

Suppression of Epidermal Growth Factor Receptor by Erlotinib Attenuates Carvacrol-induced Skin Inflammation

Yujing WANG^{1#}, Wenjie HUANG^{1#}, Haidong JIA², Qinglian TANG¹, Qingfei YIN², Yuanyuan CHEN², Wumei WANG³ and Zhengyu CAO¹

¹Department of TCM Pharmacology, School of Traditional Chinese Pharmacy, China Pharmaceutical University, Nanjing, Jiangsu, China, ²R&D Center, Shanghai Jahwa United Co., Ltd., Shanghai, China, and ³Department of Neurosurgery, Renmin Hospital of Wuhan University, School of Pharmaceutical Sciences, Wuhan University, Wuhan, China

[#]These authors contributed equally, and should be considered as first authors.

Epidermal growth factor receptors (EGFRs) regulate the growth and repair process of epithelia, as well as carcinogenesis. Activation of TRPV3 by carvacrol stimulates skin inflammation and epidermal hyperplasia; the latter can be suppressed by EGFR inhibition. However, whether EGFR signalling is responsible for skin inflammation remains elusive. The current study investigated the effect of erlotinib, an EGFR inhibitor, on skin inflammation in a carvacrol-induced atopic dermatitis mouse model. It was observed that erlotinib significantly attenuated carvacrol-induced overexpression of proinflammatory cytokines and suppressed peripheral blood mononuclear cell recruitment in HaCaT keratinocytes. In addition, it was demonstrated that erlotinib suppressed carvacrol-induced Akt and NF- κ B signalling pathways. Furthermore, inhibition of Akt and NF- κ B signalling pathways also attenuated the carvacrol-induced keratinocyte proinflammatory response. Finally, it was demonstrated that erlotinib treatment alleviated carvacrol-induced dermatitis. These data demonstrate that erlotinib ameliorates skin inflammation by regulating Akt and NF- κ B-mediated keratinocyte proinflammation, suggesting the therapeutic potential of erlotinib, a clinically used EGFR inhibitor, in skin inflammatory diseases.

Key words: carvacrol; dermatitis; EGFR; erlotinib; keratinocyte.

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Corr: Wumei Wang, PhD, Department of Neurosurgery, Renmin Hospital of Wuhan University, School of Pharmaceutical Sciences, Wuhan University, Wuhan, China, 430071, and Zhengyu Cao, PhD, Department of TCM Pharmacology, School of Traditional Chinese Pharmacy, China Pharmaceutical University, Nanjing, Jiangsu, China, 211198. E-mails: 2022103060039@whu.edu.cn; zycao@cpu.edu.cn

Skin inflammation is a common condition that affects about 20% of the population and contributes to ~1.79% of the disease burden worldwide (1). Skin inflammation can be derived from immune system dysfunction, allergic reaction, or infection by bacteria, virus, or fungi (2). Common skin inflammatory diseases include atopic dermatitis, psoriasis, and cellulitis etc. (3). Among the most common type of skin inflammatory diseases, atopic dermatitis affects up to 25% of children and

SIGNIFICANCE

Epidermal growth factor receptors regulate epithelial growth and repair as well as carcinogenesis. Recent studies have shown that epidermal growth factor receptor inhibition by erlotinib is used to treat Olmsted syndrome, a severe skin disease caused by TRPV3 gain-of-function mutants. We demonstrate that suppression of epidermal growth factor receptor by erlotinib ameliorates skin inflammation through regulation of Akt and NF- κ B-mediated keratinocyte proinflammation. Our results highlight the therapeutic potential of erlotinib, a clinically used agent, to treat skin inflammatory diseases.

2–3% of adults with symptoms that include dry, cracked, and thickened skin accompanied by severe itching (4). Despite the availability of glucocorticoids, calcineurin inhibitors, and biologics, therapeutic efficacy or safety remains unsatisfactory in clinical practice (5). Thus, novel target-engaged therapy may provide the potential to treat skin inflammatory diseases.

Carvacrol, a type of monoterpenoid, exists widely in the essential oil of oregano, thyme, pepperwort, and wild bergamot (6). Carvacrol has been shown to display a variety of beneficial effects such as anti-cancer, antimicrobial, anti-inflammatory, and pain-relieving activities (6, 7). Carvacrol has been shown to directly activate transient receptor potential ankyrin 1 (TRPA1) and transient receptor potential vanilloid 3 (TRPV3) channels as well as directly inhibiting the transient receptor potential melastatin subfamily member 7 (TRPM7) (8,9). We and others have demonstrated that topical application of 2% carvacrol for 5 consecutive days triggered skin inflammation as reflected by erythema, scaly skin lesions, pruritus, and acanthosis, as well as increased expression of inflammatory mediators (10, 11), resembling the features of atopic dermatitis (12). Such an effect was mitigated in TRPV3 knockout mice and also can be largely suppressed by the specific TRPV3 inhibitor, suggesting that carvacrol-induced skin inflammation was dependent on TRPV3 activity (10–12).

