Caffeic Acid Reduces Cutaneous Tumor Necrosis Factor Alpha (TNF-α), IL-6 and IL-1β Levels and Ameliorates Skin Edema in Acute and Chronic Model of Cutaneous Inflammation in Mice

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The authors declare no conflict of interest.

Caffeic acid (3,4-dihydroxycinnamic acid, CA) has been reported to have anti-inflammatory activity in animal models. However, the mechanisms underlying the anti-inflammatory effects of CA in skin inflammation are only partially understood. The present study was designed to investigate the effects and mechanisms of CA on acute and chronic skin inflammation in mice and the effect of CA in keratinocytes in vitro. The results showed that topical treatment with CA inhibited 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced skin edema in a dose-dependent manner, leading to substantial reductions in skin thickness and tissue weight, neutrophil-mediated myeloperoxidase activity, and various histopathological indicators. The CA treatment also significantly reduced the mRNA and protein levels of tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and IL-1β at the application site, and the TNF-α production, the TNF-α-induced IL-6 and IL-1β production, and TNF-α-induced nuclear factor-kappa B (NF-κB) activation in human keratinocytes in vitro. Furthermore, CA was effective at reducing inflammatory damage induced by chronic TPA exposure. These results demonstrate that CA has anti-inflammatory activities in both acute and chronic contact dermatitis models via blockade of the mRNA and protein synthesis of these cytokines and neutrophil-mediated myeloperoxidase activity, and can target inflammatory mediators specifically in the keratinocytes. Taken together, the present results suggest that CA might be a therapeutic agent against inflammatory skin diseases.

Key words 3,4-dihydroxycinnamic acid; pro-inflammatory cytokine; keratinocyte; 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced ear edema model

The skin is an immune organ consisting of epidermal cells, i.e., keratinocytes and Langerhans cells. It is widely recognized that the secretions of cytokines by epidermal keratinocytes, particularly tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and IL-1β, play a key role in various immunological disorders and inflammation in the skin. TNF-α released from keratinocytes would facilitate the subsequent influx of inflammatory cells via induction of expression of adhesion molecules such as endothelial leukocyte adhesion molecule-1 and intercellular adhesion molecule-1 (ICAM-1) on neighboring endothelial cells. Also, TNF-α has been shown to promote the immune/inflammatory reactions via the activation and induction of cytokines IL-6 and IL-1β. Therefore, inhibition of these cytokines could be an attractive strategy for skin diseases.

TNF-α is believed to mediate inflammation and immune responses by activating nuclear factor-κB (NF-κB) signaling pathway. The NF-κB signaling pathway is seen as a key link between the innate and adaptive immune systems. In the skin, NF-κB regulates the expression of many genes that are involved in the initiation of the inflammatory response, including cytokines, adhesion molecules and chemokines, matrix metalloproteases, and nitric oxide synthase. The aberrant activation of the signaling pathway can induce chronic inflammation and inflammatory diseases in the skin.

Caffeic acid (3,4-dihydroxycinnamic acid, CA), one of the most abundant bioactive dihydroxycinnamic acids, is a polyphenolic compound found in many fruits, vegetables, and herbs. Many studies have shown that this compound possesses anti-inflammatory activity. The study of Chao et al. revealed that CA could provide anti-inflammatory protection by down-regulated TNF-α, IL-6, and IL-1β mRNA and protein expression in cardiac tissue of diabetic mice. Khan et al. observed that CA attenuates 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced tumor promotional triggers by inhibition of oxidative stress and pro-inflammatory cytokine production. Furthermore, the study of Song et al. indicated that CA had anti-inflammatory effects by reducing myeloperoxidase (MPO) activity and phospholipase A2 activity in skin-incised mice. However, the mechanisms underlying the anti-inflammatory effects of CA in skin inflammation are so far, only partially understood. Hence, the aim of this study was to investigate the effects and mechanisms of CA in acute and chronic skin inflammation in mice and also to study the effect of CA on the TNF-α production, the TNF-α-induced IL-6 and IL-1β production, and TNF-α-induced NF-κB activation in human keratinocytes.

