3,4,5-Trihydroxycinnamic acid exerts anti-inflammatory effects on TNF-α/IFN-γ-stimulated HaCaT cells

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Abstract. 3,4,5-Trihydroxycinnamic acid (THCA) exhibits anti-inflammatory activity in acute or chronic inflammatory disorders, such as acute lung injury and asthma. The present study investigated the anti-inflammatory activity of THCA in a tumor necrosis factor-α/interferon-γ (TI) mixture-stimulated human keratinocyte cell line. The results of ELISA and reverse transcription-quantitative PCR revealed that THCA reduced the secretion and mRNA expression levels of interleukin (IL)-6, IL-8; thymus and activation-regulated chemokine; macrophage-derived chemokine; regulated upon activation, normal T cell expressed, and secreted (RANTES) and monocyte chemoattractant protein-1 (MCP-1), are closely related to the pathophysiology of AD (2-4). It is known that the combination of tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) induces the expression of inflammatory cytokines and chemokines in keratinocytes (5-7). The activation of AKT/MAPK/NF-κB is known to be associated with the production of inflammatory molecules, including IL-6, IL-8, TARC, MDC, RANTES and MCP-1, in TNF-α/IFN-γ (TI)-stimulated HaCaT cells (4, 5, 8, 9). A previous study reported that antioxidants exert protective effects in AD by regulating the activation of mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) (10). The anti-inflammatory properties of caffeic acid derivatives have been demonstrated in various studies (11,12). 3,4,5-Trihydroxycinnamic acid (THCA), a derivative of caffeic acid, exerts anti-inflammatory activity in activated BV2 microglia, RAW264.7 macrophages and A549 airway epithelial cells (13-16). In these studies, THCA also exerted protective effects in experimental animal models of sepsis, acute lung injury and allergic asthma. However, its effect and molecular mechanisms have not been examined in activated keratinocytes. Therefore, in the present study, we examined whether THCA regulates the TI mixture-induced inflammatory response in HaCaT cells.

Introduction

Atopic dermatitis (AD) is a chronic inflammatory disease characterized by severe itching and is caused by genetic and environmental factors, including microbial infection (1). Keratinocyte-derived cytokines and chemokines, such as interleukin-6 (IL-6), IL-8, thymus and activation-regulated chemokine (TARC), macrophage-derived chemokine (MDC), regulated upon activation, normal T cell expressed, and secreted (RANTES) and monocyte chemoattractant protein-1 (MCP-1), are closely related to the pathophysiology of AD (2-4). It is known that the combination of tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) induced the expression of inflammatory cytokines and chemokines in keratinocytes (5-7). The activation of AKT/MAPK/NF-κB is known to be associated with the production of inflammatory molecules, including IL-6, IL-8, TARC, MDC, RANTES and MCP-1, in TNF-α/IFN-γ (TI)-stimulated HaCaT cells (4, 5, 8, 9).

Materials and methods

Cell culture. HaCaT cells, the human keratinocyte cell line, were purchased from CLS Cell Lines Service and cultured in DMEM containing 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a 5% CO₂ atmosphere. HaCaT cells were incubated with 3,4,5-trihydroxycinnamic acid (AAPin Chemicals Limited) (2.5, 5, 10, 25, 50 and 100 µM) for 24 h. An MTT assay was used to determine cell viability based on a previous study (1).
Reverse transcription-quantitative PCR (RT-qPCR). Cells were plated in 12-well plates (1x10^5 cells/well), pretreated with THCA (5, 10, 25 and 50 µg/ml) for 30 min, and subsequently administered TNF-α (10 ng/ml, Invitrogen; Thermo Fisher Scientific, Inc.) and IFN-γ (10 ng/ml, Merck KGaA) and maintained for 6 h at 37°C. The extraction of total RNA, synthesis of cDNA and relative mRNA levels of cytokines and chemokines were determined as described previously (1). Primer sequences are listed in Table I. GAPDH was used as the housekeeping gene.

Enzyme-linked immunosorbent (ELISA) assay. Cells were plated in 96-well plates (5x10^4 cells/well), pretreated with THCA (5, 10, 25 and 50 µg/ml) for 30 min, subsequently treated with 10 ng/ml TNF-α/IFN-γ (TI) and incubated for 24 h at 37°C. The secretion levels of IL-6, IL-8, TARC, RANTES, MDC and MCP-1 in culture media were detected using commercial ELISA kits.

