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ORIGINAL ARTICLE

Antioxidant soybean tar Glyteer rescues T-helper-mediated downregulation of filaggrin expression via aryl hydrocarbon receptor

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ABSTRACT

Soybean tar Glyteer (Gly) has been widely used for the treatment of various inflammatory skin diseases in Japan since 1924 as an alternative to coal tar remedy. Recently, coal tar has been shown to induce barrier repair in atopic dermatitis via aryl hydrocarbon receptor (AhR). In this study, we demonstrated that Gly activated AhR by inducing its cytoplasmic to nuclear translocation in keratinocytes. The AhR ligation by Gly was biologically active, with significant and dose-dependent upregulation of CYP1A1 expression, which is a specific marker for AhR activation. Gly upregulated the expression of filaggrin in an AhR-dependent manner because its enhancing effect was completely abrogated in AhR-knockdown keratinocytes. T-helper (Th)2 cytokines inhibited the expression of filaggrin; however, Gly completely restored the Th2-mediated inhibition of filaggrin expression. Furthermore, Gly coordinately upregulated a series of epidermal differentiation complex genes, including involucrin, loricrin and hornerin. In addition, Gly exhibited potent antioxidant activity through the activation of nuclear factor-erythroid 2-related factor-2 (Nrf2) and downstream antioxidant enzymes such as NAD(P)H:quinone oxidoreductase 1 (Nqo1), which actually inhibited the generation of reactive oxygen species in keratinocytes treated with tumor necrosis factor-α or benzo[α]pyrene. In conclusion, antioxidant Gly rescues the downregulated expression of filaggrin (and plausibly other barrier proteins) in a Th2-skewed milieu via AhR activation, which may partly explain its empirical anti-inflammatory therapeutic effects.

Key words: aryl hydrocarbon receptor, filaggrin, Glyteer, nuclear factor-erythroid 2-related factor-2, reactive oxygen species, soybean tar.

INTRODUCTION

Because the skin is the outermost barrier of the body, it senses various external stimuli. Aryl hydrocarbon receptor (AhR), a basic helix-loop-helix/Per-ARNT-Sim (bHLH-PAS)-containing transcription factor, is known to work as a sensor for structurally diverse exogenous and endogenous ligands, such as halogenated and non-halogenated polycyclic aromatic hydrocarbons (2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD] and benzo[α]pyrene [BaP]),[1–4] various phytochemicals,[5,6] *Malassezia* metabolites[7] and tryptophan photoproducts[8–10] with a wide range of affinities. As keratinocytes possess AhR,[4,11,12] the physiological and pathological processes of epidermal homeostasis and differentiation are variably affected by the ligand-dependent activation of the AhR signal transduction pathway.[2]

Upon ligand binding, cytoplasmic AhR translocates into the nucleus and induces xenobiotic-metabolizing enzymes such as cytochrome P450 1A1 (CYP1A1).[13] Although all of the ligands for AhR induce the upregulation of CYP1A1, they are divided into two groups: those with oxidative or antioxidative capacity. For example, toxic dioxins and BaP induce cellular oxidation by generating robust reactive oxygen species (ROS),[4,11,14] while ketoconazole and quercetin, an AhR-binding phytochemical, act as antioxidants by attenuating ROS production by switching on antioxidant signaling pathways.[15,16] The mechanism behind this ligand-dependent *yin and yang* balance regarding oxidation remains unknown. However, the antioxidant activity induced by AhR activation has been proved to be mediated via downstream nuclear factor-erythroid 2-related factor-2 (Nrf2), which is a master transcription factor for protecting cells from ROS-induced oxidative damage through the

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induction of antioxidant enzymes such as NAD(P)H:quinone oxidoreductase 1 (Nqo1).[15–18]