MATERIALS AND METHODS

Animals BALB/c mice (8–10 weeks old) were purchased from HFK Bioscience Co., Ltd. (Beijing, China). All mice were housed in standard polypropylene cages at a constant temperature of 22±2°C with 12h dark/light cycles and both food and water ad libitum. All animals were treated according to “Principles of Laboratory Animal Care and Use in Research” (Ministry of Health, Beijing, China). All experimental protocols were approved by the Animal Ethics Committee of...
Materials  Caffeic acid, 12-O-tetradecanoylphorbol 13-acetate, hexadecyltrimethylammonium bromide, calcium ionophore A23187, dimethyl sulfoxide (DMSO), N,N-dimethylformamide, and tetramethylbenzidine were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Sodium acetate, hydrogen peroxide, propylene glycol, ethanol, acetic acid and hexadecyltrimethylammonium bromide (HTAB) were supplied by National Institutes for Food and Drug Control (Beijing, China). Human epidermal keratinocytes (San Diego, CA, U.S.A.) were cultured in serum-free keratinocyte growth medium (Gibco-BRL, Paisley, U.K.) supplemented with 5 ng/mL epidermal growth factor (Gibco-BRL) and 0.5% bovine pituitary extract (Gibco-BRL) at 37°C in an atmosphere of 5% CO2.

Acute Inflammatory Model in Mice  Ten BALB/c mice per group were pre-treated twice at 15-min-interval on the inner and outer surfaces of the right ear with 20 µL of CA dissolved in 2% DMSO–20% propylene glycol–78% acetone at concentrations of 20, 40, and 60 mg/mL (0.8, 1.6, and 2.4 mg/ear). The negative control received twice 20 µL of vehicle (2% DMSO–20% propylene glycol–78% acetone) on the right ear and 20 µL (vehicle) was topically applied to the right ear 6 times or acetone (vehicle) was topically applied on alternate days. On days 7–10, the mice were treated on the inner and outer surfaces of the right ear with CA (0.8, 1.6, and 2.4 mg/ear), vehicle (2% DMSO–20% propylene glycol–78% acetone, negative control), or dexamethasone (0.05 mg/ear, positive control) twice a day. Ear thickness and weight were measured using a 6 mm diameter punch at 6 h after the last TPA challenge and compound treatment, and these ear biopsy samples were collected and stored at −80°C.

Chronic Inflammatory Model in Mice  Effect of CA on TPA-induced chronic skin inflammation was examined by a previously described procedure. Briefly, 20 µL of TPA solution (2 µg/ear/6 times) or acetone (vehicle) was topically applied to the inner and outer surfaces of the right ear with a micropipette on alternate days. On days 7–10, the mice were treated on the inner and outer surfaces of the right ear with CA (0.8, 1.6, and 2.4 mg/ear), vehicle (2% DMSO–20% propylene glycol–78% acetone, negative control), or dexamethasone (0.05 mg/ear, positive control) twice a day. Ear thickness and weight were measured using a digital thickness gauge and 6-mm diameter punch at 6 h after the last TPA challenge and compound treatment, and these ear biopsy samples were collected and stored at −80°C for MPO activity assay.

Myeloperoxidase Assay  To determine the degree of neutrophil infiltration, MPO assays of ear samples were conducted as described previously. Ear biopsies were placed in an eppendorf tube with 0.75 mL of 80% phosphate-buffered saline (PBS, pH=5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB), homogenized (45 s at 0°C) and decanted into a microfuge tube. After addition of 1 s 0.75 mL aliquot of PBS/HTAB buffer, the sample was centrifuged at 12000×g at 4°C for 15 min. The resulting supernatant (30 µL×triplicate) was added to 200 µL of a mixture containing 100 µL of PBS (pH=5.4), 85 µL of 0.22 mm PBS (pH=5.4) and 15 µL of 0.017% hydrogen peroxide in a 96-well microtiter plate. The reaction was started by addition of 20 µL of 18.4 mm tetramethylbenzidine in 8% aqueous N,N-dimethylformamide. Plate was incubated at 37°C for 3 min and then placed on ice. The reaction was stopped by addition of 30 µL 1.46 mm sodium acetate in acetic acid (pH=3.0). Enzyme activity was assayed using a PARADIGM Detection platform (Beckman Coulter, Brea, CA, U.S.A.) set to measure absorbance at 620 nm.

