-cold hypotonic buffer (10 mM HEPES/KOH, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, and 0.5 mM PMSF, pH 7.9). The cells were left on ice for 10 min after which cells were lysed gently with 2.5 μl of 10% Nonidet P (NP)-40. The cell lysate was centrifuged at 15,000 x g for 3 min at 4˚C. The supernatant was carefully collected and marked as the cytoplasmic fraction. The nuclear pellets were gently resuspended in 40 μl of ice-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 left 20 min on ice. After centrifuge at 15,000 x g for 15 min at 4˚C, the supernatant was collected and marked as the nuclear fraction.

Western blot analysis. RBL-2H3 cells were incubated with THC for 1 h or 2 h prior to PMA/A23187 treatment. Cells were washed with ice-cold PBS and lysed in PRO-PREP lysis buffer (iNtRON Biotechnology). Same quantity of protein were separated on 10% SDS-polyacrylamide gel. Then, proteins were transferred to Hypond PVDF membrane (Amersham Biosciences) and blocked in 5% skim milk in TBST for 1 h at room temperature. Specific antibodies against COX-2 (Cell signaling Technology (CST), #12282), p38 (CST, #9212), p-p38 (CST, #9211), ERK1/2 (CST, #9102), p-ERK1/2 ((CST, #9101), JNK (CST, #9252), p-JNK (CST, #9251), NF-kB (p65, CST, #8242), lamin B1 (CST, #13435) (1:1,000), and β-actin (1:2,500; Sigma, #A2228) were diluted in 5% skim milk. After stringent washing with TBST, horseradish peroxidase-conjugated secondary antibodies were incubated. The blots were then developed by the enhanced chemiluminescence detection (Amersham Biosciences).

Statistical analysis. All values shown in the figures were obtained from at least three independent experiments and expressed as mean ± SD and analyzed using SPSS 20.0 (IBM

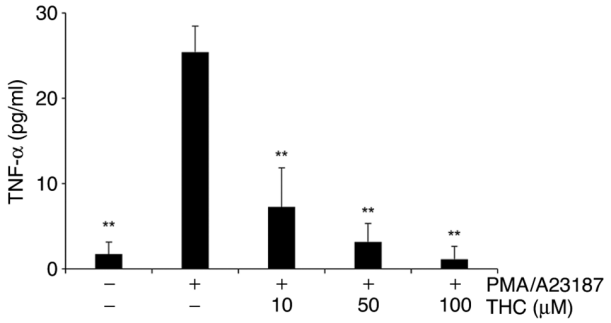

Figure 1. Effect of THC on PMA/A23187-induced extracellular secretion of TNF-α in RBL-2H3 cells. RBL-2H3 cells were incubated with indicated concentrations of THC for 1 h, then incubated with PMA/A23187 for 30 min. The concentrations of TNF-α in culture media were determined using ELISA. THC exhibited a significant inhibition of PMA/A23187-induced TNF-α secretion in a concentration-dependent manner. Data are expressed as mean ± SD from three experiments. **P<0.01 vs. PMA/A23187 alone. THC, 3,4,5-trihydroxycinnamic acid; PMA, phorbol-12-myristate-13-acetate; TNF-α, tumor necrosis factor α.

