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Suppressive effects of antimycotics on thymic stromal lymphopoietin production in human keratinocytes☆

Carren S. Hau *, Naoko Kanda, Shinichi Watanabe

Department of Dermatology, Teikyo University School of Medicine, Tokyo, Japan

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ABSTRACT

Background: Thymic stromal lymphopoietin (TSLP) is produced by epidermal keratinocytes, and it induces Th2-mediated inflammation. TSLP expression is enhanced in lesions with atopic dermatitis, and is a therapeutic target. Antimycotic agents improve the symptoms of atopic dermatitis.
Objective: The objective of this study was to examine whether antimycotics suppress TSLP expression in human keratinocytes.
Methods: Normal human keratinocytes were incubated with polyinosinic–polycytidylic acid (poly I:C) plus IL-4 in the presence of antimycotics. TSLP expression was analyzed by ELISA and real time PCR. Luciferase assays were performed to analyze NF-κB activity. IκBα degradation was analyzed by Western blot analysis.
Results: Poly I:C plus IL-4 increased the secretion and mRNA levels of TSLP, which was suppressed by an NF-κB inhibitor, and also enhanced NF-κB transcriptional activities and induced the degradation of IκBα in keratinocytes. The antimycotics itraconazole, ketoconazole, luliconazole, terbinafine, butenafine, and amorolfine suppressed the secretion and mRNA expression of TSLP, NF-κB activity, and IκBα degradation induced by poly I:C plus IL-4. These suppressive effects were similarly manifested by 15-deoxy-Δ-^{12,14}-PGJ_2 (15d-PGJ_2), a prostaglandin D_2 metabolite. Antimycotics increased the release of 15d-PGJ_2 from keratinocytes and decreased the release of thromboxane B_2, a thromboxane A_2 metabolite. Antimycotic-induced suppression of TSLP production and NF-κB activity was counteracted by an inhibitor of lipocalin type-prostaglandin D synthase.
Conclusions: Antimycotics itraconazole, ketoconazole, luliconazole, terbinafine, butenafine, and amorolfine may suppress poly I:C plus IL-4-induced production of TSLP by inhibiting NF-κB via increasing 15d-PGJ_2 production in keratinocytes. These antimycotics may block the overexpression of TSLP in lesions with atopic dermatitis.

1. Introduction

A cytokine thymic stromal lymphopoietin (TSLP) activates dendritic cells to induce proallergic Th2 cell responses [1]. TSLP activates dendritic cells to produce Th2-attracting chemokines CCL17/CCL22 and to express OX40 ligand, a key Th2 costimulatory factor [2]. TSLP-activated dendritic cells prime naïve CD4+ T cells to differentiate into inflammatory Th2 cells producing IL-4, IL-5, IL-13, and TNF-α through OX40 ligand [2,3]. In skin lesions with atopic dermatitis (AD), the expression of TSLP in epidermal keratinocytes is enhanced [2], which may trigger Th2-mediated inflammation in the skin. Compared with healthy subjects, TSLP levels in the stratum corneum, reflecting their expression levels in keratinocytes, are increased in AD patients, and correlate with severity scoring of AD and dry skin score. The stratum corneum TSLP levels as well as severity scoring of AD are reduced by topical

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Abbreviations: AD, atopic dermatitis; ANOVA, analysis of variance; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; DMSO, dimethylsulfoxide; DP, D prostanoid receptor; 15d-PGJ_2, 15-deoxy-Δ-^{12,14}-prostaglandin J_2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPGDS, hematopoietic prostaglandin D synthase; IκB, inhibitory κB; IKK, IκB kinase; KBM, keratinocyte basal medium; LPGDS, lipocalin-type prostaglandin D synthase; NEMO, NF-κB essential modifier; PG, prostaglandin; poly I:C, polyinosinic–polycytidylic acid; PPARγ, peroxisome proliferator-activated receptor γ; RIP1, receptor interacting protein 1; TAK1, TGF-β activated kinase 1; TNFR, TNF receptor; TRADD, TNF receptor-associated death domain; TRAF, TNF receptor-associated factor; TRIF, Toll/IL-1 receptor domain-containing adaptor inducing IFN-β; TSLP, thymic stromal lymphopoietin; TXA_2, thromboxane A_2; TXAS, thromboxane A_2 synthase.
* Corresponding author at: Department of Dermatology, Teikyo University School of Medicine, 11-1, Kaga-2, Itabashi-Ku, Tokyo 173-8605, Japan.
Tel.: +81 3 3964 2473; fax: +81 3 5375 5314.
E-mail address: carren.hau@gmail.com (C.S. Hau).