Epidermal growth factor receptor (EGFR) is a transmembrane receptor with signal-transducing tyrosine kinase activity, involved in the growth and repair process of epithelia, as well as carcinogenesis (13). TRPV3 and

EGFR have been proven to form a signalling complex to regulate hair morphogenesis and skin barrier formation (14). It has been demonstrated that activation of TRPV3 by carvacrol causes chemokine overexpression in human HaCaT keratinocytes (15). Our previous study has also demonstrated that TRPV3 enhances keratinocyte proliferation by transforming growth factor- α (TGF- α) autocrine activation of the EGFR signalling pathway (16). However, whether EGFR signalling is responsible for a carvacrol-induced proinflammatory response in keratinocytes remains elusive.

Although chemotherapy with the use of EGFR inhibitors frequently causes cutaneous adverse reactions, including papulopustular rash, xerosis, paronychia, and pruritus (17), recent case reports show that patients with Olmsted syndrome caused by TRPV3 gain-of-function mutants had substantial improvement in hyperkeratosis and pain on treatment with erlotinib (18–20). This discrepancy suggests that there is a need to re-evaluate the role of EGFR in cutaneous inflammation. In the current study, we aimed to investigate the effects of erlotinib on carvacrol-induced proinflammation in HaCaT cells and dermatitis in mice.

MATERIALS AND METHODS

Reagents

Carvacrol was purchased from Sigma-Aldrich (St. Louis, MO, USA). Erlotinib (ERT), gefitinib (GEF), afatinib (AFA), MK-2206, BAY11-7085, and LY294002 were obtained from MedChemExpress LLC (Shanghai, China). Protease inhibitor cocktail was from ApexBio Technology (Houston, TX, USA). Primary antibodies against phospho (p)-EGFR, EGFR, p-Akt, Akt, p-NF- κ B (p65 subunit), NF- κ B (p65 subunit), inhibitor of NF- κ B (I- κ B), p-I- κ B, β -tubulin and GAPDH were purchased from Cell Signaling Technology (Danvers, USA). IRDye[®]800CW and 680RD secondary antibodies were purchased from LI-COR (Lincoln, NE, USA).

Animal testing

All animal protocols were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and approved by the laboratory animal care and use committee of China Pharmaceutical University. WT C57BL/6 mice (6–7 weeks old) were purchased from the animal core facility of Nanjing Medical University (Nanjing, Jiangsu, China). After shaving the dorsal hair for 1 day, 100 μ L of 1% carvacrol (dissolved in 40% ethanol solution) was topically applied to the back skin of mice once daily for 4 consecutive days. To study the effect of erlotinib on skin dermatitis induced by carvacrol, a 100 μ L erlotinib solution (0.4 mg/mL) was injected subcutaneously 1 h prior to each topical application of carvacrol. The severity of dermatitis was assessed based on 4 symptoms: (1) erythema/haemorrhage, (2) scarring/dryness, (3) oedema, and (4) exfoliation/erosion. Each symptom was scored from 0 to 3 points (none, 0; mild, 1; moderate, 2; severe, 3). The sum of the scores from the 4 symptoms was used to quantify the severity of dermatitis. On the 4th day, a Canon EOS 60D camera was used to photograph the gross appearance of the dorsal skin. The degree of pruritus was calculated by counting scratching bouts in a period of 10 min after carvacrol application. A scratching bout

consisted of a series of movements when a mouse lifted its hind limb towards the dorsal skin and scratched until the limb returned to the floor or mouth. Mice were sacrificed, and the dorsal skin was dissected and fixed with 4% paraformaldehyde for 48 h at room temperature (RT) or stored in liquid nitrogen.

Histopathological examination

Histopathological examination was performed as described previously (10). In brief, fixed tissue samples were embedded in paraffin, sectioned at 2 μ m, and stained with haematoxylin and eosin (H&E). The slides were photographed using an Eclipse Ti inverted microscope (Nikon, Tokyo, Japan). Epidermal thickness was quantified using NIS-Elements (V4.3, Nikon, Tokyo, Japan).

Quantitative real-time PCR

Total RNA was extracted using TRIzol (Vazyme, Nanjing, China), cDNA was generated with the HiScript II Q RT SuperMix kit for qPCR (Vazyme, Nanjing, China), and quantitative real-time PCR was performed using the SYBR green protocol. The mouse and human primers for GAPDH, interleukin-6 (IL), and IL-8 can be found from PrimerBank (10). The mRNA expression levels were quantified using the $2^{-\Delta\Delta C_t}$ method, and normalized to GAPDH.

Cell culture

The human epidermal keratinocyte (HaCaT) cells were obtained from iCell Bioscience Inc (Shanghai, China). HaCaT keratinocytes were cultured in DMEM containing 100 U/mL penicillin and streptomycin and 10% foetal bovine serum (FBS). Cells were incubated at 37°C in an environment of 5% CO₂ and 95% humidity.