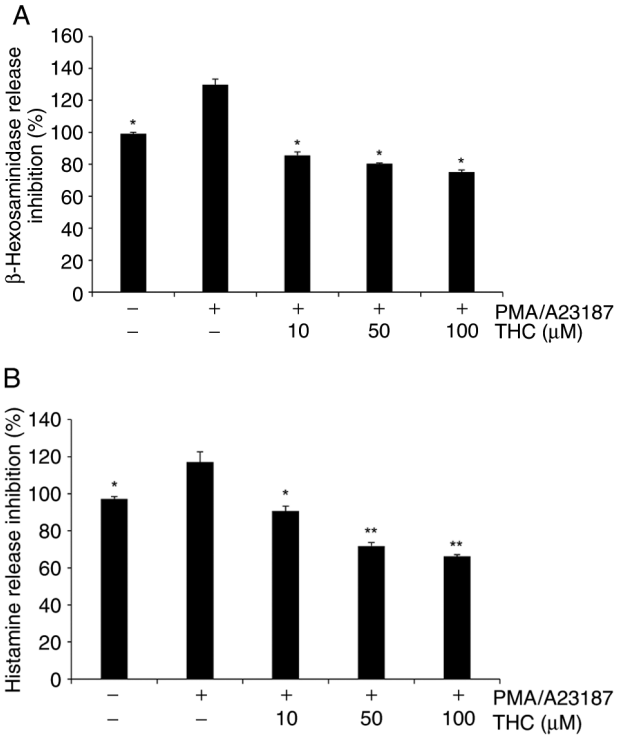

Figure 2. Effect of THC on β-hexosaminidase release and histamine release in RBL-2H3 cells. (A) β-Hexosaminidase release and (B) histamine release. Cells were treated with indicated of THC for 1 h and stimulated with PMA/A23187 for 30 min. THC significantly attenuated PMA/A23187-induced β-hexosaminidase release and also showed a significant suppression of PMA/A23187-induced histamine release in concentration-dependent manners. Results were presented as the mean ± SD from three experiments. *P<0.05 and **P<0.01 vs. PMA/A23187 alone. THC, 3,4,5-trihydroxycinnamic acid; PMA, phorbol-12-myristate-13-acetate.

Corp.). A one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was used to analyze differences between multiple groups. Data with values of P<0.05 were interpreted as statistically significant. Single */# and double **/## represent statistical significance in P<0.05 and P<0.01, respectively.

Results

THC significantly suppressed PMA/A23187-induced TNF-α secretion. Pro-inflammatory cytokines such as TNF-α have been extensively reported to play an essential role in the inflammation progression (27,28). The effects of THC on the extracellular release of TNF-α in PMA and A23187-challenged RBL-2H3 mast cells were measured. Cells were incubated with THC for 1h prior to PMA/A23187 treatment. PMA/A231897 treatment clearly increased the secretion of TNF-α in RBL-2H3 mast cells and THE significantly suppressed PMA/A23187-induced TNA-α release in a concentration-dependent manner (Fig. 1). Noticeable cytotoxicity was not observed with THC treatment in the concentrations applied in the study (data not shown).

THC significantly suppressed PMA/A23187-induced β-hexosaminidase and histamine release. The determination of the secretion of β-hexosaminidase has been widely reported to measure the level of mast cell degranulation (29). In the current study, PMA/A23187 treatment resulted in the increased the secretion of β-hexosaminidase and THC treatment significantly suppressed PMA/A23187-induced β-hexosaminidase in RBL-2H3 cells (Fig. 2A). In addition, PMA/A23187 treatment showed increased secretion of histamine and THC treatment significantly attenuated PMA/A23187-induced histamine extracellular secretion in RBL-2H3 cells in a positive association with the concentration of THC (Fig. 2B).

THC significantly attenuated PMA/A23187-induced COX-2 expression. Given the previous report that elevated levels of COX-2 is associated with mast cell activation (30), COX-2 expression was examined in PMA/A23187-challenged RBL-2H3 cells in the present study. PMA/A23187 treatment resulted in increased expression of COX-2 (Fig. 3), and THC treatment significantly attenuated PMA/A23187-induced expression of COX-2 (Fig. 3A). Quantitative analysis of COX-2 expression showed significant suppression of COX-2 expression in a positive association of the concentration of (Fig. 3B), suggesting that THC suppresses mast cell activation through the inhibition of COX-2 expression in RBL-2H3 cells.