Aryl hydrocarbon receptor also plays a key role in epidermal terminal differentiation by enhancing the expression of barrier proteins. Similarly, TCDD increases the quantity of cornified envelopes in monolayer cultures and organotypic cultures of keratinocytes.[19] It also enhances filaggrin, loricrin and involucrin expression.[20,21] Quercetin also enhances filaggrin gene expression.[22] Using organotypic skin models, van den Bogaard *et al.*[12] demonstrated that coal tar activates AhR, resulting in the induction of epidermal differentiation (i.e. upregulation of filaggrin, loricrin and hornerin expression), and thickens the cornified layer. Coal tar also restores the downregulated expression of barrier proteins in a T-helper (Th)2 cytokine milieu.[12] These AhR-mediated barrier-restoring effects may, at least in part, explain why topical coal tar remedies have been widely used to treat inflammatory skin diseases for two millennia.[23]

A delipidated soybean tar, Glyteer (Gly), licensed in 1924 in Japan, was previously used for the treatment of various inflammatory skin diseases[24,25] and is still covered under the national medical insurance system as an ointment in which it is mixed with dexamethasone. Although Gly has been shown to inhibit erythema formation induced by ultraviolet irradiation as well as contact hypersensitivity induced by picryl chloride,[26] its pharmacological mechanisms remain largely unclear. In this study, we demonstrated that Gly did indeed activate AhR-Nrf2-Nqo1 signaling and inhibited ROS production of keratinocytes treated with tumor necrosis factor (TNF)-α or BaP. Furthermore, Gly restored the Th2 cytokine-mediated downregulation of filaggrin.

METHODS

Reagents and antibodies

Glyteer was provided as an original stock solution by Fujinaga Pharm (Tokyo, Japan). Dimethylsulfoxide (DMSO) and BaP were purchased from Sigma-Aldrich (St Louis, MO, USA). Interleukin (IL)-4 was purchased from R&D Systems (Minneapolis, MN, USA). IL-13 and TNF-α were purchased from Peprotech (Rocky Hill, NJ, USA). Anti-AhR rabbit polyclonal immunoglobulin (Ig)G antibody (H-211), anti-Nrf2 polyclonal rabbit IgG antibody (H-300) and normal rabbit IgG were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-filaggrin (ab3137) antibody was purchased from Abcam (Cambridge, UK).

Cell culture

Normal human epidermal keratinocytes (NHEK) obtained from Clonetics-BioWhittaker (San Diego, CA, USA) were grown in culture dishes at 37°C in 5% CO_2. The NHEK were cultured in serum-free keratinocyte growth medium (Lonza, Walkersville, MD, USA) supplemented with bovine pituitary extract, recombinant epidermal growth factor, insulin, hydrocortisone, transferrin and epinephrine. Culture medium was replaced every 2 days. Near confluence (70–90%), cells were disaggregated with 0.25 mg/mL trypsin/0.01% ethylenediamine tetraacetic acid and subcultured. Second- to fourth-passage NHEK were used in all experiments.

Normal human epidermal keratinocytes (1×10^5) were seeded in 24-well culture plates, allowed to attach for 24 h, and then subsequently treated with or without Gly, DMSO, IL-4 or IL-13. Various concentrations of Gly (0–0.001%), TNF-α, BaP, DMSO, IL-4 (5 ng/mL) and IL-13 (5 ng/mL) were prepared in cell culture medium.

Immunofluorescence and confocal laser scanning microscopic analysis

Normal human epidermal keratinocytes (2×10^4) cultured on slides (Lab-Tek, Rochester, NY, USA) with or without Gly were washed in phosphate-buffered saline (PBS), fixed with acetone for 10 min and blocked using 10% bovine serum albumin in PBS for 30 min. Samples were incubated with primary rabbit anti-AhR or Nrf2 (1:50) in western breeze blocker diluent (Invitrogen, Carlsbad, CA, USA) overnight at 4°C. Slides were washed with PBS before incubation with antirabbit secondary antibody (Alexa Fluor 546 or 488; Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. After nuclear staining with 4′,6-diamidino-2-phenylindole, slides were mounted with UltraCruz mounting medium (Santa Cruz Biotechnology). All samples were analyzed using a D-Eclipse confocal laser scanning microscope (Nikon, Tokyo, Japan).