Analysis of Cytokine Production in Inflammatory Regions  Ear biopsy samples (n=7) were weighed (100 mg) and homogenized in 1 mL of T-PER tissue protein extraction reagent (Thermo Scientific) containing a protease inhibitor cocktail and incubated on ice for 30 min. Ear homogenates were centrifuged at 11200×g for 20 min at 4°C. After centrifugation, TNF-α, IL-6 and IL-1β in the supernatants were measured using enzyme-linked immunosorbent assay (ELISA) kits (Dakewe Biotech., Shenzhen, China) according to the manufacturer’s instructions. Cytokine levels were normalized using a bicinchoninic acid (BCA) protein assay kit (Beyotime, Jiangsu, China) and calculated as pg per mg total protein.

Quantification of TNF-α, IL-6 and IL-1β mRNAs by Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)  Ear biopsy samples (n=5) were lysed in Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.) and total RNA was extracted according to the manufacturer’s instruction. About 450 ng of total RNA was used for reverse transcription using a PrimeScript® RT reagent Kit (TaKaRa) in triplicate. The thermal cycler parameters were as follows: 30 s at 95°C for one cycle, followed by amplification for 42 cycles with melting for 10 s at 95°C, annealing for 20 s at 60°C and extension for 20 s at 72°C. Data were collected and quantitatively analyzed on an M×3000 P Real-Time PCR System (Stratagene, Austin, TX, U.S.A.). Values were normalized using β-actin as an endogenous internal standard. Results were presented as fold relative to the expression of β-actin. Sequences of PCR primer pairs were as follows: mouse TNF-α, Sense 5'-CACGCTCTTCTGTCTACTGAAAC-3', Anti-sense 5'-ATCTGATGTTGAGGCTCTGG-3'; mouse IL-6, Sense 5'-TAGTCTTCTTCTAACCACATTTC-3', Anti-sense 5'-TTGCTCTCTTTAGCCACCCTTC-3'; mouse IL-1β, Sense 5'-GAACATTGTCTGAACTCACAATC-3', Anti-sense 5'-ATCCTTTGGGGTCTCCGTAACT-3'; mouse β-actin, Sense 5'-GAGACCTTCAACCACCA GCAC-3', Anti-sense 5'-ATGTCACCGCAGATTTCCTCC-3'.

TNF-α Production by Human Keratinocytes in Vitro  Human epidermal keratinocytes were seeded on 96 well collagen I-coated flat bottom plate at a density of 5×103 cells/well in 200 µL for 18 h before the experiment. After removing the supernatant and adding the fresh medium, the cells were pre-treated for 30 min with CA at different concentrations prior to 8 h of stimulation with TPA (10 ng/mL) and calcium ionophore A23187 (1 µg/mL). Cell culture supernatants were assayed by ELISA for TNF-α using human TNF-α ELISA kit (Dakewe Biotech., Shenzhen, China) according to the manufacturer’s instructions.

TNF-α-Induced IL-6 and IL-1β Production in Human Keratinocytes  Human epidermal keratinocytes were seeded in triplicate into a 24-well plate (1×105 per well). When the cell confluence in each well reached about 60%, the culture supernatants were removed and the fresh medium was added. The cells were then treated with 10 ng/mL TNF-α (Peprotech, Rochy Hill, NJ, U.S.A.) in the presence of 0.5–10 µM CA for 24 h at 37°C. Culture supernatants were analyzed using IL-6
Transfection and Luciferase Assay Human keratinocytes were seeded in a 6-well plate (2×10^5 cells/well). When the cell confluence in each well reached about 60%, the cells were transfected with a total of 4 µg of pNF-κB-Luc plasmid (Stratagene, La Jolla, California, U.S.A.) DNA using Fugene6 (Roche, Indianapolis, Indiana) according to the manufacturer’s protocols. At 24h post-transfection, the cells were treated for 30min with CA at different concentrations and then were pre-treated for 24h with 10ng/mL TNF-α (Peprotech). The cells were then lysed, and their luciferase activities were measured using a LS55 luminescence spectrometer (Perkin-Elmer, Boston, MA, U.S.A.).

