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Protective role of 6-formylindolo[3,2-b]carbazole (FICZ), an endogenous ligand for arylhydrocarbon receptor, in chronic mite-induced dermatitis

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ABSTRACT

Background: Chronic eczema such as atopic dermatitis imposes significant socio-econo-psychologic burdens on the affected individuals. In addition to conventional topical treatments, phototherapy is recommended for patients with extensive lesions. Although immunosuppression is believed to explain its primary effectiveness, the underlying mechanisms of phototherapy remain unsolved. Ultraviolet irradiation generates various tryptophan photoproducts including 6-formylindolo[3,2-b]-carbazole (FICZ). FICZ is known to be a potent endogenous agonist for aryl hydrocarbon receptor (AHR); however, the biological role of FICZ in chronic eczema is unknown.
Objective: To investigate the effect of FICZ on chronic eczema such as atopic dermatitis.
Methods: We stimulated HaCaT cells and normal human epidermal keratinocytes (NHEKs) with or without FICZ and then performed quantitative reverse transcriptase polymerase chain reaction, immunofluorescence, and siRNA treatment. We used the atopic dermatitis-like NC/Nga murine model and treated the mice for 2 weeks with either Vaseline® as a control, FICZ ointment, or betamethasone 17-valerate ointment. The dermatitis score, transepidermal water loss, histology, and expression of skin barrier genes and proteins were evaluated.
Results: FICZ significantly upregulated the gene expression of filaggrin in both HaCaT cells and NHEKs in an AHR-dependent manner, but did not affect the gene expression of other barrier-related proteins. In addition, FICZ improved the atopic dermatitis-like skin inflammation, clinical scores, and transepidermal water loss in NC/Nga mice compared with those of control mice. On histology, FICZ significantly reduced the epidermal and dermal thickness as well as the number of mast cells. Topical FICZ also significantly reduced the gene expression of *Il22*.
Conclusion: These findings highlight the beneficial role of FICZ-AHR and provide a new strategic basis for developing new drugs for chronic eczema.

1. Introduction

The skin is a sensory organ that recognizes physiologic and pathologic chemical stimuli to ensure self-defense and homeostasis. Aryl hydrocarbon receptor (AHR) is a chemical receptor that can bind and be activated by structurally diverse external and internal chemicals including dioxins as well as various phytochemicals and photo-induced chemicals with a wide range of affinities [1–4]. Upon ligand binding, the ligand-AHR complex translocates from the cytoplasm to the nucleus, binds to its specific DNA recognition site, and then upregulates the transcription of various responsive genes such as *CYP1A1*, which is a member of a multigene family of xenobiotic-metabolizing enzymes [1,2].

AHR is an evolutionarily-conserved and widely-expressed multifunctional transcription factor that profoundly modulates the functions of the epidermis and immune system [2,4,5]. A recent study by Di Meglio et al. demonstrated a significant enhancement of imiquimod-induced psoriasis-like skin inflammation in *Ahr*-null mice [6]. Moreover, their investigation stressed the importance of nonhematopoietic cell types such as epidermal cells, rather than hematopoietic cell types, in recapitulating the exaggerated skin

Abbreviations: FICZ, 6-formylindolo[3,2-b]-carbazole; AHR, aryl hydrocarbon receptor; NHEKs, normal human epidermal keratinocytes; TEWL, transepidermal water loss; FLG, filaggrin; UV, ultraviolet.

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inflammation under the *Ahr*-deleted condition [6]. In addition, the activation of AHR by the intraperitoneal injection of 6-formylindolo[3,2-b]-carbazole (FICZ, a ligand for AHR) potently inhibited the imiquimod-induced skin inflammation [6]. FICZ was originally identified as a tryptophan metabolite that forms after exposure to ultraviolet (UV) or visible light in humans [7,8]. Because of its high-affinity binding to AHR and its specific induction of *CYP1A1*, FICZ is now considered as one of the essential endogenous and physiologic ligands for AHR alongside other dietarily-supplied indoles and flavonoids [7–9]. Although sun-exposed skin is one of the major sources of FICZ, the role of FICZ in skin inflammation is not fully understood.