Western blot analysis

Western blotting was performed as described by Li *et al.* (21). Briefly, HaCaT cells were lysed using the RIPA buffer (in millimoles: 150 NaCl, 20 Tris-HCl, 2.5 sodium pyrophosphate, 1 EGTA, 2 Na₂EDTA, 1 sodium orthovanadate, 1% Nonidet P40, 1% sodium deoxycholate, 1 β -glycerophosphate, pH 7.4) supplemented with a cocktail of protease inhibitors. After quantification of the protein concentration using a BCA kit (Beyotime, Shanghai, China), a 30 μ g of protein from each sample was loaded to SDS-PAGE for separation. Separated proteins were then transferred onto nitrocellulose membranes. After blocking with skimmed milk, primary antibodies against p-EGFR (1:500), EGFR (1:500), p-Akt (1:500), Akt (1:500), p-NF- κ B (1:1000), NF- κ B (1:1000), p-I- κ B (1:1000), I- κ B (1:1000), and β -tubulin (1:10,000) were added. After incubation overnight at 4°C, the membranes were incubated with the secondary antibodies for 1 h at RT. The blot images were acquired and the densitometry was quantified using the LI-COR Odyssey Infrared Imaging System (LI-COR Biotechnology).

Peripheral blood mononuclear cells (PBMC) recruitment assay

Isolation and purification of PBMCs from healthy donors were performed as described previously (10). The study was approved (protocol code: 2019-ky013) by the research ethics board of the Institute of Dermatology and Hospital for Skin Diseases, Chinese Academy of Medical Sciences, and informed consent of donors was obtained. To evaluate PBMC recruitment by HaCaT cells, 200 μ L PBMC suspension (5×10^5 cells/mL in RPMI-1640) was loaded onto the upper chamber of a Transwell. A volume of 600 μ L supernatant from HaCaT cells treated with the respective drug for 6 h was added to the lower chamber. After incubating in 5% CO₂ at 37°C for another 3 h, cells on the membrane of the inlet were fixed with 10% methanol for 10 min and stained with crystal violet

for 30 min. Cell numbers were counted blindly from 5 random fields photographed at 200× magnification using a digital colour camera (Nikon, Tokyo, Japan) attached to an inverted microscope.

Statistical analysis

GraphPad Prism version 6.0 (San Diego, CA, USA) was used to generate graphs. Data were presented as mean ± SEM. The statistical significance was analysed using an unpaired Student's *t*-test or one-way ANOVA followed by Dunnett's multiple comparisons test. A *p*-value less than 0.05 is considered to be statistically significant.

RESULTS

Erlotinib attenuated carvacrol-induced proinflammatory response in keratinocytes

We previously have demonstrated that in human HaCaT keratinocytes, carvacrol induced a proinflammatory response that was nearly abolished by a selective TRPV3 inhibitor, scutellarein (10). We have also demonstrated that carvacrol promoted proliferation of HaCaT cells through TRPV3-mediated EGFR activity (16). We therefore examined whether EGFR activity was also involved in the regulation of carvacrol-induced proinflammatory

response. Consistent with previous demonstration, carvacrol (100 μM) treatment for 3 h increased mRNA expression levels of IL-6 and IL-8 to 3.61 ± 0.40 ($p < 0.01$) and 10.48 ± 1.29 -fold ($p < 0.01$) of vehicle-treated HaCaT cells, respectively (Fig. 1A, B). The carvacrol-enhanced IL-6 and IL-8 expression were reduced by erlotinib in a dose-dependent manner (10–100 nM). The reductions were $14.3 \pm 6.1\%$ (10 nM), $80.2 \pm 5.6\%$ (30 nM, $p < 0.01$), and $99.0 \pm 1.5\%$ (100 nM, $p < 0.01$), and $47.1 \pm 3.4\%$ (10 nM, $p < 0.01$), $70.8 \pm 1.5\%$ (30 nM, $p < 0.01$), and $69.0 \pm 0.3\%$ (100 nM, $p < 0.01$) for IL-6 and IL-8, respectively (Fig. 1A, B). Similarly, treatment with gefitinib dose-dependently reduced carvacrol-enhanced IL-6 and IL-8 expression by $16.7 \pm 15.7\%$ (10 nM), $71.5 \pm 9.5\%$ (30 nM, $p < 0.01$), and $93.4 \pm 4.0\%$ (100 nM, $p < 0.01$), and $85.5 \pm 2.0\%$ (10 nM, $p < 0.01$), $79.4 \pm 0.2\%$ (30 nM, $p < 0.01$), and $91.4 \pm 0.4\%$ (100 nM, $p < 0.01$), respectively (Fig. S1A, B). However, afatinib caused a more robust inhibition of carvacrol-induced IL-8 expression but a remarkable promotion of carvacrol-induced IL-6 expression, which would contribute to adverse drug reactions in the clinic (Fig. S1C, D). We next investigated the chemotactic effect of the conditional medium collected