THC significantly suppressed PMA/A23187-induced MAPKs phosphorylation. Signaling pathways of MAPKs have been extensively demonstrated to be involved in the degranulation of activated mast cells (30,31). In the present study, the phosphorylation of MAPKs was examined in the presence of PMA/A23187 to determine the effect of mast cell activation on MAPK phosphorylation and then the role of THC was measured on the PMA/A23187-induced MAPKs phosphorylation in RBL-2H3 cells. PMA/A23187 challenge showed an increased phosphorylation of all three MAPKs in RBL-2H3 cells (Fig. 4). THC treatment significantly suppressed the PMA/A23187-induced phosphorylation of MAPKs (Fig. 4). Quantitative analyses of p-p38 and p-JNK immunoblots showed a concentration-dependent inhibition (Fig. 4B and D) whereas phosphorylation level of p-ERK1/2 was significantly attenuated in low concentration of THC and maintained through the tested concentrations (Fig. 4C).

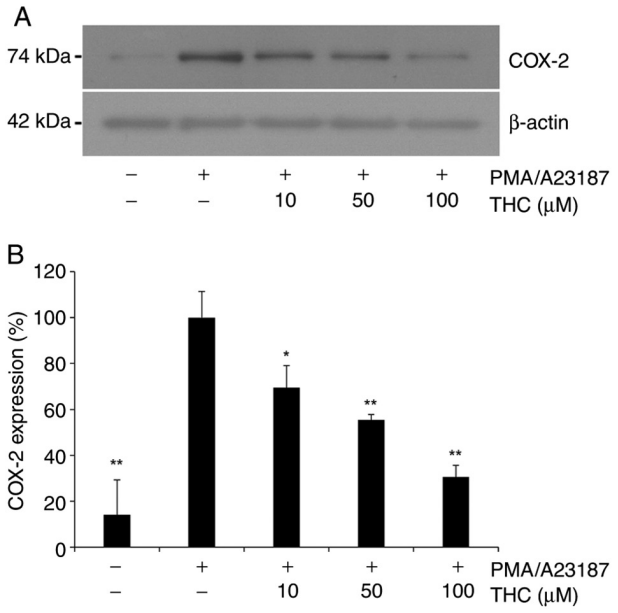

Figure 3. Effect of THC on PMA/A23187-induced COX-2 expression in RBL-2H3 cells. (A) Representative immunoblots. (B) Quantitative immunoblot analyses of COX-2. Cells were pretreated with THC (10, 50 and 100 μM) for 2 h and then stimulated with PMA/A23187 for 30 min. The expression of COX-2 was measured using western blotting. THC exhibited a significant attenuation of PMA/A23187-induced COX-2 expression was positively associated with the concentration of THC. The data were presented as means ± SD from three independent experiments. *P<0.05 and **P<0.01 vs. PMA/A23187 alone. THC, 3,4,5-trihydroxycinnamic acid; PMA, phorbol-12-myristate-13-acetate; COX-2, cyclooxygenase 2.

THC significantly attenuated PMA/A23187-induced nuclear translocation of NF-κB. In the present study, the effect of THC on PMA/A23187 induced nuclear translocation of NF-κB was examined in RBL-2H3 cells as NF-κB is a major transcription factor of pro-inflammatory genes in mast cells (28). PMA/A23187 challenge showed significantly increased nuclear translocation of NF-κB in RBL-2H3 cells (Fig. 5). Representative immunoblot showed almost complete translocation of NF-κB upon PMA/A23187 treatment. However, THC treatment significantly attenuated PMA/A23187-induced nuclear translocation of NF-κB in a concentration-dependent manner (Fig. 5). With THC treatment, the level of nuclear NF-κB was reversely associated with the level of cytosolic NF-κB (Fig. 5).

Discussion

The present results clearly demonstrate that THC significantly inhibits PMA/A23187-induced allergic responses in RBL-2H3 mast cells. THC significantly suppressed PMA/A23187-induced secretion of TNF-α, hexosaminidase, and histamine in PMA/A23187-challenged RBL-2H3 cells. THC significantly attenuated PMA/A23187-induced COX-2 expression, MAPKs phosphorylation, and nuclear translocation of NF-κB in RBL-2H3 cells.

