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Suppressive effects of antimycotics on tumor necrosis factor-α-induced CCL27, CCL2, and CCL5 production in human keratinocytes

Naoko Kanda *, *Shinichi Watanabe*

Department of Dermatology, Teikyo University School of Medicine, 11-1, Kaga-2, Itabashi-Ku, Tokyo 173-8605, Japan

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ABSTRACT

Antimycotic agents are reported to improve cutaneous symptoms of atopic dermatitis or psoriasis vulgaris. Keratinocytes in these lesions excessively produce chemokines, CCL27, CCL2, or CCL5 which trigger inflammatory infiltrates. Tumor necrosis factor-α (TNF-α) induces production of these chemokines via activating nuclear factor-κB (NF-κB). We examined in vitro effects of antimycotics on TNF-α-induced CCL27, CCL2, and CCL5 production in human keratinocytes. Antimycotics ketoconazole and terbinafine hydrochloride suppressed TNF-α-induced CCL27, CCL2, and CCL5 secretion and mRNA expression in keratinocytes in parallel to the inhibition of NF-κB activity while fluconazole was ineffective. Anti-prostaglandin E_2 (PGE_2) antiserum or antisense oligonucleotides against PGE_2 receptor EP2 or EP3 abrogated inhibitory effects of ketoconazole and terbinafine hydrochloride on TNF-α-induced NF-κB activity and CCL27, CCL2, and CCL5 production, indicating the involvement of endogenous PGE_2 in the inhibitory effects. Prostaglandin H_2, a precursor of PGE_2 can be converted to thromboxane A_2. Ketoconazole, terbinafine hydrochloride and thromboxane A_2 synthase (EC 5.3.99.5) inhibitor, carboxyheptyl imidazole increased PGE_2 release from keratinocytes and reduced that of thromboxane B_2, a stable metabolite of thromboxane A_2. Carboxyheptyl imidazole also suppressed TNF-α-induced NF-κB activity and CCL27, CCL2, and CCL5 production. These results suggest that ketoconazole and terbinafine hydrochloride may suppress TNF-α-induced NF-κB activity and CCL27, CCL2, and CCL5 production by increasing PGE_2 release from keratinocytes. These antimycotics may suppress thromboxane A_2 synthesis and redirect the conversion of PGH_2 toward PGE_2. These antimycotics may alleviate inflammatory infiltration in atopic dermatitis or psoriasis vulgaris by suppressing chemokine production.

1. Introduction

Atopic dermatitis (AD) and psoriasis vulgaris (PV) are chronic inflammatory dermatoses with massive infiltrates of T cells or macrophages [1]. Such infiltrates may be attributable to the excessive production of chemokines by lesional keratinocytes [1]. CCL27 secreted by keratinocytes attracts cutaneous lymphocyte-associated antigen-positive memory T cells into skin lesions with AD and PV [2,3]. CCL5 secreted by keratinocytes recruits type 1 T cells or macrophages into skin lesions with both diseases, and is involved in the infiltration and degranulation of eosinophils in the early phase of AD [4,5]. CCL2 secreted by keratinocytes may recruit macrophages into skin lesions with PV and AD [1,6]. A pro-inflammatory

* *Corresponding author*. Tel.: +81 3 3964 2473; fax: +81 3 5375 5314.
E-mail address: nmk@med.teikyo-u.ac.jp (N. Kanda).
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cytokine tumor necrosis factor-α (TNF-α) is released from mast cells, macrophages, or dendritic cells in the skin, and induces the production of these chemokines in keratinocytes at least partially via activating nuclear factor-κB (NF-κB) [4,7–9]. Promoter regions of these chemokine genes contain NF-κB elements which confer transcription of these genes [7,9,10]. We previously found that a lipid mediator prostaglandin E_2 (PGE_2) suppressed TNF-α-induced CCL27, CCL2 and CCL5 production via the suppression of NF-κB activity [8]. Prostaglandin E_2 is intracellularly produced from arachidonic acid through two rate-limiting steps [11]: firstly arachidonic acid is converted to PGH_2 by cyclooxygenase (COX; EC 1.14.99.1). Secondly, PGH_2 is converted to PGE_2 by prostaglandin E_2 synthase (PGES; EC 5.3.99.3). Prostaglandin H_2 is also converted to thromboxane A_2 (TXA_2) by thromboxane A_2 synthase (TXAS; EC 5.3.99.5).

Systemic or topical treatment with antimycotic agents is reported to improve cutaneous symptoms of AD or PV especially in the seborrheic region [12–15]. This is partly caused by the fungicidal or fungistatic effects of these agents; several *Malassezia* species are isolated from AD or PV skin lesions [16,17], and may act as allergens and exacerbate AD [18]. *Malassezia furfur* enhances the expression of integrins or 70 kDa heat shock protein in keratinocytes [19], which may activate dendritic cells and exacerbate PV [20]. Treatment with antimycotics may thus reduce *Malassezia* colonization and the severity of AD or PV. However, besides antifungal effects, the direct immunomodulatory effects of antimycotics may mediate their therapeutic efficacy on AD or PV. Antimycotic azole derivative ketoconazole or non-azole terbinafine hydrochloride suppressed interleukin-4 (IL-4) and IL-5 production in anti-CD3 plus anti-CD28-stimulated T cells from patients with AD [21]. Ketoconazole suppressed IL-4 plus anti-CD40-induced IgE class switching in surface IgE^- B cells from patients with AD [22]. Oral ketoconazole treatment also reduced serum IgE values in patients with AD [12]. Topical miconazole on guinea pig skin reduced the proliferation of keratinocytes, indicating the suppressive effects of this drug on keratinocyte hyperproliferation in PV [23]. It is thus plausible that antimycotics may attenuate overexpression of chemokines by keratinocytes in AD or PV lesions and thus relieve skin inflammation. However, the direct effects of antimycotics on chemokine production by keratinocytes have not yet been investigated.

In this study, we investigated the in vitro effects of antimycotics on TNF-α-induced CCL27, CCL2, and CCL5 production in human keratinocytes. We identified the inhibitory effects of azole antimycotic ketoconazole and non-azole terbinafine hydrochloride, and the involvement of PGE_2 in those effects.

2. Materials and methods

2.1. Reagents

Ketoconazole was donated by Janssen Pharmaceutica NV (Beerse, Belgium). Terbinafine hydrochloride was from Novartis Pharma (Tokyo, Japan). Fluconazole was from Wako Pure Chemical (Osaka, Japan). Ketoconazole, terbinafine hydrochloride, and fluconazole were dissolved in DMSO at 20 mM to create stock solutions and were subsequently diluted into experimental media to yield final concentrations. The DMSO concentration as vehicle control was 0.1% (v/v). (E)3-[(4-methylphenyl)sulfonyl]-2-propenitrile (Bay11-7082) was purchased from Calbiochem (San Diego, CA). Actinomycin D, parthenolide, rabbit anti-PGE_2 antiserum, and rabbit non-immune serum (NIS) were from Sigma (St. Louis, MO). Recombinant human TNF-α was from R&D Systems (Minneapolis, MN). Thromboxane A_2 mimetic, 9,11-dideoxy-9α,11α-methanoepoxy $PGF_{2\alpha}$ (U46619) and TXAS inhibitor, carboxyheptyl imidazole were from BIOMOL (Plymouth Meeting, PA).