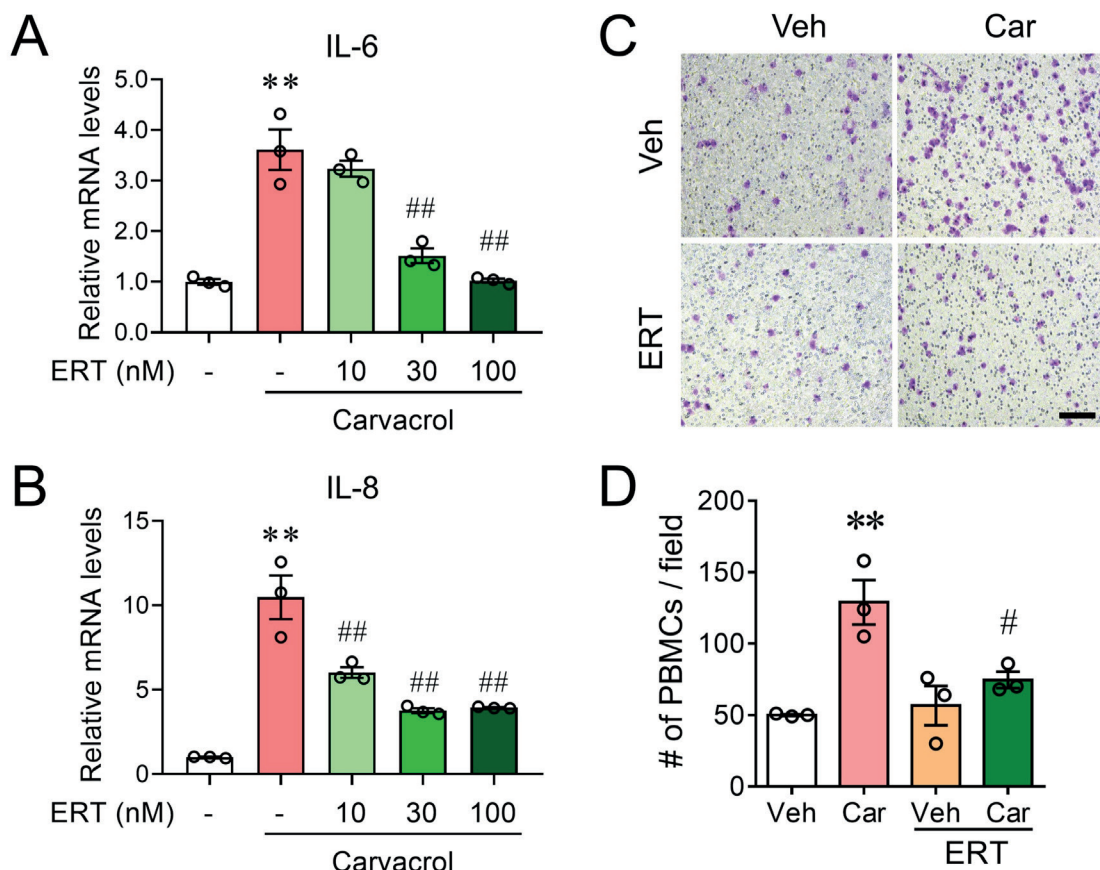


Fig. 1. Erlotinib attenuates carvacrol-induced proinflammatory responses in keratinocytes. (A–B) Relative mRNA levels of IL-6 (A) and IL-8 (B) in HaCaT cells treated with 100 μM carvacrol (Car) in the absence and presence of erlotinib (ERT) at different concentrations for 3 h. Data were normalized to the vehicle (Veh) control. (C) Representative microphotographs showing fixed and stained PBMCs on the bottom side of the invasion membrane. Scale bar, 50 μm. (D) Quantification of PBMC chemotaxis induced by media harvested from cultured HaCaT cells treated with Car in the absence and presence of ERT for 6 h. Data in (A), (B), and (D) are mean ± SEM from 3 independent experiments. ** $p < 0.01$, vs vehicle (Veh); * $p < 0.05$, ** $p < 0.01$ vs carvacrol (Car) by one-way ANOVA followed by Dunnett's multiple comparison tests.

from the HaCaT cell cultures. The culture medium collected from carvacrol (100 μ M)-treated HaCaT cells for 6 h significantly increased the number of chemotactic PBMCs from 50.0 ± 0.57 cells/field to 129.0 ± 15.5 cells/field ($p < 0.01$) (Fig. 1C, D). The medium from erlotinib and carvacrol-treated HaCaT cells recruited significantly fewer PBMCs (74.7 ± 5.7 cells/field) ($p < 0.05$).