Aberrant mast cell activation plays a detrimental role in the allergic response in a variety of allergic diseases including asthma, anaphylaxis and autoimmune disorders (4,5). Allergic response is initiated by a variety of inflammatory mediators

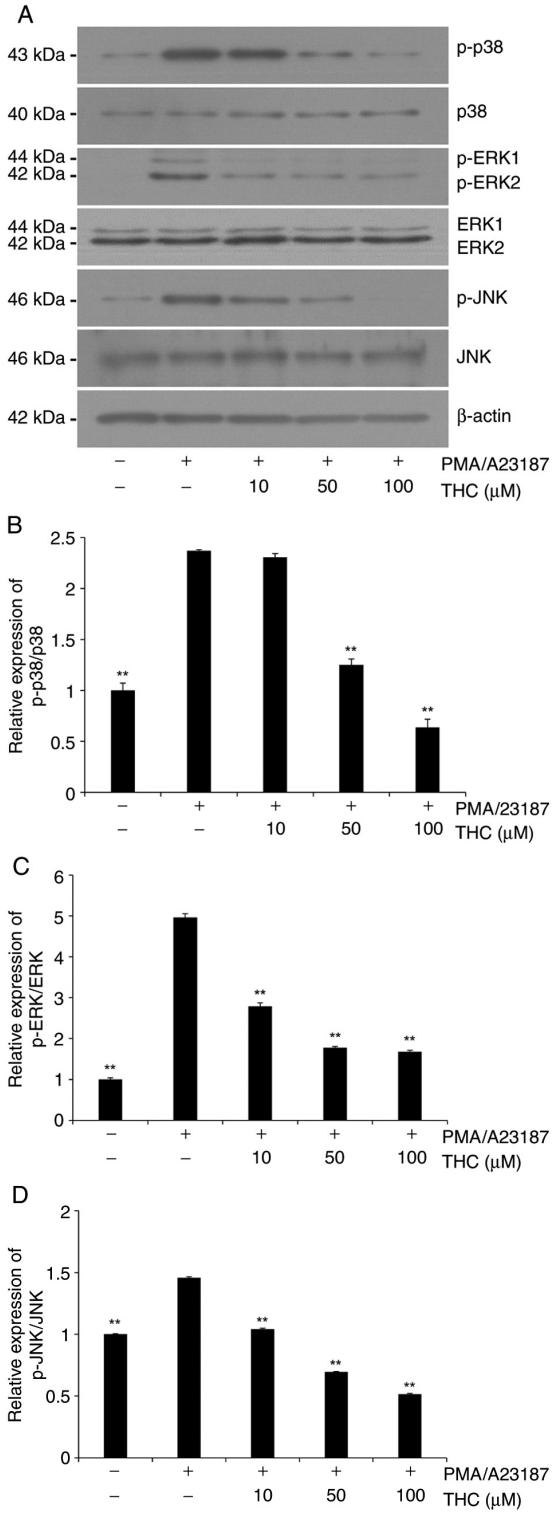

Figure 4. Effect of THC on PMA/A23187-induced phosphorylation of MAPKs in RBL 2H3 cells. Cells were pretreated with THC (10, 50 and 100 μM) for 1 h and then stimulated with PMA/A23187 for 30 min. (A) Representative immunoblots of MAPKs. (B) Quantitative analyses of immunoblots of p-p38. (C) Quantitative analyses of immunoblots of p-ERK1/2. (D) Quantitative analyses of immunoblots of p-JNK. THC exhibited a significant suppression of PMA/A23187-induced MAPKs activation. p-p38 and p-JNK showed significant suppression in a positive association with the concentration of THC whereas p-ERK1/2 showed a significant suppression with all tested concentration of THC. The data are presented as means ± SD from three independent experiments. **P<0.01 vs. PMA/A23187 alone. THC, 3,4,5-trihydroxycinnamic acid; PMA, phorbol-12-myristate-13-acetate; MAPKs, mitogen-activated protein kinases; ERK1/2, extracellular signal-regulated kinase 1/2; JNK, c-Jun N-terminal kinase; p38, p38 mitogen-activated protein kinase; p-, phosphorylated.