2.2. Culture of keratinocytes

Human neonatal foreskin keratinocytes (Clonetics, Walkersville, MD) from four different donors were cultured as described [8] in serum-free KGM (Clonetics) consisting of basal medium KBM supplemented with 0.5 μg/ml hydrocortisone, 5 ng/ml epidermal growth factor, 5 μg/ml insulin, and 0.5% bovine pituitary extract. Cells in the third passage were used.

2.3. Chemokine secretion

Keratinocytes (5×10^4 per well) were seeded in triplicate into 24-well plates in 0.4 ml KGM, adhered overnight, washed, and incubated with KBM for 24 h. The cells were then treated with 10 ng/ml TNF-α in the presence of vehicle (DMSO) or indicated concentrations of antimycotics in 0.4 ml KBM for 48 h. Culture supernatants were assayed by ELISA for CCL27 (R&D), CCL2 and CCL5 (Biosource, Camarillo, CA). In some experiments, keratinocytes were incubated with 1000-fold diluted anti-PGE_2 antiserum or NIS, or 10 μM U46619 for 30 min prior to the addition of TNF-α and antimycotics.

2.4. Reverse transcription (RT)-polymerase chain reaction (PCR)

Keratinocytes were incubated as above for 8 h to analyze CCL27, CCL2, and CCL5 mRNA levels. Total cellular RNA was extracted and reverse-transcribed to produce cDNA. The cDNA was thermocycled for PCR using primers for CCL27, CCL2, CCL5, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described [4,6,8]. PCR products were analyzed by electrophoresis, and densitometric analysis was performed by ATTO lane analyzer ver.3 (ATTO, Osaka, Japan). CCL27, CCL2 or CCL5 mRNA levels were normalized to those of GAPDH, and are shown as a fold induction.

2.5. mRNA stability analysis

mRNA stability was analyzed as described [8]. Keratinocytes were incubated as above for 8 h. Actinomycin D (5 μg/ml) was then added, and RNA was isolated 0, 0.5, 1, 2, 4, 8, 16, and 32 h later. RT-PCR was performed as described above, and the decay of mRNA was determined from band density ratios of CCL27, CCL2 or CCL5/GAPDH.

2.6. Plasmid and transfection

pNF-κB-luc containing four copies of NF-κB elements in front of the TATA box upstream of firefly luciferase reporter was

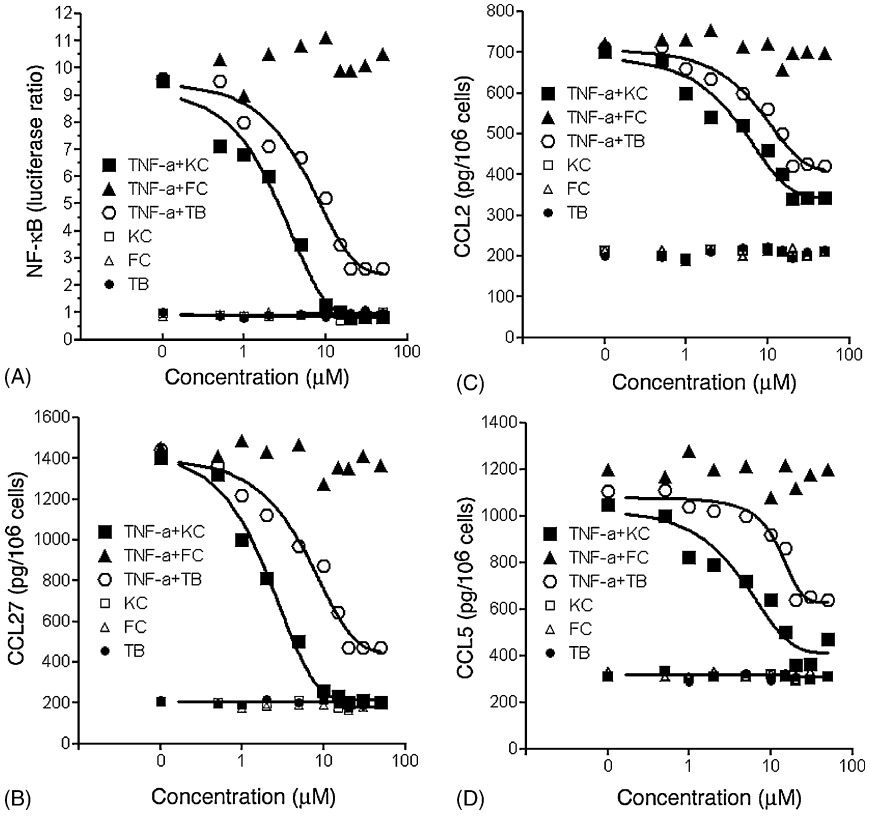

Fig. 1 – Inhibition by antimycotics on TNF-α-induced NF-κB transcriptional activity (A) and CCL27 (B), CCL2 (C) or CCL5 (D) secretion. (A) Keratinocytes were transfected with pNF-κB-luc and pRL-tk for 6 h, washed and starved in KBM for 24 h, then incubated with 10 ng/ml TNF-α in the presence of vehicle or indicated concentrations of ketoconazole (KC), fluconazole (FC) or terbinafine hydrochloride (TB). At 18 h, NF-κB transcriptional activity was analyzed by dual luciferase assays. (B–D) Keratinocytes without transfection were incubated with TNF-α and antimycotics as described above. At 48 h, chemokine amounts in culture supernatants were determined by ELISA. Data are means of triplicate cultures and represent four separate experiments.

Table 1 – The effects of antimycotics on the stability of chemokine mRNA[a]

Conditions	Estimated mRNA half-life (h)		
	CCL27	CCL2	CCL5
Controls	5.1 ± 0.5	4.0 ± 0.5	3.5 ± 0.4
KC	4.9 ± 0.5	4.1 ± 0.5	3.4 ± 0.4
FC	5.3 ± 0.6	3.9 ± 0.4	3.6 ± 0.4
TB	4.8 ± 0.5	3.8 ± 0.4	3.7 ± 0.4
TNF-α	8.2 ± 0.9*	9.1 ± 1.0*	7.1 ± 0.8*
TNF-α + KC	8.3 ± 0.9*	9.2 ± 0.9*	7.3 ± 0.8*
TNF-α + FC	7.9 ± 0.8*	9.1 ± 0.9*	6.8 ± 0.7*
TNF-α + TB	8.1 ± 0.9*	8.9 ± 0.9*	7.2 ± 0.8*

[a] Keratinocytes were incubated with 10 ng/ml TNF-α in the presence of vehicle or 20 μM ketoconazole (KC), fluconazole (FC) or terbinafine hydrochloride (TB) for 8 h. Actinomycin D (5 μg/ml) was added, and RNA was isolated 0, 0.5, 1, 2, 4, 8, 16 and 32 h later. RT-PCR was performed, and the decay of mRNA was determined from band density ratios of each chemokine/GAPDH. Results are means ± S.E.M. ($n = 4$).