In the present study, we found that 1) FICZ augmented the expression of filaggrin (FLG) in cultured keratinocytes, and 2) the topical application of FICZ reduced the mite antigen-induced dermatitis and transepidermal water loss (TEWL) in NC/Nga mice.

2. Materials and methods

2.1. Reagents and antibodies

FICZ was purchased from Enzo Life Sciences (Farmingdale, NY) and was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) to the concentration of 5 mM. Anti-AHR rabbit polyclonal IgG antibody (H-211) and normal mouse IgG were obtained from Santa Cruz Biotechnology (Dallas, TX). Anti-FLG (ab3137) antibody was purchased from Abcam (Cambridge, UK).

2.2. Cell culture

Human immortalized HaCaT cells were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum and antibiotics at 37 °C in 5% CO_2. HaCaT cells (2×10^5 cells/well) were seeded in 6-well culture plates, allowed to attach for 24 h, and subsequently treated with or without FICZ when they reached 70–90% confluence.

Normal human epidermal keratinocytes (NHEKs) obtained from Lonza (Walkersville, MD) were maintained at 37 °C in 5% CO_2. The NHEKs were cultured in serum-free keratinocyte growth medium (Lonza) containing bovine pituitary extract, recombinant epidermal growth factor, insulin, hydrocortisone, transferrin, GA-1000, and epinephrine. The culture medium was replaced every 2 days. Third-to-fifth-passage NHEKs were used in all experiments. NHEKs (1×10^5 cells/well) were seeded in 24-well culture plates, allowed to attach for 24 h, and then treated with or without FICZ when they reached 80–90% confluence.

2.3. Immunofluorescence

HaCaT cells or NHEKs (1×10^4 cells/well) were plated on an 8-well μ-slide (Ibidi, Munich, Germany) for 48 h, and treated with or without FICZ for 3 or 1 h, respectively. Then, the cells were washed with phosphate-buffered saline (PBS), fixed with acetone for 10 min, and blocked with 5% (w/v) bovine serum albumin in PBS for 30 min. The cells were then incubated with primary rabbit anti-AHR antibody (1:100) overnight at 4 °C. Specific binding was detected using a horseradish peroxidase-conjugated goat anti-rabbit antibody followed by tyramide labeling with green-fluorescent Alexa Fluor® 488 (Molecular Probes, Eugene, OR) for 1 h at room temperature in accordance with the manufacturer's instructions. The treated cells were covered with UltraCruz™ mounting medium containing 4′,6-diamidino-2-phenylindole (Santa Cruz Biotechnology). The fluorescence images were analyzed using an EVOS® FL cell imaging system (Life Technologies, Carlsbad, CA).

NHEKs ($2.5–4.0 \times 10^4$ cells/well) were cultured on an 8-well slide (Lab-Tek, Rochester, NY) with or without FICZ for 24 h. The cells were incubated with primary mouse anti-FLG antibody (1:100) in WesternBreeze® blocker diluent (Invitrogen, Carlsbad, CA) overnight at 4 °C. Slides were washed in PBS before incubation with an anti-mouse secondary antibody (Alexa Fluor® 546, Life Technologies) for 1 h at room temperature. All samples were analyzed using a D-Eclipse confocal laser scanning microscope (Nikon, Tokyo, Japan).

2.4. Real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

NHEKs or HaCaT cells were treated with or without FICZ for 3 or 5 h, respectively. Total RNA was extracted using the RNeasy® Mini kit (Qiagen, Hilden, Germany). The dorsal skin of NC/Nga mice affected by mite-induced dermatitis was treated with each ointment for 2 weeks, and then total RNA was extracted using the RNeasy® fibrous tissue mini kit (Qiagen). qRT-PCR was performed with PrimeScript™ RT-reagent and SYBR® Premix Ex Taq™II (Takara Bio, Ōtsu, Japan) according to the manufacturer's instructions. qRT-PCR amplifications were performed with the following cycling conditions: 95 °C for 30 s, then 40 cycles at 95 °C for 5 s (denaturation) and 60 °C for 20 s (annealing/extension). The mRNA level of each target gene was normalized to the cycle threshold of β-actin (*ACTB*; internal control). The primer sequences are listed in Table 1.