Erlotinib suppressed carvacrol-evoked Akt and NF- κ B signalling in HaCaT keratinocytes

Given that erlotinib robustly suppressed the carvacrol-induced chemokine expression and chemotactic effect to PBMC, we evaluated the signalling pathways responsible for erlotinib effect. Carvacrol treatment (30 min) increased EGFR phosphorylation to 2.28 ± 0.67 -fold of vehicle control ($p < 0.01$) in HaCaT cells (Fig. 2). Erlotinib (100 nM), which did not affect the basal level of EGFR phosphorylation, but abolished carvacrol-induced EGFR phosphorylation (Fig. 2). Similarly, carvacrol treatment also increased the phosphorylation levels of Akt, I- κ B, and NF- κ B to 1.98 ± 0.07 ($p < 0.05$), 4.07 ± 0.55 ($p < 0.01$), and 1.94 ± 0.27 -fold ($p < 0.05$) of respective vehicle control (Fig. 2). Erlotinib treatment abolished carvacrol-induced Akt, I- κ B, and NF- κ B phosphorylation by $96.1 \pm 13.1\%$ ($p < 0.05$), $99.5 \pm 0.6\%$ ($p < 0.01$), and $76.9 \pm 7.3\%$ ($p < 0.05$), respectively (Fig. 2).

Inhibition of PI3K/Akt and I- κ B/NF- κ B suppressed carvacrol-induced proinflammatory response in keratinocytes

Given that erlotinib suppressed Akt and NF- κ B phosphorylation, we examined the role of Akt and NF- κ B on

carvacrol-induced inflammatory response. The NF- κ B inhibitor, BAY11-7085 (10 μ M), which had no effect on basal IL-6 and IL-8 mRNA expression, decreased carvacrol-enhanced IL-6 and IL-8 expression by $80.0 \pm 2.2\%$ ($p < 0.01$), and $84.3 \pm 0.9\%$ ($p < 0.01$), respectively (Fig. 3A, B). Interestingly, inhibition of PI3K appeared to be less effective on carvacrol-enhanced IL-6 and IL-8 expression. LY294002 (10 μ M) treatment inhibited carvacrol-enhanced IL-6 and IL-8 expression by $60.2 \pm 3.5\%$ ($p < 0.01$) and $25.8 \pm 1.2\%$ ($p < 0.01$), respectively (Fig. 3A, B). We further investigated the effects of Akt inhibition on carvacrol-enhanced cytokines expression. MK-2206 (100 nM) abolished carvacrol-enhanced IL-6 and IL-8 expression (Fig. S2). Moreover, PBMC recruitment assay showed that BAY11-7085 abolished the PBMC recruitment and LY294002 decreased the PBMC recruitment by $71.6 \pm 7.7\%$ ($p < 0.01$) (Fig. 3C, D).

Erlotinib ameliorates carvacrol-induced skin inflammation in C57BL/6 mice

Given that erlotinib suppressed the proinflammation in HaCaT keratinocytes and inhibited the chemotaxis of PBMCs, we therefore examined the potential therapeutic effect of erlotinib against carvacrol-induced dermatitis in C57BL/6 mice. Consistent with our previous finding, topical application of 2% carvacrol once daily for 4 consecutive days evoked inflamed and scaly lesions. Subcutaneous injection of erlotinib alleviated atopic dermatitis-like symptoms including redness, oedema, and thickening (Fig. 4A, upper panel). Erlotinib treatment reduced the dermatitis score to $43.6 \pm 4.7\%$ ($p < 0.01$) (Fig. 4B). Carvacrol treatment induced remarkable acanth-