* $P < 0.05$ vs. controls without TNF-α.

purchased from Clontech (San Jose, CA). Transient transfection was performed with FuGENE® 6 (Roche, Indianapolis, IN) as described [8]. Keratinocytes were seeded in 24-well plates and grown to about 60% confluence. pNF-κB-luc and herpes simplex virus thymidine kinase promoter-linked renilla luciferase vector (pRL-tk) (Clontech) mixed with FuGENE® 6 were added to keratinocytes. After 6 h, cells were washed and incubated in KBM for 24 h, then treated with 10 ng/ml TNF-α in the presence of vehicle, indicated concentrations of antimycotics, Bay11-7082, or parthenolide. After 18 h, firefly and renilla luciferase activities of cell extracts were quantified by the dual luciferase assay system (Promega, Madison, WI). NF-κB transcriptional activities are expressed as ratios of firefly/renilla luciferase activity.

2.7. *Measurement of PGE_2 and thromboxane B_2 (TXB_2) release*

Keratinocytes were incubated with 10 ng/ml TNF-α in the presence of vehicle or indicated concentrations of antimycotics for 48 h. The supernatant PGE_2 or TXB_2 amounts were measured by ELISA (R&D or Cayman, Ann Arbor, MO, respectively).

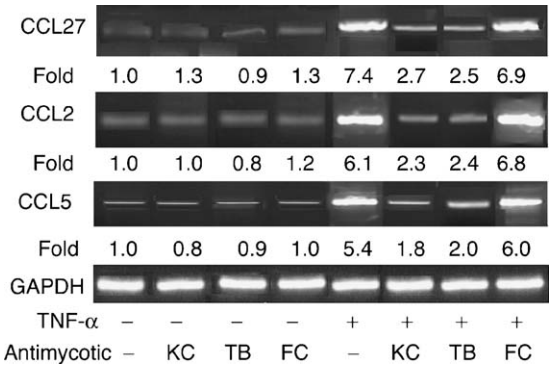

Fig. 2 – Inhibition by antimycotics on TNF-α-induced CCL27, CCL2 or CCL5 mRNA expression. Keratinocytes were incubated with 10 ng/ml TNF-α in the presence of vehicle or 20 μM ketoconazole (KC), fluconazole (FC) or terbinafine hydrochloride (TB) for 8 h. RNA was isolated and chemokine and GAPDH mRNA levels were analyzed by RT-PCR. Results represent four separate experiments. Chemokine mRNA levels were normalized to those of GAPDH and are shown as a fold induction.

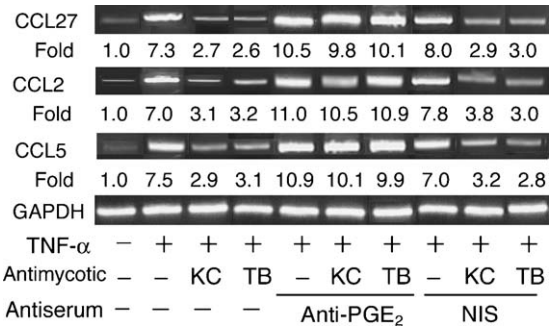

Fig. 4 – Effects of anti-PGE_2 antiserum on antimycotic-mediated inhibition of CCL27, CCL2 or CCL5 mRNA expression induced by TNF-α. Keratinocytes were preincubated with 1000-fold diluted anti-PGE_2 antiserum or non-immune serum (NIS) for 30 min, then incubated with 10 ng/ml TNF-α in the presence of vehicle or 20 μM ketoconazole (KC) or terbinafine hydrochloride (TB). mRNA levels were analyzed at 8 h. Results represent four separate experiments. Chemokine mRNA levels were normalized to those of GAPDH and are shown as a fold induction.

2.8. *Western blotting*

Western blotting was performed as described [24]. Keratinocytes were incubated with 10 ng/ml TNF-α in the presence of vehicle, indicated concentrations of antimycotics, Bay11-7082, or parthenolide for 24 h. Cellular proteins extracted in lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, 10 μg/ml each of aprotinin, leupeptin, and pepstatin, 1% Nonidet P-40, 1 mM Na_3VO_4, and 1 mM NaF) were subjected to SDS-PAGE, and transferred to polyvinylidene difluoride membranes. The membranes were blocked, and incubated with primary antibodies, then with horseradish

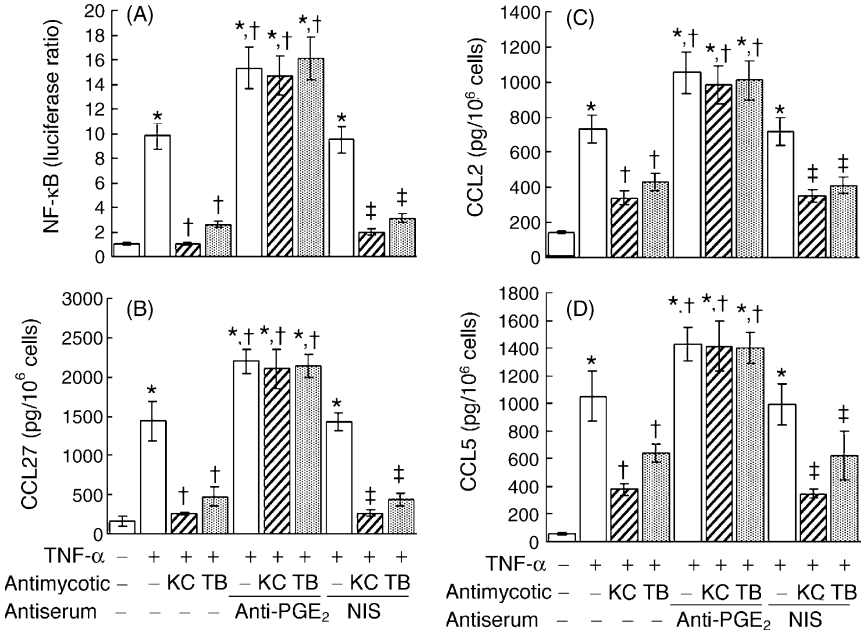

Fig. 3 – Effects of anti-PGE_2 antiserum on antimycotic-mediated inhibition of NF-κB activity (A) and CCL27 (B), CCL2 (C) or CCL5 (D) secretion induced by TNF-α. (A) Keratinocytes were transfected with pNF-κB-luc and pRL-tk, starved in KBM, preincubated with 1000-fold diluted anti-PGE_2 antiserum or non-immune serum (NIS) for 30 min, then incubated with 10 ng/ml TNF-α in the presence of vehicle or 20 μM ketoconazole (KC) or terbinafine hydrochloride (TB). At 18 h, NF-κB activity was analyzed. (B–D) Keratinocytes without transfection were sequentially incubated with antiserum and TNF-α plus antimycotics, as described above. Chemokine secretion was analyzed at 48 h. $^{*}P < 0.05$ vs. controls without TNF-α; $^{\dagger}P < 0.05$ vs. TNF-α plus vehicle; $^{\ddagger}P < 0.05$ vs. TNF-α plus NIS plus vehicle. Data are presented as means ± S.E.M. ($n = 4$).