Histology Ear biopsies were fixed in 10% neutral-buffered formalin, embedded in paraffin wax, sectioned at a thickness of 5 µm, and stained with hematoxylin and eosin (H&E). A representative area was selected for qualitative light microscopic analysis and leukocyte accumulation and edema were evaluated using a magnification of 200×. To minimize bias, the sample analysis was blinded.

Statistical Analysis Results are expressed as mean±standard error of mean (S.E.M.). The statistical analyses were performed by one-way ANOVA followed by Dunnett’s multiple comparison test. A p-value <0.05 was considered statistically significant.

**RESULTS**

**Effect of the CA on Skin Edema in TPA-Induced Ear Edema Model** We first assessed the anti-inflammatory activity of CA in a murine model of TPA-induced acute irritant contact dermatitis. Increased skin thickening is often the first hallmark of skin irritation and local inflammation. This parameter is indicative of a number of processes that occur during skin inflammation, including increased vascular permeability, edema and swelling within the dermis and proliferation of the epidermal keratinocytes. As shown in Fig. 1, topical exposure to TPA resulted in a marked increase in ear thickness. Topical application of 2% DMSO–20% propylene glycol–78% acetone (negative control) alone did not alter the skin thickness significantly. However, CA (0.8, 1.6 and 2.4 mg/ear), or a dexamethasone positive control (DEX, 0.05 mg/ear) significantly reduced the increase in ear thickening, indicating the therapeutic effect of CA.

Next, we investigated hematoxylin and eosin (H&E) stained sections of ear tissue from TPA-treated mice. TPA application resulted in a marked increase in ear thickness, with clear evidence of edema and substantial numbers of inflammatory cells infiltrating the dermis (Figs. 2A, B). CA treatment reduced ear thickness and the associated pathological indicators significantly. These results provided further evidence that CA ameliorated TPA-induced acute irritant contact dermatitis.

**Effect of Topical Application of CA on the Protein and mRNA Expressions of Pro-inflammatory Cytokine in Inflammatory Regions** Previous studies have shown that topical exposure to TPA resulted in marked increases in TNF-α, IL-6, and IL-1β protein levels in mouse ear biopsy homogenates. As shown in Figs. 3A, 3B, and 3C, TPA challenge resulted in dramatic increases in TNF-α, IL-6, and IL-1β protein levels in ear biopsy homogenates. In contrast, treatment with TPA plus CA (0.8, 1.6, and 2.4 mg/ear) significantly reduced protein levels of these cytokines. Notably, the effectiveness of CA (1.6 and 2.4 mg/ear) on IL-6 was comparable to DEX (Fig. 3B).

To get better insights into molecular mechanisms involved in the development of cutaneous inflammation, we further examined the effect of CA on inflammatory cytokine gene expression in the ear skin by real-time polymerase chain reaction (RT-PCR). Treatment with CA (2.4 mg/ear) markedly inhibited TPA-induced mRNA expression of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) (Fig. 4), and the inhibitory effects of CA (0.8 and 2.4 mg/ear) on TNF-α and IL-1β were comparable with DEX (Figs. 4A, C). These data indicated that CA might act to reduce cutaneous inflammation by inhibiting the mRNA and protein synthesis of TNF-α, IL-6, and IL-1β.
cytokines.

**Effect of CA on Pro-inflammatory Mediators in Human Keratinocytes** Since the topical application of CA on the ear skin comes in contact mainly with the epidermis, the cytokine-inhibitory effect of the drug was also investigated specifically on keratinocytes (85% of the cells in the epidermis). As shown in Fig. 5A, human keratinocytes produced TNF-α at barely detectable levels without stimulation, but the TNF-α production was significantly induced when the cells were activated with TPA plus calcium ionophore A23187. CA markedly inhibited the TPA/A23187-induced TNF-α production at 1 µM and higher concentration.