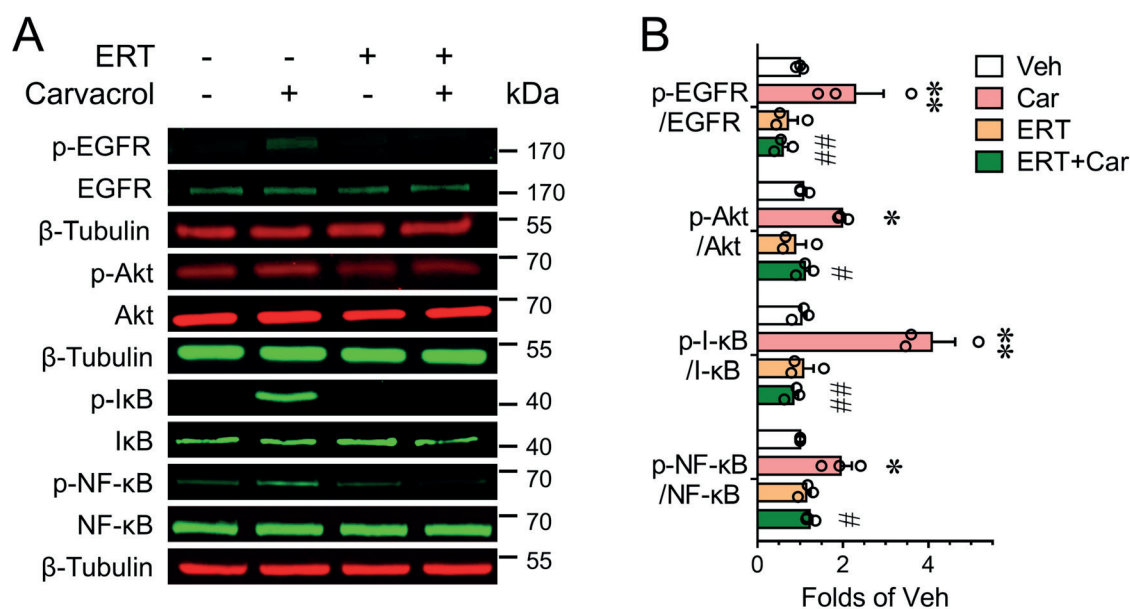


Fig. 2. Erlotinib suppressed carvacrol-evoked EGFR signalling in keratinocytes. (A–B) Representative western blots (A) and quantification (B) for phosphorylation of EGFR, Akt, I- κ B, and NF- κ B in HaCaT cells treated with 100 μ M carvacrol (Car) in the absence or presence of 100 nM erlotinib (ERT) for 30 min. * $p < 0.05$, ** $p < 0.01$, vs vehicle (Veh); * $p < 0.05$, ** $p < 0.01$ vs carvacrol (Car) by one-way ANOVA followed by Dunnett's multiple comparison tests.

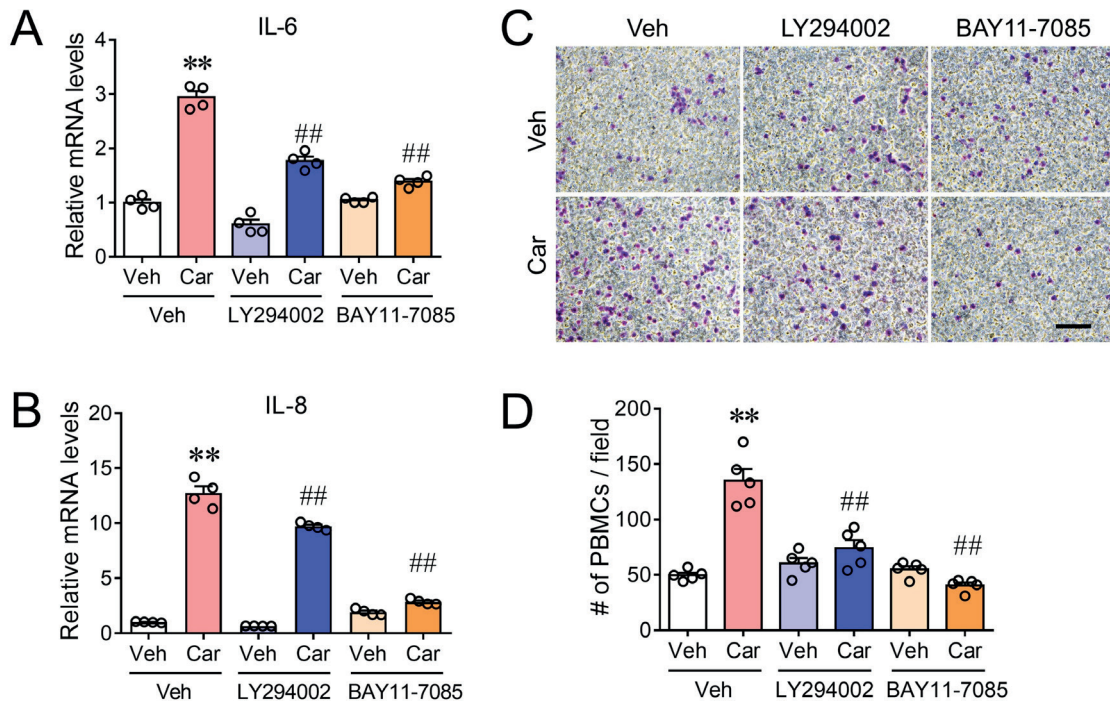


Fig. 3. Inhibition of PI3K/Akt and I- κ B/NF- κ B signalling suppressed carvacrol-induced proinflammatory responses in keratinocytes. (A–B) Relative mRNA levels of IL-6 (A) and IL-8 (B) in HaCaT cells treated with 100 μ M carvacrol (Car) in the absence and presence of 1 μ M BAY11-7085 or 10 μ M LY294002 for 3 h. Data were normalized to the vehicle (Veh) control. (C) Representative microphotographs showing fixed and stained PBMCs on the bottom side of the invasion membrane. Scale bar, 50 μ m. (D) Quantification of PBMC chemotaxis induced by media harvested from cultured HaCaT cells treated with Car in the absence and presence of BAY11-7085 or LY294002 for 6 h. Data in (A), (B), and (D) are mean $\pm$ SEM from 3 independent experiments. ** p < 0.01, vs vehicle (Veh); ** p < 0.01 vs carvacrol (Car) by one-way ANOVA followed by Dunnett's multiple comparison tests.