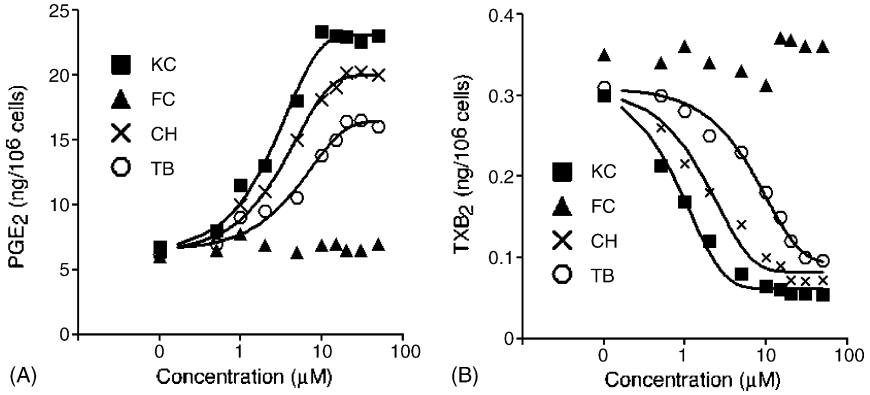

Fig. 5 – Effects of antimycotics on TNF-α induced release of PGE_2 (A) and TXB_2 (B). Keratinocytes were incubated with 10 ng/ml TNF-α in the presence of vehicle or indicated concentrations of ketoconazole (KC), fluconazole (FC), carboxyheptyl imidazole (CH) or terbinafine hydrochloride (TB) for 48 h. Prostaglandin E_2 or TXB_2 amounts in culture supernatants were determined by ELISA. Data are means of triplicate cultures and represent four separate experiments. Prostaglandin E_2 or TXB_2 release in cultures without TNF-α were means ± S.D. 3.32 ± 0.33 or 0.21 ± 0.02 ng/10^6 cells, respectively.

peroxidase-conjugated secondary antibodies, washed and developed by enhanced chemiluminescence (Amersham, Arlington Heights, IL). Primary antibodies used were anti-COX-1, COX-2, anti-microsomal PGES-1 (mPGES-1), anti-cytosolic PGES (cPGES), anti-TXAS (Cayman), and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA). Cyclooxygenase-1, COX-2, mPGES-1, cPGES, and TXAS protein levels were normalized to those of GAPDH, and are shown as a fold induction. The protein levels of PGE_2 receptors, EP1, EP2, EP3 or EP4 were examined by Western blotting using whole cell-lysates, anti-EP1, EP2, EP3, or EP4 antibodies (Cayman) or GAPDH antibody, and secondary antibodies as described above.

2.9. *Treatment with antisense oligonucleotide*

Antisense oligonucleotides against EP1, EP2, EP3 or EP4 or control scrambled oligonucleotide were synthesized as phosphorothioate-modified oligos and were HPLC-purified as described [25,26]. The oligonucleotides were EP1, 5′-GCAAGGGCTCATGTCAGG-3′; EP2, 5′-GCCTGGAGTCATTGA-3′; EP3, 5′-GTCTCCTTCATGTTGGC-3′; EP4, 5′-GACTCCGGG-GATGGA-3′; control scrambled, 5′-AGTACCAGGACTTC-GAATGTGCACT-3′. The keratinocytes were transfected with a final volume of 0.2 μM of the indicated oligonucleotides premixed with FuGENE® 6 in KGM for 24 h. The medium was aspirated, and the cells were cultured with KBM for 24 h, then incubated with 10 ng/ml TNF-α in the presence of vehicle or antimycotics (20 μM). In some experiments, these antisense oligonucleotides were transfected together with pNF-κB-luc and phRL-tk.

2.10. *Statistical analyses*

Dose response curves in Figs. 1 and 5 were fitted by non-linear regression analyses. One-way ANOVA with Scheffe's multiple comparison test was used in Figs. 3, 6, 8B–E and 9B, and Table 1. A P-value <0.05 was considered significant.

3. Results

3.1. *Inhibition of TNF-α-induced CCL27, CCL2 and CCL5 production by antimycotics*

We firstly examined the effects of azole or non-azole antimycotics on TNF-α-induced NF-κB transcriptional activity. Azole antimycotic ketoconazole or non-azole antimycotic terbinafine hydrochloride dose-dependently suppressed TNF-α-induced NF-κB transcriptional activity while azole antimycotic fluconazole was ineffective (Fig. 1A). The inhibitory effect of ketoconazole was greater than that of terbinafine hydrochloride; IC_{50} was 2.9 or 7.5 μM for ketoconazole or terbinafine hydrochloride, respectively. Basal NF-κB activity in the absence of TNF-α was modest and was not altered by these antimycotics. These antimycotics (≤100 μM) did not alter the viability of keratinocytes (>96% viable).

We then examined whether antimycotics suppressed TNF-α-induced production of CCL27, CCL2 or CCL5 whose expression is dependent on NF-κB activity. In parallel to the inhibition of NF-κB activity, ketoconazole and terbinafine hydrochloride dose-dependently suppressed TNF-α-induced CCL27, CCL2 and CCL5 secretion while fluconazole was ineffective (Fig. 1B–D). The inhibitory effects of ketoconazole were greater than those of terbinafine hydrochloride; the IC_{50} values for CCL27, CCL2 or CCL5 secretion were 2.9, 5.2 or 5.3 μM in ketoconazole and 6.3, 8.3 or 11.4 μM in terbinafine hydrochloride, respectively. Basal levels of CCL27, CCL2 or CCL5 secretion were marginal and were not altered by these antimycotics. In parallel to protein secretion, ketoconazole and terbinafine hydrochloride suppressed TNF-α-induced CCL27, CCL2 or CCL5 mRNA expression while fluconazole was ineffective (Fig. 2). Basal mRNA levels of these chemokines were marginal and were not altered by these antimycotics. These results suggest that ketoconazole and terbinafine hydrochloride may suppress TNF-α-induced CCL27, CCL2 or CCL5 production at least partially via the suppression of NF-κB