We also measured the inhibitory effect of CA on the production of cytokine IL-1β and IL-6 in keratinocytes exposed to 10 ng/mL TNF-α. As shown in Figs. 5B and 5C, keratinocytes released barely detectable levels of IL-6 and very low levels of IL-1β without TNF-α treatment, whereas the production of IL-6 and IL-1β by keratinocytes was specifically increased upon stimulation. CA markedly attenuated the TNF-α-induced IL-6 and IL-1β production in a dose dependent manner. These results indicated CA could inhibit the production of these pro-inflammatory cytokines in the human keratinocytes.

Since CA was demonstrated to have an ability to reduce TNF-α-induced vascular inflammation by targeting pathways that result in the activation of the nuclear transcription factor NF-κB, we further investigated whether CA treatment decreased the NF-κB activity in human keratinocytes. Keratinocyte cells were transiently transfected with pNF-κB-Luc reporter plasmid, stimulated with 10 ng/mL TNF-α in the presence or absence of CA, and then measured the luciferase activity, which served as an indicator of NF-κB activation. As shown in Fig. 6, the transcriptional activity of NF-κB was decreased by CA treatment.

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**Fig. 3. Effect of CA on TPA-Induced Expression of TNF-α (A), IL-6 (B), and IL-1β (C) Protein Levels**

Ear punch biopsies were taken at 6 h after TPA application, and tissue homogenates were examined for cytokine production by ELISA. Negative control animals received acetone (C) or the vehicle (V). Positive control animals received dexamethasone (DEX). The data were expressed as mean±S.E.M. (n=7). *p<0.05, **p<0.01, and ***p<0.001 compared with vehicle. #p<0.001 compared with control.

**Fig. 4. Effect of CA on TPA-Induced Expression of TNF-α (A), IL-6 (B), and IL-1β (C) mRNA Levels**

Ear punch biopsies were taken at 6 h after TPA application. Total RNA was extracted from ear biopsies. The mRNA expression levels of these cytokines were quantified by real-time RT-PCR. Data were presented as fold induction relative to the ears of control mice received acetone alone. Negative control animals received acetone (C) or the vehicle (V). Positive control animals received dexamethasone (DEX). The data were expressed as mean±S.E.M. (n=5). *p<0.01 and ***p<0.001 compared with vehicle.
markedly induced with the TNF-α stimulation in the absence of CA. Notably, CA (5 and 10 µM) inhibited the transcriptional activity of NF-κB. Our finding is consistent with the findings in human umbilical vein endothelial cells and HaCaT cells.10,20

Effect of CA on Chronic Inflammation Induced by Repeated Application of TPA As a second in vivo evaluation of the anti-inflammatory activity of CA, CA was administered in a chronic skin inflammation model induced by multiple topical applications of TPA to mouse ears. A inflammatory response characterized by increases in ear weight, inflammatory cell infiltration and epidermal hyperplasia is persistent in this model.15) As shown in Fig. 7, exposure to TPA resulted in significant increase in both ear thickness and ear weight. 2% DMSO–20% propylene glycol–78% acetone (vehicle) alone did not significantly alter the skin thickness. Interestingly, CA (0.8, 1.6, and 2.4 mg/ear) markedly inhibited the increases in ear thickness and weight. The TPA-induced chronic inflammatory response in skin is associated with an increase in the

![Fig. 5. Effect of CA on the Production of Pro-inflammatory Mediators in Human Keratinocytes](image)

In (A), keratinocytes were pre-treated for 30 min with 0.5–10 µM CA prior to 8 h of stimulation with TPA (100 ng/mL) and calcium ionophore A23187 (1 µg/mL). In (B) and (C), keratinocytes were treated with 10 ng/mL TNF-α in the presence of 0.5–10 µM CA for 24 h. The concentration of TNF-α (A), IL-6 (B), and IL-1β (C) in the culture supernatant was determined by ELISA. The data were expressed as mean ± S.E.M. of triplicate cultures. *p<0.05, **p<0.01, and ***p<0.001, compared with the group in which keratinocytes were not treated with TPA plus calcium ionophore or TNF-α.