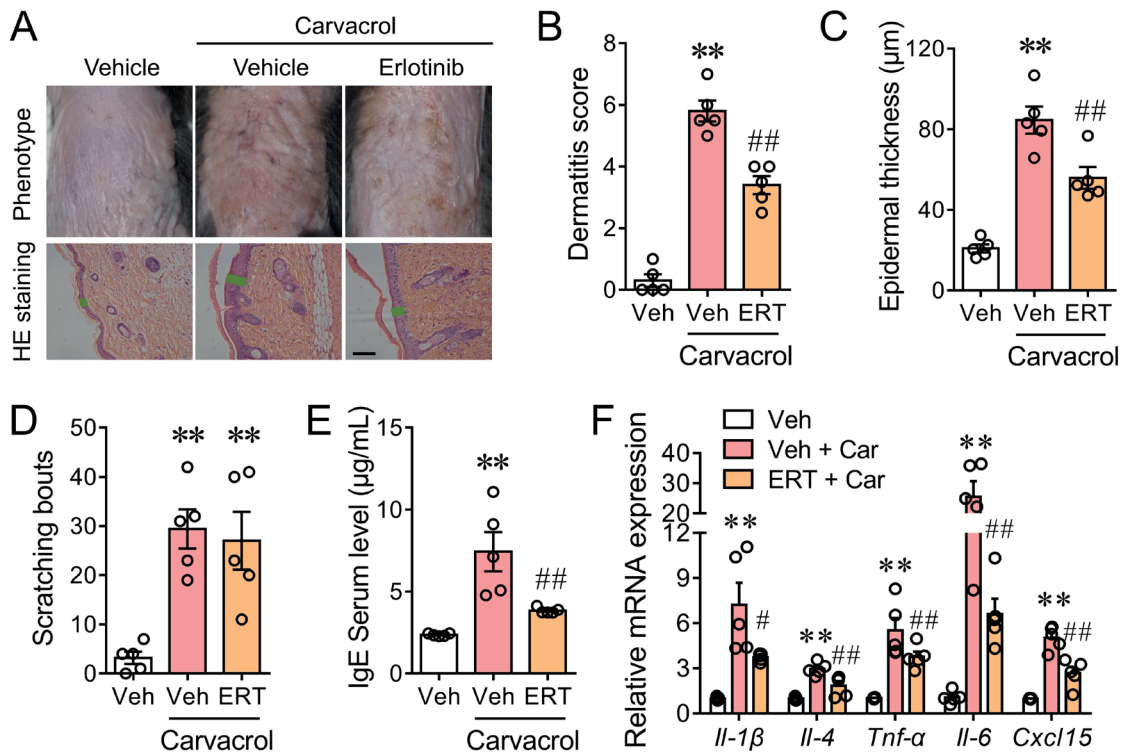


Fig. 4. Erlotinib ameliorates carvacrol-induced skin inflammation in C57BL/6 mice. (A) Representative images of the gross appearance (upper panel) and H&E-stained sections (bottom panel) of the back skins of WT C57BL/6J mice treated with vehicle (Veh), 2% carvacrol (Car), and Car plus 2.0 mg/kg erlotinib (ERT). Black scale bar, 50 μ m. Green lines indicate the thickness of stratum epidermis. (B–E) Quantification of dermatitis score (B), epidermal thickness (C), scratching bouts (D), and serum IgE level (E) in mice treated with Veh, Car, and Car plus ERT. (F) Relative mRNA levels of *Il-1 β* , *Il-4*, *Tnf- α* , *Il-6*, and *Cxcl15* in the back skins of the indicated groups, normalized to that of the Veh group. All bar graphs represent mean $\pm$ SEM (n = 5 mice). ** p < 0.01, vs Veh; * p < 0.05, ** p < 0.01 vs Car, by one-way ANOVA followed by Dunnett's multiple comparison tests.

osis, as indicated by the increased epidermal thickness (Fig. 4A, bottom panel and C). Erlotinib decreased the carvacrol-induced acanthosis by $45.0 \pm 7.6\%$ ($p < 0.01$) (Fig. 4C) without affecting the gross appearance and the skin thickness in vehicle-treated mice (Fig. S3). Carvacrol also triggered scratching behaviour, quantified by the increased number of scratching bouts from 3.20 ± 1.24 to 29.40 ± 3.98 per 10 min ($p < 0.01$). Erlotinib did not affect carvacrol-induced pruritus (27.00 ± 5.86 vs 29.40 ± 3.98 per 10 min, $p > 0.05$) (Fig. 4D). The serum IgE level in carvacrol-treated mice was increased from 2.37 ± 0.04 $\mu\text{g/mL}$ to 7.44 ± 1.20 $\mu\text{g/mL}$ ($p < 0.01$), which was decreased by $70.9 \pm 1.4\%$ with erlotinib treatment ($p < 0.01$) (Fig. 4E). The mRNA levels of inflammatory cytokines including *Il-1 β* , *Il-4*, *Tnf- α* , *Il-6*, and *Cxcl15* were increased by carvacrol treatment to 7.22 ± 1.47 ($p < 0.01$), 2.96 ± 0.19 ($p < 0.01$), 5.51 ± 0.83 ($p < 0.01$), 25.52 ± 5.18 ($p < 0.01$), and 5.03 ± 0.35 -fold ($p < 0.01$) of respective control of the vehicle-treated group. Erlotinib caused reductions on the carvacrol-induced mRNA levels by $56.0 \pm 1.9\%$ for *Il-1 β* ($p < 0.01$), $57.2 \pm 15.2\%$ for *Il-4* ($p < 0.05$), $39.0 \pm 7.7\%$ for *Tnf- α* ($p < 0.01$), $77.3 \pm 4.1\%$ for *Il-6* ($p < 0.01$), and $58.4 \pm 9.9\%$ for *Cxcl15* ($p < 0.01$) (Fig. 4F), respectively. These results suggest that erlotinib can alleviate the pathological hallmarks in the carvacrol-induced dermatitis.