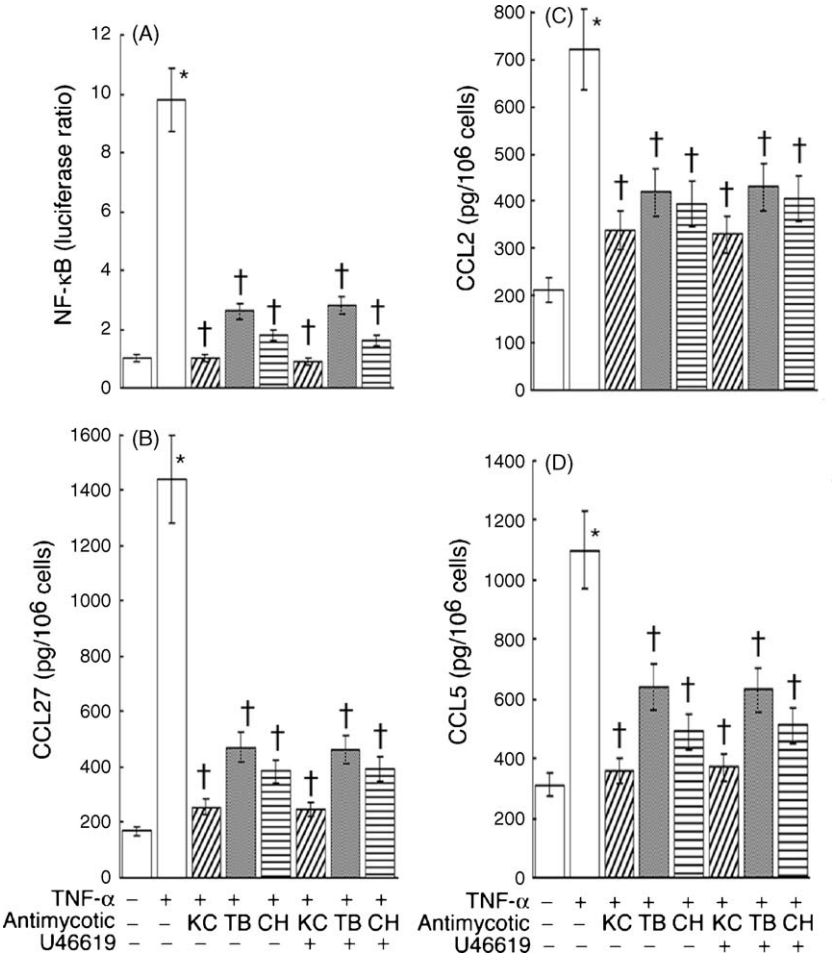

Fig. 6 – Effects of U46619 on antimycotic-mediated inhibition of NF-κB activity (A), CCL27 (B), CCL2 (C) or CCL5 (D) secretion induced by TNF-α. (A) Keratinocytes were transfected with pNF-κB-luc and pRL-tk, starved in KBM, preincubated with 10 μM U46619 for 30 min, then incubated with 10 ng/ml TNF-α in the presence of vehicle or 20 μM ketoconazole (KC), terbinafine hydrochloride (TB) or carboxyheptyl imidazole (CH). At 18 h, NF-κB activity was analyzed. (B–D) Keratinocytes without transfection were sequentially incubated with U46619 and TNF-α plus antimycotics as described above. Chemokine secretion was analyzed at 48 h. *$P < 0.05$ vs. controls without TNF-α; †$P < 0.05$ vs. TNF-α plus vehicle. Data are presented as means ± S.E.M. ($n = 4$).

activity. We next examined whether antimycotics reduced the stability of CCL27, CCL2 or CCL5 mRNA. TNF-α increased the mRNA half-lives of these chemokines (Table 1). Ketoconazole, fluconazole, and terbinafine hydrochloride did not alter the mRNA half-lives of these chemokines either in the presence or absence of TNF-α. Thus, these antimycotics did not reduce the stability of CCL27, CCL2 or CCL5 mRNA, indicating the suppression of the chemokine production at transcriptional levels.

3.2. *Involvement of PGE_2 in the suppression of CCL27, CCL2 and CCL5 production by antimycotics*

We previously found that PGE_2 suppressed TNF-α-induced CCL27, CCL2 and CCL5 production via the suppression of NF-κB activity [8]. Prostaglandin E_2 can be released from keratinocytes [24]. We thus examined the involvement of keratinocyte-derived PGE_2 in the suppression of chemokine production by antimycotics. Anti-PGE_2 antiserum increased TNF-α-induced NF-κB activity (Fig. 3A), CCL27, CCL2 or CCL5 secretion (Fig. 3B–D) and mRNA expression (Fig. 4) while these were not altered by control NIS. These results indicate that endogenous PGE_2 from keratinocytes may suppress TNF-α-induced NF-κB activity and CCL27, CCL2, and CCL5 production. In the presence of anti-PGE_2, ketoconazole and terbinafine hydrochloride did not suppress TNF-α-induced NF-κB activity (Fig. 3A) and CCL27, CCL2 or CCL5 secretion (Fig. 3B–D) and mRNA expression (Fig. 4). In contrast, in the presence of control NIS, TNF-α-induced CCL27, CCL2 or CCL5 production and NF-κB activity was suppressed by ketoconazole and terbinafine hydrochloride, as well as those in the absence of antiserum (Figs. 3 and 4). These results indicate that endogenous PGE_2 from keratinocytes may be involved in the suppression of TNF-α-induced NF-κB activity and CCL27, CCL2 or CCL5 production by ketoconazole and terbinafine hydrochloride.

3.3. Antimycotics enhance PGE_2 synthesis and inhibit that of TXA_2

We then examined whether antimycotics altered PGE_2 release from keratinocytes. Tumor necrosis factor-α increased PGE_2 release from keratinocytes; means ± S.E.M. ($n = 4$) 3.41 ± 0.32 or 6.08 ± 0.69 ng/10^6 cells at 48 h in control or TNF-α (10 ng/ml)-treated keratinocytes, respectively ($P < 0.05$ by paired t-test). Ketoconazole and terbinafine hydrochloride dose-dependently increased the TNF-α-induced PGE_2 release while fluconazole was ineffective (Fig. 5A). The stimulatory effect of ketoconazole was greater than that of terbinafine hydrochloride; EC_{50} was 3.0 or 7.5 μM for ketoconazole or terbinafine hydrochloride, respectively. Inhibitors of TXAS are reported to increase PGE_2 synthesis by redirecting the conversion of common substrate PGH_2 toward PGE_2 from TXA_2 [27]. We then analyzed whether ketoconazole or terbinafine hydrochloride reduced TXA_2 synthesis concomitantly with increasing that of PGE_2, by examining the release of TXB_2, a stable metabolite of TXA_2. Though the amount of TXB_2 released from keratinocytes was much lower than that of PGE_2, TNF-α significantly increased the TXB_2 release; means ± S.E.M. ($n = 4$) 0.20 ± 0.03 or 0.32 ± 0.03 ng/10^6 cells at 48 h in control or TNF-α (10 ng/ml)-treated keratinocytes, respectively ($P < 0.05$ by paired t-test). Ketoconazole and terbinafine hydrochloride significantly reduced the TNF-α-induced TXB_2 release (Fig. 5B). The inhibitory effect of ketoconazole (IC_{50} 1.2 μM) was greater than that of terbinafine hydrochloride (IC_{50} 6.4 μM), which positively correlated with the stimulatory effects on PGE_2 release (Fig. 5A). As well as ketoconazole or terbinafine hydrochloride, specific TXAS inhibitor carboxyheptyl imidazole reduced TNF-α-induced TXB_2 release (Fig. 5B) and coincidentally increased that of PGE_2 (Fig. 5A). Moreover carboxyheptyl imidazole suppressed TNF-α-induced NF-κB activity (Fig. 6A) and CCL27, CCL2 or CCL5 secretion (Fig. 6B–D) and mRNA expression (Fig. 7) as did ketoconazole or terbinafine hydrochloride. These results indicate that ketoconazole or terbinafine hydrochloride may suppress TNF-α-induced NF-κB activity and CCL27, CCL2 or CCL5 production by a mechanism similar to that of the TXAS inhibitor.