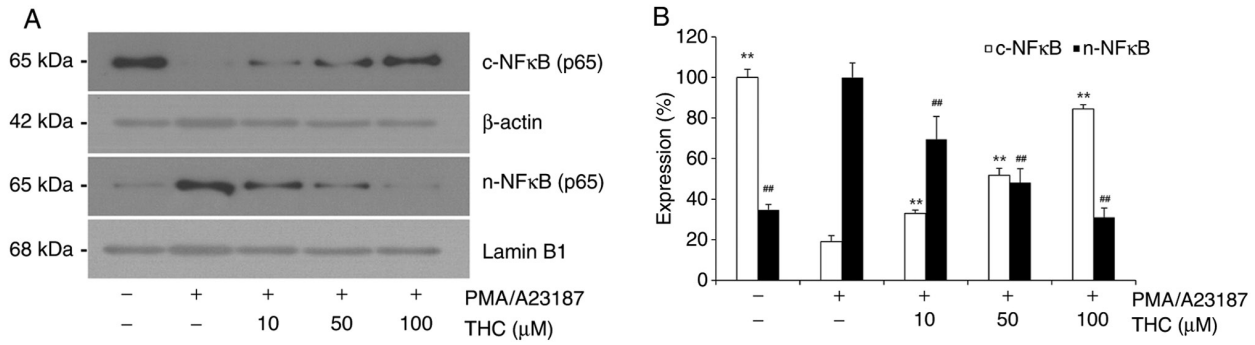

Figure 5. Effects of THC on PMA/A23187-induced n-NFκB and c-NFκB. (A) Representative immunoblots. (B) Quantitative analyses of immunoblots of n-NFκB and c-NFκB. Cells were pretreated with THC (10, 50 and 100 μM) for 1 h and then stimulated with PMA/A23187 for 30 min. THC significantly suppressed nuclear translocation of NFκB in a positive association with the concentration of THC. The data are presented as means ± SD from three independent experiments. **P<0.01 vs. PMA/A23187 alone in cytosolic fraction; $^{\#\#}$P<0.01 vs. PMA/A23187 alone in nuclear fraction. THC, 3,4,5-trihydroxycinnamic acid; PMA, phorbol-12-myristate-13-acetate; NF-κB, nuclear factor-κB; n-NFκB, nuclear NFκB; c-NFκB, cytosolic NFκB.

such as histamine, proteases and various cytokines from aberrantly activated mast cells. Released inflammatory mediators consequently cause, vasodilation, increased vascular permeability, leukocyte recruitment and bronchoconstriction and severe symptoms such as asthmatic attack and anaphylactic shock might happen (32,33). Especially, cytokines such as TNF-α play a key role in leukocyte recruitment (34), The secretion of TNF-α was examined in PMA/A23187-challenged RBL-2H3 cells in the present study. THC significantly suppressed PMA/A23187-induced TNF-α secretion in a concentration-dependent manner. The degranulation response of mast cells has been reported to be quantitatively determined by measuring the level of released β-hexosaminidase (35) and histamine is the most well characterized and most potent vasoactive mediator in activated mast cells (6). Secretion of β-hexosaminidase and histamine was quantitatively determined in the present study. PMA/A23187-challenged RBL-2H3 cells resulted in the increased release of β-hexosaminidase and histamine and THC significantly attenuated PMA/A23187-induced secretion of β-hexosaminidase and histamine in RBL-2H3 cells. The increased expression of COX-2 in activated mast cells results in the production of prostaglandin E2, which causes increased vascular permeability contributing to the aggravation of inflammation (36). THC significantly attenuated PMA/A23187-induced increased COX-2 expression in PMA/A23187-challenged RBL-2H3 cells.