2.5. Transfection with siRNA against AHR

Anti-*AHR* siRNA (s1200) and control siRNA (negative control #1) were purchased from Ambion (Austin, TX). NHEKs and HaCaT cells were transfected with siRNA using the Lipofectamine® RNAiMAX transfection reagent in accordance with the manufacturer's protocol. The efficiency of siRNA transfection for down-regulating *AHR* expression was $93.507 \pm 0.308\%$ as assessed by measuring *AHR* expression in NHEKs.

2.6. 3D-cultured NHEKs as human skin equivalent model

The human epidermal three-dimensionally (3D) model (Epi-Dem EPI-200: MatTek, Ashland, MA, USA) was incubated with or without FICZ (1 μM) for 72 h at 37 °C.

Table 1
Primers for real-time quantitative RT-PCR.

Gene (human)	Primer sequence (5′–3′)	
	Sense	Antisense
CYP1A1	TAGACACTGATCTGGCTGCAG	GGGAAGGCTCCATCAGCATC
FLG	TGAAGCCTATGACACCACTGA	TCCCCTACGCTTTCTTGTCCT
LCE1A	CCTGCAAGAGTGGCTGAGATG	GGCAGCAGATAGGTTTGTGG
LCE1B	TCTGGAGGCTGCTGCTAAAGTG	GGCCTCTGAACTCCAAGACAGAA
LCE3D	TTGATGCATGAGTTCCCAGATAC	TGACATCCTGGACATCAGACA
LCE3E	TCCAGATCCTGATGCTGAGACAA	AGCTCAGCCTGTGAAAGTCAGAA
KRT1	AGAGTGGACCAACTGAAGAGT	ATTCTCTGCATTTGTCCGCTT
KRT10	ATGTCTGTTCGATACAGCTCAAG	CTCCACCAAGGGAGCCTTTG
S100A7	AATTACCTCGCCGATGTCTTTGA	ATGGCTCTGCTTGTGGTAGTC
S100A13	CCTGAGGCTCCAGCTCACTCTA	GTCAGTGGTTCTGCTGCCATTA
S100A15	CTTCAATCCATCGCTACAGTCC	TGCCAATTGGACGGAATATTATCAG
CRNN	ATGCCTCAGTTACTGCAAAACA	TCACATCGGCAAACTCTTGCT
RPTN	ATGGGGACTGTGCCTTACTAT	TCTCGGTCTTGATCTAAGAGGTT
TCHH	CGGAGACCACATGACCCTAAG	AATCGACACGCCCATTACTGT
TCHHL1	AGACAGGTGACTAAACCAGAGAA	TCTTGAGTTGGTGAAGTTCCCA
HRNR	TCGAGACCATAACAAGAAAGTGG	GTGAGTGTCATCTCTCAGCTTTG
SPRR1B	CATTCTGTCTCCCCCAAAAA	ATGGGGGTATAAGGGAGTTG
SPRR2D	TCTGTGACAGCAAAAGATTC	ATTCAGGGAGTGAAAGATAAA
SPRR2E	ATTGGCTCACCTTGTTCCAC	TGGGAACTGACACTGCTGAG
SPRR2G	TCCACCATGCCAGGATAAAT	GCTGAAGGGAAGATGATGGA
IVL	TCCTCCAGTCAATACCCATCAG	CAGCAGTCATGTGCTTTTCCT
β-actin	ATTGCCGACAGGATGCAGA	GAGTACTTGCGCTCAGGAGGA
G6PD	CTACAGGTTCAGATGATGTC	CAGCTTCTCCTTCTCCATTG

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