![Fig. 6. The Effect of CA on the Transcriptional Activity of NF-κB in Human Keratinocytes](image)

Keratinocyte cells were transiently transfected with pNF-κB-Luc reporter plasmid. At 24 h post-transfection, the cells were treated for 30 min with CA at different concentrations and then were pre-treated for 24 h with 10 ng/mL TNF-α. The luciferase activities were measured and were expressed as fold-increase relative to unstimulated and untreated cells. Values represent the mean ± S.E.M. from three independent experiments. *p<0.05 compared with the group in which keratinocytes were not treated with CA.

![Fig. 7. Effect of CA on TPA Multiple Application-Induced Mouse Ear Thickness and Weight Changes](image)

Ear thickness and weight were measured at 6 h after the last TPA challenge and compound treatment. Values are the mean ± S.E.M. of the differences in ear thickness between before and after challenge (A) or in weight between right and left ear of mice (n=10) (B). Control animals received acetone (C) or the vehicle (V). *p<0.05, **p<0.01, and ***p<0.001 compared with vehicle. *p<0.001 compared with control.
infiltration of activated neutrophils into the dermis. These activated neutrophils abundantly expressed MPO, which can be quantitated as a measure of the magnitude of neutrophil activation. We found significant reductions in MPO activity levels after CA treatment in the ears of mice exposed to TPA, and treatment with CA (1.6 or 2.4 mg/ear) was comparable with the potent topical DEX (Fig. 8).

We next examined H&E-stained ear sections from multiple TPA-treated mice. Multiple TPA application resulted in a marked increase in ear thickness, epidermal hyperplasia, and substantial inflammatory cells infiltrating in the dermis (Figs. 9A, B). CA treatment (2.4 mg/ear) reduced ear thickness, epidermal hyperplasia, and inflammatory cells infiltrating to a level comparable with the positive control DEX (Figs. 9C, D). These findings support the ability of CA to resolve an existing, persistent inflammatory lesion with multiple topical applications.

DISCUSSION

Previously CA has been reported to have anti-inflammatory, anti-oxidant, and anti-allergic activities in animal models. Recent study indicated that CA had anti-inflammatory effects by reducing MPO activity and phospholipase A2 activity in skin-incised mice. However, the mechanisms underlying the anti-inflammatory effects of CA in skin inflammation are only partially understood. TPA-induced mouse ear inflammation model mimics several aspects of skin diseases, and this model is reliable in vivo model system to evaluate compounds for both acute and chronic effects in skin inflammation.

Our study demonstrated that topical treatment with CA markedly reduced the TPA-induced increase in ear thickness and weight, and ameliorated several histopathological indicators in animal models of both acute and chronic irritant contact dermatitis.

Our study also showed that topical exposure to TPA resulted in increased secretion of TNF-α, IL-6, and IL-1β in mouse ear biopsy homogenates, suggesting that these cytokines mediate inflammatory signaling and play a pivotal role in TPA-induced mouse ear inflammation model. These results were supported by the findings from others. We further demonstrated that CA inhibited the production of TNF-α, IL-6, and IL-1β in a dose-dependent manner at the mRNA and protein expression levels. It was likely that this inhibition of cytokine secretion at least partially contributed to the anti-inflammatory response of CA treatment in acute skin inflammation. Furthermore, pro-inflammatory molecules like cytokines TNF-α, IL-6, and IL-1β act as cell-to-cell signalling messengers and play a key role in the promotion stage of cancer development. The study of Khan et al. also indicated that CA significantly reduced TNF-α protein expression level in mouse skin using the double TPA application model, which was a well-known model to understand the role of reactive oxygen species, inflammation and hyperplasia in the promotion stage of carcinogenesis. The result showed that CA also might attenuate TPA induced tumor promotional triggers by inhibition of TNF-α protein expression.