Isolation of nuclear and cytoplasmic proteins. Cells were seeded in growth medium at a density of 1x10^5 cells/well in 60-mm cell culture dishes and incubated with THCA for 30 min. Then, TI mixture was administered to each well, and the plates were maintained for 1 h. Nuclear and cytoplasmic proteins were isolated using a protein extraction kit (cat. no. 71183; Merck) according to the manufacturer's instructions.

Western blotting. To determine the phosphorylation of AKT, MAPK and NF-κB, cells were plated in 60-mm cell culture dishes (1x10^5 cells/well), pretreated with THCA for 30 min, and subsequently maintained with TI mixture for 1 h. To detect the phosphorylation of nuclear factor erythroid 2-related factor 2 (Nrf2) and the expression of HO-1/NQO1, cells were plated in 60-mm cell culture dishes (1x10^5 cells/well) and maintained with THCA for 1 or 16 h. Cell lysates were prepared using lysis buffer (C-3228; Sigma-Aldrich; Merck KGaA). The protein equivalents of samples were separated by 10-12% SDS polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. Five percent skim milk was used as a blocking solution for each membrane. Then, membranes were probed with primary antibodies as follows: Anti-phosphorylated (p)-AKT (4060S), anti-p-ERK (9101S), anti-p-JNK (4691S), anti-p-p38 (9211S), anti-p-NF-κB p65 (3033S), AKT (4691S), ERK (9102S), JNK (9252S), p38 (9212S), NF-κB p65 (8242S), anti-β-actin (4967S; all, 1:1,000; Cell Signaling Technology, Inc.), anti-HO-1 (2733S), anti-p-IκB (1:1,000; 15087; Invitrogen; Thermo Fisher Scientific, Inc.), anti-LaminA/C (1:1,000; sc-376248; Santa Cruz Biotechnology, Inc.), anti-NQO1, (1:1,000; N5288; Sigma-Aldrich), anti-p-Nrf2 (1:1,000; NBP2-67465; Novus Biologicals) and anti-Nrf2 (1:1,000; 137550; Abcam). Subsequently, each membrane was exposed to HRP-conjugated secondary antibodies and developed with an ECL solution (Thermo Fisher Scientific, Inc.) The visualization of all bands was performed using the image analyzer, and the density of each band was determined using ImageJ.

Immunocytochemistry. The conditions of cell seeding and fixation were based on a previous study (1). Cells were washed with PBS, blocked in 3% BSA for 1 h at RT and then maintained with the anti-NF-κB p65 subunit (1:250; Cell Signaling Technology, Inc.) or anti-Nrf2 (1:250; Abcam) for 24 h at 4°C. Then, the cells were washed with PBS and maintained with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:250; Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h at RT. Finally, cells were stained with Gold Antifade reagent containing DAPI (Invitrogen; Thermo Fisher Scientific, Inc.) for 5 min and were subsequently visualized using a confocal microscope.

Statistical analysis. One-way analysis of variance followed by Tukey's post hoc test was used to determine significant differences between the TI group and the THCA treated groups (SPSS Statistics 20; IBM Corp.). Data are expressed as the means ± standard deviation (SD).

Results

Effect of THCA on IL-6 and IL-8 in activated HaCaT cells. To examine the inhibitory effect of THCA treatment on IL-6 and IL-8, MTT assays were first conducted. As presented in Fig. 1A, no noticeable reduction in cell viability was observed following treatment with 2.5-50 µg/ml THCA. The secretion levels of IL-6 and IL-8 were evaluated with an ELISA. As presented in Fig. 1B, the production of these molecules was notably increased by TI mixture administration in HaCaT cells, whereas THCA pretreatment exerted inhibitory activity on TI mixture-induced upregulation of IL-6 and IL-8. As shown in Fig. 1D, RT-qPCR revealed that the administration of TI resulted in significant upregulation of IL-6 and IL-8 mRNA expression in HaCaT cells. However, THCA pretreatment reduced this upregulation.

Effects of THCA on TARC and MDC in activated HaCaT cells. The TI mixture markedly increased the secretion of TARC and MDC, whereas this increase was reduced by THCA pretreatment (Fig. 2A and B). Next, the mRNA levels of these molecules were determined using RT-qPCR. As shown in Fig. 2C and D, the increases in the mRNA levels of each molecule were confirmed following administration of the TI mixture. THCA pretreatment decreased this increase (Fig. 2C and D).