MAPK cascade is a crucial signaling pathway involved in various immune response (37,38). Previous reports have demonstrated that the production of inflammatory cytokines and mediators during the mast cell activation is associated with MAPKs signaling (7,30,31). The addition of PMA and A21387 to mast cells results in the phosphorylation of MAPK cascade including p38, ERK1/2, and JNK pathways and subsequent expression of cytokines (39,40). It has been reported that coumarin derivative attenuated PMA/A21378-induced allergic response by suppressing ERK1/2 signaling pathway in RBL-2H3 cells (41) and CAME, a caffeic acid derivate, has been reported to suppress allergic response by inhibiting JNK activation in HMC-1 human mast cells (6). In addition, bisdemmethoxycoumarin has been reported to inhibit all three MAPKs and suppress allergic response in PMA/A21378-induced HMC-1 human mast cells (7). These reports strongly suggest that MAPKs are involved in the propagation of allergic response during the mast cell activation. In the present study, PMA/A21378 treatment caused clear activation of all three MAPKs and THC treatment resulted in the significant suppression of MAPKs phosphorylation. Especially, activation of p38 and JNK was significantly attenuated in a concentration-dependent manner. However, phosphorylation of ERK1/2 was significantly inhibited with low concentration of THC.

NF-κB is the major transcription factor for the inflammatory responses (28). NF-κB is involved in the production of pro-inflammatory mediators such as iNOS, interleukins, and TNF-α and the upregulation of adhesion molecules (42,43). Inflammatory stimuli such as lipopolysaccharide cause nuclear translocation of NF-κB in various immune cells (21,22). In the present study, nuclear translocation of NF-κB was observed with PMA/A21387 treatment in RBL-2H3 cells. THC treatment significantly suppressed PMA/A21387-induced nuclear translocation of NF-κB. With THC treatment, NF-κB was retained in the cytosol in a concentration-dependent manner. Nuclear translocation of NF-κB has been demonstrated to be regulated by MAPKs, which regulate the degradation of IκB, NF-κB inhibitory protein (44).

Caffeic acid and its derivatives have been reported to possess a variety of biological actions including anti-tumor, anti-inflammatory, immunosuppressive, antibiotic and neuroprotection actions (17-19). THC showed anti-inflammatory action via the inhibition of NF-κB activation in LPS-stimulated BV2 microglial cells (28). We demonstrated that THC exhibited the significant attenuation of LPS-induced inflammatory response via the activation of cytoprotective Nrf2/HO-1 signaling in RAW264.7 macrophage cells (45). THC inhibited LPS-induced macrophage infiltration to kidney and showed improved survival of animal in LPS-induced endotoxemia mouse model (45,46). Recently, THC has been also reported to possess anti-inflammatory action on atopic dermatitis model in human keratinocyte cell line, HaCat cells (47).

In conclusion, in addition to our previous studies that THC exerts anti-inflammatory response in microglial, macrophage, and keratinocyte cells (45-47), and endotoxemia animal model (45,46), the present study clearly demonstrates that

THC significantly inhibits PMA/A23187-induced mast cell activation through the suppression of MAPKs and NF-κB signaling pathways in BRL-2H3 cells, suggesting that THC might be an important therapeutic agent in the treatment of allergy-related various disorders. However, to clearly evaluate anti-allergic effect of THC, further examinations might be necessary in various study models including animal models.

Acknowledgements

Not applicable.

Funding

This work was supported by the National Research Foundation of Korea grant funded by the Korean government (grant no. 2021-R1A4A1031574).

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

JYP was involved in the experimental operation and data analysis. HJL, ETH, JHH and WSP were involved in statistical analysis and data interpretation and in the study methodology. YSK was involved in the design and result discussion of the study. WC was involved in the conceptualization and the writing, reviewing and editing of the manuscript. YSK and WC confirm the authenticity of all the raw data. 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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