Since the topical application of CA on the ear skin comes in contact mainly with the epidermis, the cytokine-inhibitory effect of the drug was also investigated specifically on keratinocytes, which consists around 8% of living epidermal cells. TNF-α, an autocrine stimulator as well as a potent inducer of other inflammatory cytokines, is produced by keratinocytes, epidermal Langerhans cells (LCs) and macrophages in the papillary dermis, which has been considered a pivotal cytokine and a key mediator in inflammation in the skin.

In the present paper we further demonstrated that CA markedly inhibited TPA/A23187-induced TNF-α production in human keratinocytes. IL-1β, a pro-inflammatory cytokine, activates neutrophils, monocytes, eosinophils and basophils. Its main source is keratinocytes in the skin stored in the form of precursor particles. IL-6 is involved in the growth and differentiation of dermal and epidermal cells, and acts as a chemotactic factor for T cells. Stimulation of keratinocytes with TNF-α leads to expression of pro-inflammatory cytokines, such as IL-6 and IL-1β, and these cytokines also play dominant roles in mediating the progression of inflammation in the skin. In this study, we demonstrated that stimulation...
of human keratinocytes by TNF-α resulted in the production of IL-6 and IL-1β, and CA significantly attenuated the TNF-α-induced IL-6 and IL-1β production. TNF-α is believed to mediate immune responses and inflammation by activating nuclear factor-κB (NF-κB) signaling pathways. The TNF-α-induced NF-κB activation subsequently leads to increased expression of other inflammatory cytokines, such as IL-6 and IL-1β, and endothelial adhesion molecules.\(^\text{32}\) The study of Moon et al. demonstrated that CA reduced TNF-α-induced endothelial adhesiveness to human umbilical vein endothelial cells by inhibiting NF-κB activation.\(^\text{19}\) Our findings also demonstrated that CA reduced TNF-α-induced NF-κB activation in human keratinocytes. The findings suggested that CA might effectively modulate the transcriptional activity of NF-κB in cutaneous inflammatory responses, which is critical for the transcriptional regulation of many pro-inflammatory cytokines.

Neutrophils are key players in the recognition and elimination of pathogens, but they are a two-edged sword. Improper activation of neutrophils is thought to induce tissue lesions and contribute to the pathophysiology of various inflammatory diseases.\(^\text{33}\) MPO can be quantitatively as a measure of the magnitude of neutrophil activation, thus the inhibition of MPO activity is indicative of anti-inflammatory activity in the chronic or acute inflammation model. Multiple applications of TPA produced a higher MPO activity than a single application of TPA.\(^\text{15}\) Therefore, we only assayed MPO content in the chronic skin inflammation model (5 applications of TPA), and we found that CA markedly inhibited MPO activation. Khan et al. also observed that CA significantly inhibited MPO activity in mouse skin using the double TPA application model.\(^\text{13}\) Furthermore, the study of Song et al. indicated that CA significantly reduced MPO activity in incised-skin.\(^\text{14}\) These findings indicated that CA could inhibit MPO activity in several mouse skin models.

In conclusion, our results showed that CA has anti-inflammatory activities in both acute and chronic irritant contact dermatitis, and the inhibitory effect of CA might due, at least in part, to reducing the mRNA and protein synthesis of TNF-α, IL-6, and IL-1β cytokines and neutrophil-mediated MPO activity, and to the subsequent blockade of leucocyte accumulation. CA can also target inflammatory mediators specifically in the keratinocytes. The present data suggested that CA might modulate the expression of these cytokines at both transcription and post-transcription levels in acute irritant contact dermatitis. Our results also suggested that CA might be a good candidate for the treatment of inflammatory skin diseases.

Acknowledgments The study was supported by Grants from the National Natural Science Foundation of China (No. 31100653), Scientific and Technological Special Major Project for “Significant New Drug Creation” (No. 2012ZX09103-101-080), the Fundamental and Advanced Research Projects of Chongqing (No. cstc2013jcyyA10098), and the Key Science and Technology Program of Chongqing (No. CSTC, 2010AB5099).

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