Effects of THCA on RANTES and MCP-1 in activated HaCaT cells. The TI mixture-induced notable increases in RANETS and MCP-1 secretion were significantly blocked by THCA pretreatment (Fig. 3A and B). In addition, THCA inhibited the increased mRNA expression levels of RANETS and MCP-1 in TI mixture-stimulated HaCaT cells (Fig. 3C and D).

Effects of THCA on AKT and MAPK phosphorylation in activated HaCaT cells. Western blotting results showed that AKT and MAPK (ERK, JNK and p38) phosphorylation levels were increased by administration of the TI mixture in HaCaT cells (Fig. 4A and B). Pretreatment with THCA resulted in suppression of AKT and ERK phosphorylation in TI-stimulated HaCaT cells. However, the phosphorylation levels of JNK and p38 were not affected by THCA pretreatment.

Effect of THCA on NF-κB p65 phosphorylation in activated HaCaT cells. Next, the effects of THCA on NF-κB p65 and
Figure 1. Effect of THCA on TI-induced IL-6 and IL-8 in HaCaT cells. (A) Cell viability was determined with the MTT assay. HaCaT cells were treated with THCA (2.5, 5, 10, 25, 50 and 100 µg/ml) for 24 h. The secretion levels of (B) IL-6 and (C) IL-8 were determined using ELISAs. HaCaT cells were pretreated with THCA (5, 10, 25 and 50 µg/ml) 30 min prior to incubation with 10 ng/ml TI for 24 h. The mRNA levels of (D) IL-6 and (E) IL-8 were determined using reverse transcription-quantitative PCR assays. HaCaT cells were pretreated with THCA 30 min prior to incubation with 10 ng/ml TI. Data are expressed as the mean ± standard deviation. *P<0.05 vs. negative control group; #P<0.05 vs. TI only group. TI, TNF-α/IFN-γ; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; THCA, 3,4,5-trihydroxycinnamic acid; IL, interleukin-6.

Table I. Primer sequences used for reverse transcription-quantitative PCR.

| Gene     | Forward primer sequence (5'→3') | Reverse primer sequence (5'→3') |
|----------|---------------------------------|---------------------------------|
| IL-6     | GACAGCCACTCACCTCTTCA            | AGTGCTTCTTTGCTGCTTTTC          |
| IL-8     | ATGACTTCCAAGCTGGCCCTGGCT       | TTATGATTCTCAGCCCTCTTCAAAAA     |
| TARC     | CACGCAGCTCGAGGGACCAATGTG       | TCAAGACCTCCTCAAGGCTTTGCGAGG    |
| MDC      | AGGACAGAGCATGATGGCTCGCTCACAGA  | TAATGGCAGGGAGTGGAGGGCTCTCTGA   |
| RANTES   | CTGCCCTCCCATATTCCCTCGG          | GAGTGTGATGACTCCCCGAACCC        |
| MCP-1    | TCTGTGCCCTGTCGTCATAG           | CAGATCTCTTTGGCCACACAT          |
| GAPDH    | CCTCCAAATATCGAGTGG             | CCATCCACGTCTCCTCGG             |

IL, interleukin; TARC, thymus and activation-regulated chemokine; MDC, macrophage-derived chemokine; RANTES, regulated upon activation, normal T cell expressed and secreted; MCP-1, monocyte chemoattractant protein-1.
Figure 2. Effects of THCA on TI-induced TARC and MDC in HaCaT cells. The secretion levels of (A) TARC and (B) MDC were determined using ELISAs. HaCaT cells were pretreated with THCA 30 min prior to incubation with 10 ng/ml TI for 24 h. The mRNA levels of (C) TARC and (D) MDC were determined using reverse transcription-quantitative PCR assays. HaCaT cells were pretreated with THCA 30 min prior to incubation with 10 ng/ml TI for 6 h. Data are expressed as the mean ± standard deviation. #P<0.05 vs. negative control group; *P<0.05 vs. TI only group. TI, TNF-α/IFN-γ; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; THCA, 3,4,5-trihydroxycinnamic acid; TARC, thymus and activation-regulated chemokine; MDC, macrophage-derived chemokine.