3.4. Keratinocyte-derived PGE_2 may mediate suppressive effects of antimycotics via EP2 or EP3 receptors

To examine whether a decrease in TXA_2 per se was responsible for the suppression of TNF-α-induced NF-κB activity and CCL27, CCL2 or CCL5 production by antimycotics, we examined whether the addition of U46619, a TXA_2 receptor agonist [28], counteracted the suppressive effects. The addition of U46619 did not counteract the suppression of TNF-α-induced NF-κB activity (Fig. 6A) and CCL27, CCL2 or CCL5 secretion (Fig. 6B–D) and mRNA expression (Fig. 7) by ketoconazole, terbinafine hydrochloride or carboxyheptyl imidazole. These results indicate that endogenous TXA_2 may not contribute to TNF-α-induced NF-κB activity and CCL27, CCL2 or CCL5 production and that the decrease in endogenous TXA_2 by antimycotics may not directly mediate the suppression of TNF-α-induced NF-κB activity and CCL27, CCL2 or CCL5 production.

We recently found that exogenous PGE_2 suppressed NF-κB activity via cell surface EP2 and EP3 receptors on human keratinocytes [8]. We thus examined if PGE_2 released from keratinocytes may suppress NF-κB activity and NF-κB-dependent CCL27, CCL2 or CCL5 production via these receptors, by using antisense oligonucleotides against individual EP receptors. Antisense oligonucleotide against EP1, EP2, EP3, or EP4 selectively suppressed the expression of respective receptor protein (Fig. 8A). Antisense EP2 or EP3 partially blocked suppressive effects of ketoconazole or terbinafine hydrochloride on NF-κB activities (Fig. 8B) or CCL27, CCL2, or CCL5 secretion (Fig. 8C–E), and addition of both oligonucleotides completely abrogated the suppressive effects. On the other hand, antisense EP1 or EP4 were ineffective. These results suggest that EP2 and EP3 receptors on keratinocytes may mediate the suppressive effects of keratinocyte-derived PGE_2 whose release is induced by antimycotics.

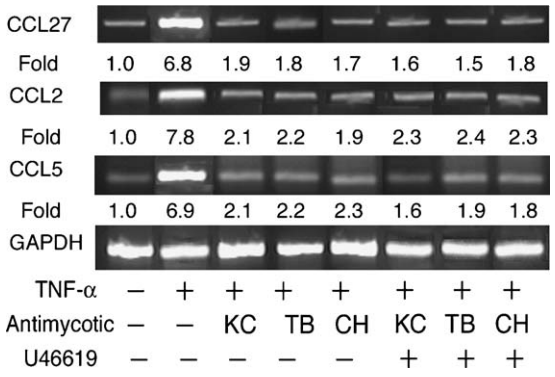

Fig. 7 – Effects of U46619 on antimycotic-mediated inhibition of CCL27, CCL2 or CCL5 mRNA expression induced by TNF-α. Keratinocytes were preincubated with 10 μM U46619 for 30 min, then incubated with 10 ng/ml TNF-α in the presence of vehicle or 20 μM ketoconazole (KC), terbinafine hydrochloride (TB) or carboxyheptyl imidazole (CH). mRNA levels were analyzed at 8 h. Results represent four separate experiments. Chemokine mRNA levels were normalized to those of GAPDH and are shown as a fold induction.

3.5. Antimycotics do not alter protein levels of enzymes involved in the syntheses of PGE_2 or TXA_2

We then examined whether ketoconazole, terbinafine hydrochloride or carboxyheptyl imidazole altered the expression of enzymes involved in the syntheses of PGE_2 or TXA_2. Tumor necrosis factor-α increased the protein levels of COX-2, converting arachidonic acid into PGH_2, and of mPGES-1, converting PGH_2 into PGE_2 (Fig. 9A). Ketoconazole, terbinafine hydrochloride or carboxyheptyl imidazole did not further enhance the TNF-α-induced expression of COX-2 or mPGES-1. Protein levels of COX-1, cPGES, or TXAS, converting PGH_2 into TXA_2, were not increased by TNF-α, and were not altered by ketoconazole, terbinafine hydrochloride or carboxyheptyl imidazole. Thus these agents did not appear to alter the protein levels of enzymes involved in the syntheses of PGE_2 or TXA_2. It is also indicated that the inhibition of NF-κB activity or chemokine production by these agents is not caused by the generalized depression of protein synthesis.