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application of moisturizer [4]. It is known that TSLP production is induced by the stimulation of Toll-like receptors (TLRs), such as TLR3 or TLR2, or several cytokines like TNF-α, IL-1, IFN-α, TGF-α, or IL-4 [5–7], and that the activation of NF-κB is required for the TSLP production [5].

Antimycotic agents, azole or non-azole, are reported to be therapeutically effective in treating patients with AD [8,9]. One possible mechanism is that antimycotics suppress the colonization of fungi like *Malassezia*, acting as allergens for AD patients [10], by exerting fungicidal or fungistatic effects [9]. The effectiveness of antimycotics is also reported for patients with seborrheic dermatitis whose pathogenesis may involve *Malassezia* [11]. Another proposed mechanism is that antimycotics may directly act on keratinocytes or lymphocytes of AD patients and manifest anti-inflammatory effects [12,13]. We have found that an antimycotic ketoconazole reduces IL-4 and IL-13 production in human T cells [13], and that ketoconazole and another antimycotic terbinafine suppress CCL27 production in human keratinocytes [12]. However, the effects of antimycotics on TSLP production in keratinocytes are unknown.

We previously found that the antimycotics itraconazole and terbinafine increase the secretion of prostaglandin D_2 (PGD_2) from human keratinocytes [14]. PGD_2 is intracellularly converted from PGH_2 by lipocalin-type PGD synthase (LPGDS) or hematopoietic PGD synthase (HPGDS) [15,16]. PGH_2 is also converted to thromboxane A_2 (TXA_2) by thromboxane A_2 synthase (TXAS) [14]. Itraconazole and terbinafine appear to suppress the activity of TXAS, and thus, redirect the conversion of PGH_2 from TXA_2 to PGD_2 [14]. PGD_2 is intracellularly dehydrated into 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) non-enzymatically [15,16]. 15d-PGJ_2 is known to suppress the activity of NF-κB and NF-κB-dependent gene expression, dependent or independent of intracellular receptor peroxisome proliferator-activated receptor γ (PPARγ) [15,17]. 15d-PGJ_2 binds PPARγ, and the liganded PPARγ (i) induces the expression of NF-κB inhibitory subunit, inhibitory κB (IκB) or (ii) competes with NF-κB for the transcriptional coactivators like p300/CBP [15]. Independent of PPARγ, 15d-PGJ_2 covalently binds to IκB kinase (IKK) and inhibits its function to phosphorylate and inactivate IκB [17].

In this study, we aimed to examine if antimycotics suppress TSLP production in human keratinocytes and to elucidate the mechanism for the effects related to the induction of 15d-PGJ_2. The antimycotics used were itraconazole, ketoconazole, fluconazole, voriconazole and luliconazole (azole class), terbinafine (allylamine class), butenafine (benzylamine class), and amorolfine (morpholine class) (Supplementary Fig. S1) [18]. The azole class is potent against both *Trichophyton* spp. and *Candida albicans* and inhibits C14α-lanosterol demethylase in the biosynthetic pathway of ergosterol, an essential component of fungal cell membranes. In contrast, the allylamine (terbinafine) and benzylamine (butenafine) classes inhibit squalene epoxidase, an early step in the ergosterol biosynthesis, show stronger anti-fungal activity against *Trichophyton* spp., but less effective against *C. albicans*. On the other hand, the morpholine class (amorolfine) inhibits both C14-reductase and C7–C8 isomerase, later stages in the ergosterol biosynthesis, shows comparable potency against *Trichophyton* spp., and *C. albicans*. The azole class also shows potent antifungal activity against *Malassezia* spp. [18]. Among azole antimycotics, itraconazole and ketoconazole, fluconazole and voriconazole are structurally alike, respectively, while luliconazole is distinct; among non-azole antimycotics, terbinafine and butenafine are structurally similar while amorolfine is dissimilar (Supplementary Fig. S1). The differential effects of these drugs on TSLP production were compared.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jdermsci.2013.04.023.