Figure 3. Effects of THCA on TI-induced RANTES and MCP-1 in HaCaT cells. The secretion levels of (A) RANTES and (B) MCP-1 were determined using ELISAs. HaCaT cells were pretreated with THCA 30 min prior to incubation with 10 ng/ml TI for 24 h. The mRNA levels of (C) RANTES and (D) MCP-1 were determined using reverse transcription-quantitative PCR assays. HaCaT cells were pretreated with THCA 30 min prior to incubation with 10 ng/ml TI for 6 h. Data are expressed as the mean ± standard deviation. #P<0.05 vs. negative control group; *P<0.05 vs. TI only group. TI, TNF-α/IFN-γ; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; THCA, 3,4,5-trihydroxycinnamic acid; RANTES, regulated upon activation, normal T cell expressed and secreted; MCP-1, monocyte chemoattractant protein-1.
IκBα activation were examined by western blotting. As shown in Fig. 5A and B, THCA treatment attenuated the phosphorylation of NF-κB p65/IκBα in TI mixture-stimulated HaCaT cells. THCA exerted a suppressive effect on the nuclear translocation of NF-κB p65 (Fig. 5C and D). This effect of THCA on nuclear translocation was confirmed using immunocytochemistry (ICC) (Fig. 5E).

**Effects of THCA on HO-1 and NQO1 induction in HaCaT cells.** As presented in Fig. 6, THCA treatment significantly upregulated the expression of heme oxygenase-1 (HO-1) in HaCaT cells (Fig. 6A and B). THCA treatment also resulted in upregulation of NAD(P)H:quinone oxidoreductase 1 (NQO1) expression in HaCaT cells. Furthermore, Nrf2 activation and nuclear translocation were also increased by THCA treatment (Fig. 6A-D). This ability of THCA on nuclear translocation was confirmed using ICC (Fig. 6E).

**Discussion**

In the present study, the inhibitory effect of THCA on AD-like markers and its molecular mechanism were studied in vitro using HaCaT human keratinocytes. Accumulating evidence has reported that an increased levels of IL-6 and IL-8 are involved in the pathogenesis of AD and is closely associated with the promotion of keratinocyte proliferation and migration (3,17,18). Inflammatory chemokines have been reported to control the migration of inflammatory cells to sites of infection and inflammation; however, sustained levels of chemokines, such as TARC, MDC, RANTES and MCP-1, are associated with the initiation and disease severity of AD (5,19-22). Considering all these reports, the suppression of inflammatory cytokines and chemokines could lead to the amelioration of AD symptoms. As mentioned earlier, the mixture of TI has been used to induce an inflammatory response in human keratinocytes, such as HaCaT cells (5,6); therefore, we selected the TI mixture as an inducer and evaluated the anti-inflammatory effect of THCA. In the present study, the experimental results showed that THCA ameliorates the production of IL-6, IL-8, TARC, MDC, RANTES and MCP-1 in activated HaCaT cells (Figs. 1-3). These results indicate that THCA exerts anti-inflammatory activity in activated HaCaT cells.

Accumulated evidence has shown that the production of inflammatory cytokines and chemokines is associated with the AKT/MAPK/NF-κB signaling pathways in experimental atopic models (1,4,7). Thus, these signaling pathways represent therapeutic targets for the improvement of AD. Previous studies have shown the inhibitory activity of THCA on NF-κB in lipopolysaccharide-stimulated BV2 microglia and RAW264.7 cells (13,14). Recently, its inhibitory effect was confirmed on AKT/MAPK/NF-κB in activated A549 cells (15). In this study, THCA effectively decreased the activation of AKT, ERK, JNK, p38 and NF-κB in stimulated-A549 cells and exerted inhibitory effects on AKT, ERK, JNK and NF-κB in lung of asthmatic mice (15). THCA also significantly reduced the activation of ERK and p38 in lung of COPD-like mice (16). In these studies, the regulatory effects of THCA on AKT and MAPK was excellent. Furthermore, the regulatory effects of THCA on MAPK activation were confirmed in both in vitro and in vivo. Thus, we expected this effect of THCA on AKT, MAPK and NF-κB to be confirmed in this study. However, no modulatory effects were found on p-JNK and p-38. Considering that THCA did not totally exert the regulatory effect in both JNK (16) and p38 (15) activation in previous studies, it was not surprising that THCA only exerts an inhibitory effect on ERK activation in the present study. Thus, this result indicated that THCA have inhibitory effect on AKT, ERK and NF-κB activation in TNF-α/IFN-γ stimulated HaCaT cells. Collectively, regulatory ability of THCA on AKT/ERK/NF-κB may contribute the amelioration
of inflammatory response induced by TI stimulation in the present study (Figs. 4 and 5).