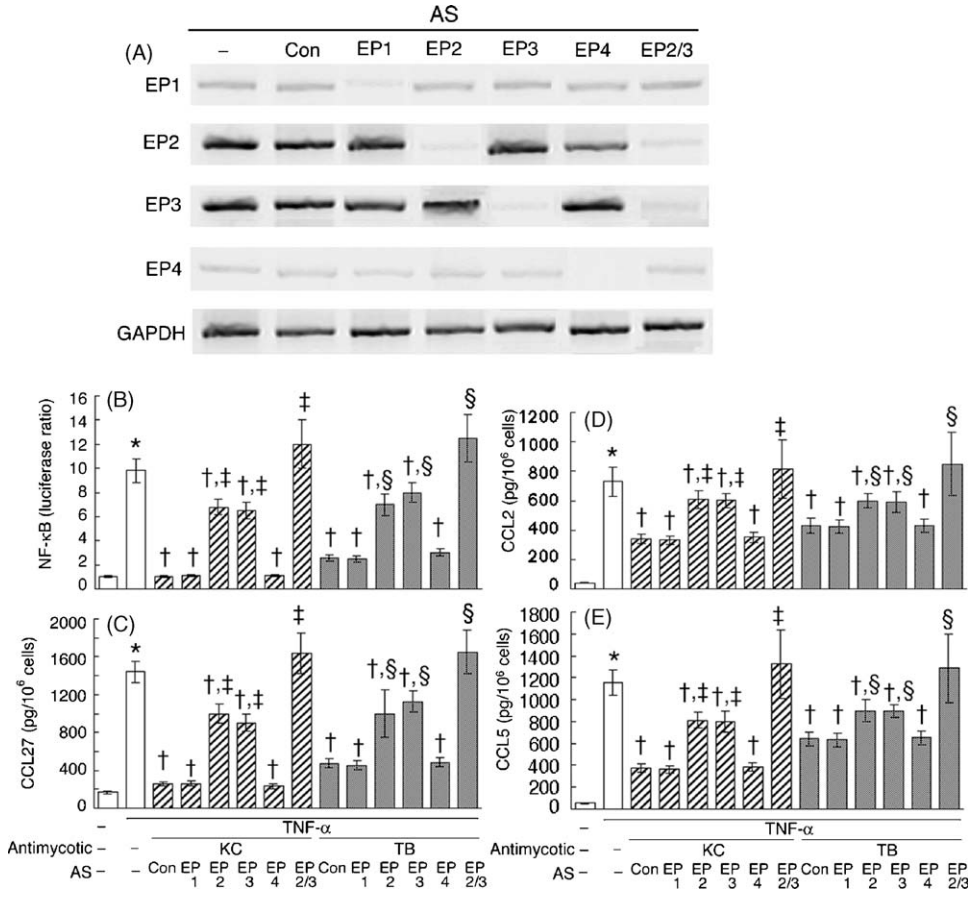

Fig. 8 – Effects of antisense EP2 or EP3 on antimycotic-mediated inhibition of NF-κB activity (B), CCL27 (C), CCL2 (D) or CCL5 (E) secretion induced by TNF-α. (A) Keratinocytes were transfected with antisense oligonucleotides (AS) against EP1, EP2, EP3, EP4, control scrambled oligonucleotide (Con) (each 0.2 μM) or antisense EP2 plus antisense EP3 (EP2/3). At 24 h, the whole cell-lysates were analyzed for EP1, EP2, EP3, EP4, or GAPDH expression by Western blotting. The results are representative of four separate experiments. (B) Keratinocytes were transfected with pNF-κB-luc and pRL-tk with above-mentioned antisense oligonucleotides, starved in KBM, then incubated with 10 ng/ml TNF-α in the presence of vehicle or 20 μM ketoconazole (KC), or terbinafine hydrochloride (TB). At 18 h, NF-κB activity was analyzed. (C–E) Keratinocytes transfected with above-mentioned antisense oligonucleotides were incubated with TNF-α plus antimycotics as described above. Chemokine secretion was analyzed at 48 h. $^{*}P < 0.05$ vs. controls without TNF-α; $^{\dagger}P < 0.05$ vs. TNF-α plus vehicle; $^{\ddagger}P < 0.05$ vs. TNF-α plus KC plus control AS; $^{\S}P < 0.05$ vs. TNF-α plus TB plus control AS. Data are presented as means ± S.E.M. ($n = 4$).

It is reported that COX-2 promoter contains several NF-κB elements and its transcription is dependent on NF-κB activity in certain cell types, such as murine embryonic fibroblasts [29]. Thus it is rather discrepant that kerotonazole or terbinafine hydrochloride did not suppress TNF-α-induced COX-2 expression in human keratinocytes though these agents suppressed NF-κB activities in these cells. We examined if specific NF-κB inhibitors, Bay11-7082 or parthenolide may suppress TNF-α-induced COX-2 expression in parallel to the suppression of NF-κB activities in human keratinocytes. These agents did not suppress TNF-α-induced COX-2 protein expression at concentrations effectively suppressing NF-κB activities in human keratinocytes (Fig. 9B). These results indicate that TNF-α-induced COX-2 expression in human keratinocytes may be independent from NF-κB activity, indicating cell-type specific regulation.

4. Discussion

The antimycotics ketoconazole and terbinafine hydrochloride suppress TNF-α-induced NF-κB activity and CCL27, CCL2 or CCL5 production in keratinocytes. These agents increase PGE_2 release, and the released PGE_2 may suppress TNF-α-induced NF-κB activity and NF-κB-dependent production of these chemokines via EP2 or EP3 receptors. Immediately after PGE_2 is released, PGE_2 concentrations on the surface of keratinocytes may be much higher than those of culture supernatants. Thus compared to exogenously added PGE_2, keratinocyte-derived PGE_2 may act more effectively on cell surface EP2 or EP3 receptors and thus more potently suppress TNF-α-induced CCL27, CCL2 or CCL5 production. In our previous study, PGE_2 induced cyclic AMP (cAMP) or Ca^{2+} signals through EP2 or EP3 receptors on keratinocytes, respectively, and both signals suppressed TNF-

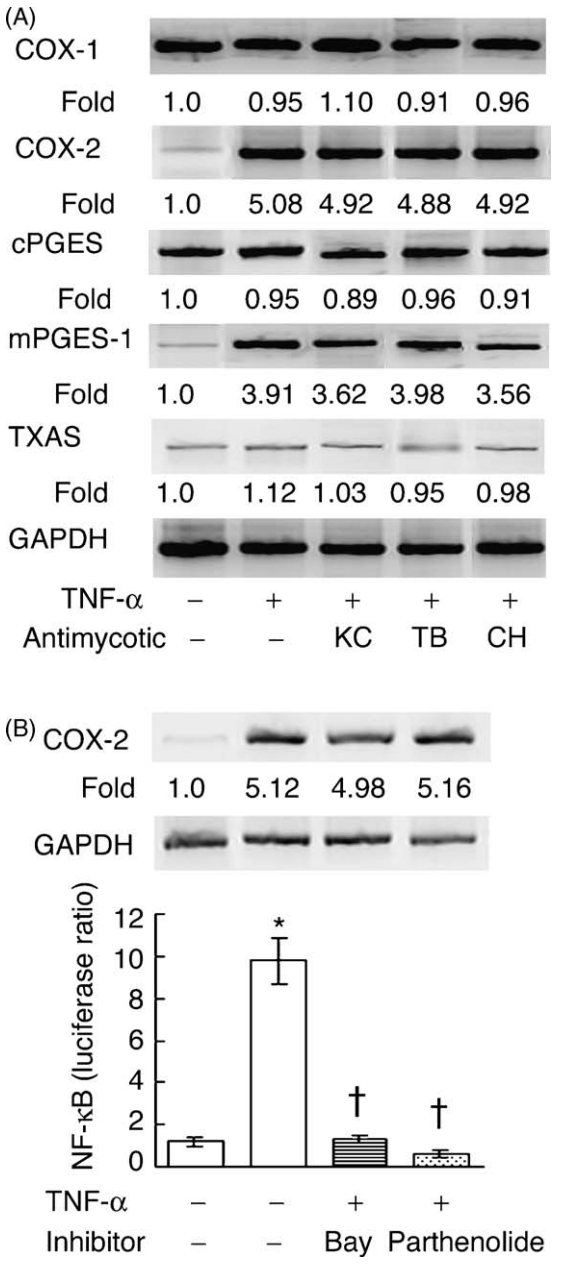

Fig. 9 – Effects of antimycotics on protein levels of COX-1, COX-2, cPGES, mPGES-1 and TXAS (A) and effects of specific NF-κB inhibitors on COX-2 levels (B). (A and B upper half) Keratinocytes were incubated with 10 ng/ml TNF-α in the presence of vehicle or 20 μM ketoconazole (KC), terbinafine hydrochloride (TB) or carboxyheptyl imidazole (CH) (A) or 10 μM Bay11-7082 (Bay) or 20 μM parthenolide (B). At 24 h, total proteins were extracted and Western blot was performed to detect COX-1, COX-2, cPGES, mPGES-1 and TXAS. The levels of these proteins were normalized to those of GAPDH and are shown as a fold induction. Results represent four separate experiments. (B lower half) Keratinocytes were transfected with pNF-κB-luc and pRL-tk, starved in KBM, then incubated with TNF-α in the presence of vehicle or Bay or parthenolide at above-mentioned concentrations. At 18 h, NF-κB activity was analyzed. *P < 0.05 vs. controls without TNF-α; †P < 0.05 vs. TNF-α plus vehicle. Data are presented as means ± S.E.M. (n = 4).