2. Materials and methods

2.1. Reagents

Helenalin was purchased from Calbiochem (La Jolla, CA, USA). Recombinant human IL-4, TNF-α, IFN-α, and TGF-α were purchased from R&D Systems (Minneapolis, MN, USA). 15d-PGJ_2, PGE_2, $PGF_{2\alpha}$, rosiglitazone, BW245C, 13,14-dihydro-15-keto-PGD_2, AT-56, and HQL-79 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Pam_3Cys-Ser-$(Lys)_4$·3HCl was purchased from IMGENEX (San Diego, CA, USA). β-Glucan and zymosan from Baker's yeast (*Saccharomyces cerevisiae*) and polyinosinic–polycytidylic acid (poly I:C) potassium salt were purchased from Sigma–Aldrich (St. Louis, MO, USA). Mite extract from *Dermatophagoides pteronyssinus* was purchased from Cosmo Bio (Tokyo, Japan). Lysophosphatidic acid sodium salt was from Enzo Life Sciences (Farmingdale, NY, USA). Itraconazole, ketoconazole, fluconazole, voriconazole, and terbinafine hydrochloride were from Wako Pure Chemical (Osaka, Japan). Luliconazole, butenafine hydrochloride, and amorolfine hydrochloride were donated by POLA PHARMA (Tokyo, Japan), Kaken Pharmaceutical Co. (Tokyo, Japan), and Kyorin Pharmaceutical Co. (Tokyo, Japan), respectively. The antimycotics were dissolved in dimethylsulfoxide (DMSO) at 100 mM to create solutions and subsequently diluted in the experimental media to yield the final concentrations. The DMSO concentration as a vehicle control was 0.1% (v/v).

2.2. Keratinocyte culture

Human neonatal foreskin keratinocytes were purchased from Clonetics (Walkersville, MD, USA). The keratinocytes were cultured in serum-free keratinocyte growth medium (Clonetics) containing keratinocyte 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. The cells in the third passage were used. Each experiment was performed four times using the same lot of keratinocytes from a single donor.

2.3. ELISA

Keratinocytes (5×10^4 cells/well) were seeded in triplicate in 24-well plates in 0.4 ml keratinocyte growth medium, and allowed to adhere overnight. The cells were washed and incubated with supplement-free KBM for 24 h. The cells were washed and preincubated with the vehicle (DMSO) or the indicated concentrations of antimycotics or prostanoids, or 1 μM helenalin for 30 min, and subsequently incubated with IL-4 (10 ng/ml) and/or poly I:C (10 μg/ml), TNF-α (1, 10, or 100 ng/ml), IL-1β (1, 10, or 100 ng/ml), TGF-α (1, 10, or 100 ng/ml), IFN-α (1, 10, or 100 ng/ml), Pam_3Cys-Ser-$(Lys)_4$, β-glucan, zymosan, or lysophosphatidic acid (each 1, 10 or 100 μg/ml) in KBM for 48 h. The culture supernatants were analyzed by performing ELISA for TSLP (R&D Systems), 15d-PGJ_2 (Abnova, Taipei, Taiwan), PGD_2, TXB_2, PGE_2, or $PGF_{2\alpha}$ (Cayman Chemical), according to the manufacturers' instructions. In some experiments, the cells were pretreated with HQL-79 or AT-56 (each 10 μM) for 30 min before the addition of antimycotics.

2.4. Real-time PCR

The keratinocytes were incubated for 24 h as described above, and the total cellular RNA was extracted using RNeasy Mini kits (SABiosciences, Frederick, MD, USA). cDNA was synthesized using Superscript III First Strand synthesis kits (Invitrogen, Carlsbad, CA, USA). TSLP mRNA levels were quantified by performing TaqMan gene expression assay, using a probe set specific for the long form of the *TSLP* transcript (Hs01572933 m1; Applied Biosystems,