DISCUSSION

EGFRs are prominently expressed in the epithelial cells, where they regulate the growth and repair process of epithelia (22). Mutation and/or overexpression of EGFR leads to carcinoma such as non-small-cell lung cancer (NSCLC), squamous cell carcinoma, and breast cancer (23). Previous studies have shown that activation of TRPV3 by carvacrol triggers cell proliferation and chemokine release in HaCaT cells (15, 16). Such cell proliferative effect is dependent on the EGFR activation through Ca^{2+} -mediated $\text{TGF}\alpha$ release (16). A study has also demonstrated that EGFR activation in keratinocytes induces expression of granulocyte/macrophage colony-stimulating factor (GM-CSF), a cytokine associated with psoriasis or allergic contact dermatitis progression (24). $\text{TNF-}\alpha$ -induced EGFR transactivation results in increased expression of keratinocyte-derived thymic stromal lymphopoietin (TSLP) (25). In the current study, we showed that erlotinib, an EGFR inhibitor, suppressed the expression of chemokines in human HaCaT keratinocytes and inhibited the chemotaxis of PBMCs, unambiguously demonstrating that in addition to cell proliferation EGFR also regulated the inflammatory response in keratinocytes. Moreover, we demonstrated that erlotinib suppressed carvacrol-induced dermatitis score, acanthosis, and inflammatory mediator expression in the mouse. These data demonstrated that erlotinib, a clinically used drug compound for non-small-cell lung

carcinoma therapy may have the potential to treat skin inflammatory diseases. To support our finding, erlotinib has been used to treat Olmsted syndrome, a severe skin disease resembling the features of psoriasis, with satisfactory efficacy (18). Interestingly, erlotinib has no effect on carvacrol-induced purpures, suggesting that erlotinib does not affect the neuronal firing.

Our study also demonstrated that erlotinib suppressed proinflammation in HaCaT keratinocytes through decreased activity of EGFR with subsequent attenuated activities of PI3K/Akt and I- κB /NF- κB . As a downstream signalling effector of EGFR receptor tyrosine kinase activity, PI3K/Akt is generally responsible for the proliferative effect by upregulation of a variety of cell growth and proliferative genes (26). Interestingly, we found that pharmacological suppression of PI3K/Akt robustly suppressed the carvacrol-induced chemokine expression, and chemotaxis of PBMCs suggesting that, in addition to its role in the proliferation, the PI3K/Akt signalling pathway was also involved in keratinocyte proinflammation. Erlotinib suppressed the carvacrol-induced phosphorylation level of Akt, suggesting that erlotinib-suppressed proinflammation in HaCaT keratinocytes was through decreased PI3K/Akt activity. We also demonstrated that pharmacological suppression of I- κB /NF- κB suppressed the carvacrol-induced chemokine expression and chemotaxis of PBMCs. Moreover, erlotinib suppressed carvacrol-induced phosphorylation level of I- κB and NF- κB . Together, these data suggested the involvement of I- κB /NF- κB in the erlotinib suppression of proinflammation in keratinocytes. Although it has been widely reported that NF- κB regulates the expression of an array of inflammatory mediators, how EGFR signalling to NF- κB occurs has not been fully clarified. Whether I- κB /NF- κB depends on PI3K/Akt activity needs further elucidation.

In conclusion, we demonstrate that erlotinib alleviates carvacrol-induced proinflammation responses in HaCaT keratinocytes through inhibition of the EGFR-mediated PI3K/Akt and I- κB /NF- κB signalling pathway. We also demonstrated that erlotinib ameliorates carvacrol-induced atopic dermatitis-like skin inflammation. Erlotinib, a clinically used agent, has the potential to treat skin inflammatory diseases.

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IRB approval status: All animal protocols were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised

1978) and approved by the laboratory animal care and use committee of China Pharmaceutical University.

The authors have no conflicts of interest to declare.

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