α-induced NF-κB activity [8]. Several mechanisms are suggested for the cAMP-mediated inhibition of NF-κB activity and which mechanism works may depend on cell types or sources of cAMP-inducing stimuli; firstly, cAMP may induce protein kinase A to phosphorylate and activate another transcription factor, cAMP response element-binding protein (CREB) and the activated CREB may compete with NF-κB p65 for limiting the amounts of transcriptional coactivator CREB-binding protein [30]. Secondly, cAMP-activated protein kinase A may suppress the phosphorylation of TATA-binding protein, a transcriptional coactivator for NF-κB [31]. Alternatively, cAMP may stabilize inhibitory NF-κB, which constitutively binds and inactivates NF-κB p50/p65 [32]. The Ca^{2+} signal either inhibits or stimulates TNF-α-induced NF-κB activity depending on target cell types; Ca^{2+}-dependent phosphatase calcineurin prevents phosphorylation and degradation of inhibitory NF-κB and thus inhibits the activation of NF-κB in chick embryo forebrain [33], which is indicative of the events occurring in keratinocytes while calcineurin manifests completely opposite effects in Jurkat T cells [34]. Further studies should define the mechanism(s) for PGE_2-mediated suppression of NF-κB activity in keratinocytes.

Ketoconazole and terbinafine hydrochloride may suppress TXA_2 synthesis and redirect the conversion of common substrate PGH_2 toward PGE_2 away from TXA_2. Thromboxane A_2 per se did not appear to mediate NF-κB activity and CCL27, CCL2 or CCL5 production induced by TNF-α (Figs. 6 and 7). In human platelets in vitro, ketoconazole is reported to decrease the release of TXB_2, a stable metabolite of TXA_2 and coincidentally increase that of PGE_2 [35]. In vivo treatment with ketoconazole also decreased TXB_2 synthesis while increasing that of PGE_2 in mice [24,36]. Thromboxane A_2 synthase, catalyzing conversion of PGH_2 into TXA_2, is the cytochrome P450-dependent enzyme, CYP5A1 [37,38]. Ketoconazole suppresses ergosterol synthesis in fungi by inhibiting cytochrome P450-dependent sterol C-14α demethylase (CYP51) [39] and acts as a competitive inhibitor of several other cytochrome P450-dependent enzymes [40]. This agent may inhibit TXAS possibly by binding to the active site of TXAS through the interaction of azole nitrogen with heme iron of the cytochrome P-450 component [41–43]. Though terbinafine hydrochloride suppresses cytochrome P450-independent squalene epoxidase in fungi [44], inhibition of TXAS by this agent has not yet been reported. However, this agent suppresses several cytochrome P450-dependent enzymes such as CYP2D6 [45], and may also suppress TXAS in a manner similar to that of ketoconazole. On the other hand, another azole derivative fluconazole did not reduce TXB_2 release from keratinocytes (Fig. 5). The results indicate that this agent may not suppress TXAS though it suppresses CYP51 and several other cytochrome P450-dependent enzymes like CYP2C9, CYP2C19, and CYP3A4 [46]. Since the inhibitor-binding site of TXAS contains a hydrophobic domain [41,42] and fluconazole has hydrophilic side chains [47], the binding affinity of fluconazole to TXAS may be much lower than that of ketoconazole. This hypothesis may be supported by our preliminary data that other relatively hydrophilic azole derivatives, neticonazole hydrochloride or croconazole hydrochloride, neither decreased TNF-α-induced TXB_2 release nor increased that of PGE_2 from human keratinocyte (supplementary data). In contrast, other hydrophobic azole derivatives,

clotrimazole or miconazole decreased TNF-α-induced TXB_2 release and simultaneously increased that of PGE_2 in human keratinocytes (supplementary data).

Tumor necrosis factor-α enhanced the expression of both COX-2 and mPGES-1 in keratinocytes (Fig. 9A). It is reported that COX-2 and mPGES-1 are functionally coupling and the expression of these enzymes is induced by pro-inflammatory cytokines like TNF-α, IL-1, or IL-17 [48,49]. Tumor necrosis factor-α is reported to induce the expression of transcription factor Egr-1 which mediates mPGES-1 transcription [50,51]. We also recently found that TNF-α increased COX-2 transcription and mRNA stability in keratinocytes by activating p38 mitogen-activated protein kinase [24]. Thus in TNF-α-treated keratinocytes, the conversion of arachidonic acid into PGH_2 by COX-2 and that of PGH_2 into PGE_2 by mPGES-1 may be simultaneously induced, which may lead to the increase in PGE_2 synthesis. Ketoconazole and terbinafine hydrochloride may further enhance TNF-α-induced PGE_2 synthesis by redirection of PGH_2 metabolism toward PGE_2 from TXA_2 without altering the protein levels of these enzymes. In addition to antimycotics, specific NF-κB inhibitors, Bay11-7082 or parthenolide did not suppress TNF-α-induced COX-2 expression in human keratinocytes (Fig. 9B). These results indicate that TNF-α-induced COX-2 expression in human keratinocytes may be mediated by transcription factors other than NF-κB, such as CCAAT/enhancer-binding protein β or CREB and/or activating transcription factor 1, binding sites of which reside on COX-2 promoter [24].

Ketoconazole or terbinafine hydrochloride suppressed in vitro CCL27, CCL2 or CCL5 production by keratinocytes at 1–20 μM, which is close to the concentrations obtained by oral administration of these drugs; peak serum or plasma concentrations in humans were 7.5 μM by oral 200 mg ketoconazole [52] or 2 μM by oral 125 mg terbinafine hydrochloride [53], respectively. It is thus indicated that these antimycotics may suppress TNF-α-induced CCL27, CCL2 or CCL5 production by keratinocytes in vivo at physiological concentrations. Thus our results in vitro suggest possible mechanisms for the therapeutic efficacy of antimycotics on AD or PV; treatment with ketoconazole or terbinafine hydrochloride may suppress overexpression of CCL27, CCL2 or CCL5 by keratinocytes in AD or PV lesions, and thus attenuate the infiltration of T cells, macrophages, or eosinophils induced by these chemokines. Further study is required to examine whether in vivo treatment with these antimycotics reduces the production of CCL27, CCL2 or CCL5 in keratinocytes of lesions with AD or PV.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2006.05.001.

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