Antioxidant proteins, such as HO-1 and NQO1, exert anti-inflammatory properties in HaCaT cells (23,24). Researchers have reported that the induction of HO-1 ameliorates the inflammatory response in activated HaCaT cells by reducing TARC and MDC expression levels and regulating NF-κB nuclear translocation (25,26). NQO1 was shown to be related to antimelanogenic efficacy in UVA-irradiated keratinocytes (27). Thus, HO-1 and NQO1 are recognized as protective molecules in AD (28). Nrf2 has an important role in HO-1 and NQO1 expression (29). In this study, THC showed inhibitory effects on inflammatory molecules and AKT/ERK/NF-κB phosphorylation in activated HaCaT cells (Figs. 1-5). Based on these results, we studied whether THCA leads to the induction of HO-1/NQO1 and the activation of Nrf2. We confirmed that THCA induces HO-1/NQO1 expression and Nrf2 activation in HaCaT cells (Fig. 6). Thus, this ability of THCA may be associated with amelioration of the inflammatory response in activated HaCaT cells. However, it is uncertain whether THCA directly affect NF-κB nuclear translocation.

In the previous studies, THCA exerted strong anti-inflammatory properties in LPS-stimulated RAW264.7 macrophages (14) and this study also showed the protective effect of THC on LPS-induced endotoxemia mice.
Figure 6. Effects of THCA on the expression of HO-1/NQO1 and the activation of Nrf2 in HaCaT cells. (A) Levels of HO-1 and NQO1 expression and Nrf2 phosphorylation were determined using western blot analysis. HaCaT cells were treated with THCA for 16 h to detect the levels of HO-1 and NQO1 expression. Cells were also treated with THCA for 1 h to detect the levels of Nrf2 activation. (B) Quantitative analysis of HO-1, NQO1 and p-Nrf2 was performed using ImageJ. (C) The level of Nrf2 nuclear translocation was determined using western blot analysis. HaCaT cells were treated with THCA for 1 h. Then, nuclear and cytosolic fractions were obtained. (D) Quantitative analysis of Nrf2 expression was performed using ImageJ. Data are expressed as the means ± standard deviation. (E) Levels of Nrf2 nuclear translocation were determined using immunocytochemistry. Scale bar, 20 µm. *P<0.05 vs. negative control group. THCA, 3,4,5-trihydroxycinnamic acid; HO-1, heme oxygenase-1; NQO1, NAD(P)H quinone dehydrogenase 1; Nrf2, nuclear factor erythroid 2-related factor 2; DAPI, 4',6-diamidino-2-phenylindole.
In addition, THCA had anti-inflammatory properties in PMA-stimulated airway epithelial cells (15) and protective effects on COPD-like mice and allergic asthma mice (15,16). In the present study, we confirmed that THCA has anti-inflammatory effects in TNF-α/IFN-γ-stimulated HaCaT cells. Collectively, these results indicated that THCA could ameliorate the inflammatory response in various inflammatory diseases. Thus, this experimental approach will support the validity that THCA has a variety of anti-inflammatory actions.

In summary, THCA exerted anti-inflammatory activities on TI-stimulated HaCaT cells (Fig. 7). In particular, its inhibitory effects on important AD markers were excellent, and its regulatory effects on AKT/ERK/NF-κB activation were also remarkable. Furthermore, THCA upregulated the activation of Nrf2 and the expression of antioxidant proteins. Therefore, our results suggest that THCA can be used as an adjuvant in AD. However, further experiments are needed to prove the ameliorative effect in AD animal models.

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Availability of data and materials

All data generated and/or analyzed during the present study are included in this published article.

Authors’ contributions

JWP designed the present study, performed the in vitro experiments, and made substantial contributions to the analysis and interpretation of data. JHO and DH performed the in vitro experiments and made substantial contributions to the analysis and interpretation of data. SMK, JHM and JYS contributed to data analysis. WC, HJL and SRO made substantial contributions to the conception and design of the present study. JWL and KSA designed the present study, wrote the manuscript and was involved in revising it critically for important intellectual content. All authors discussed the results, read and approved the final version of the 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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