Reverse transcription polymerase chain reaction (RT–PCR) and quantitative (q)RT–PCR analyses

Total RNA was extracted using the RNeasy Mini kit (Qiagen, Courtaboeuf, France). RT was performed using a PrimeScript RT-reagent kit (Takara Bio, Otsu, Japan). qRT–PCR was performed on the Mx3000p real-time system (Stratagene, La Jolla, CA, USA) using SYBR Premix Ex Taq (Takara Bio). Amplification was started at 95°C for 30 s as the first step, followed by 40 cycles of qRT–PCR at 95°C for 5 s and at 60°C for 20 s. mRNA expression was measured in triplicate and normalized to the β-actin expression level. The sequences of primers from Takara Bio and SA Biosciences (Frederick, MD, USA) are shown in Table 1.

Detection of ROS production by microscopy

Dichloro-dihydro-fluorescein diacetate (DCFH-DA; Molecular Probes) is a cell-permeable non-fluorescent probe that is deesterified intracellularly and oxidized to highly fluorescent 2′,7′-dichlorofluorescein in the presence of ROS. After treatment with or without TNF-α (10 ng/mL) for 30 min or BaP (1 μmol/L) for 3 h in the presence or absence of 0.001% Gly, NHEK were incubated with DCFH-DA (5 μmol/L) for 30 min at 37°C, and the fluorescence signal of 2′,7′-dichlorofluorescein (excitation, 490 nm), the oxidation product of DCFH-DA, was analyzed using a D-Eclipse confocal laser scanning microscope (Nikon).

3-D epidermal equivalents and immunohistochemical analysis

The human epidermal 3-D model (EpiDem EPI-200; MatTek, Ashland, MA, USA) was treated with or without Gly or Th2 cytokines (IL-4 [60 ng/mL] and IL-13 [60 ng/mL]) for 6 days.

Table 1. Primers for quantitative polymerase chain reaction

Gene	Forward primer	Reverse primer	SA Biosciences ID
Nqo1	5′-GGATTGGACCGAGCTGGAA-3′	5′-AATTGCAGTGAAGATGAAGGCAAC-3′	
Nrf2	5′-CTTGGCCTCAGTGATTCTGAAGTG-3′	5′-CCTGAGATGGTGACAAGGGTTGTA-3′	
CYP1A1			PPH01271E
FLG	5′-CATGGCAGCTATGGTAGTGCAGA-3′	5′-ACCAAACGCACTTGCTTTACAGA-3′	
SPRR1A	5′-CTGGCCACTGGATACTGAACACC-3′	5′-GCACCCGAGCAACAAGAAGA-3′	
SPRR1B	5′-CCTTGCAATTAGCATTCTGTCTCC-3′	5′-ACCTTCAGCTTCATTCAGAGACTCA-3′	
SPRR2A	5′-CCCCACCCTGCCAGTCAAAGTA-3′	5′-GCAGTATGGCAGCCTCAGAAAAGA-3′	
SPRR2B	5′-CCCACCCTGCCAGCCAAAGTA-3′	5′-CATGCCCAGGTGAAAGACAGACA-3′	
SPRR2D	5′-TCCCTGGGAACCATCAGACAA-3′	5′-GTGGTAGAAGCTCATGACCAGGTG-3′	
SPRR2F	5′-CAAAGCCATCCAGGGATACACAG-3′	5′-GTGGCAGTATGGCAGCCTCA-3′	
SPRR3	5′-CATGAGTTCTTACCAGCAGAAGCAG-3′	5′-TTCCAGGTTGTGGAACCTTTGAG-3′	
SPRR4	5′-CAGCTAAGGCCCATCCATTTC-3′	5′-CACCAGCCTCTGTGACCCTA-3′	
LCE1A	5′-CCTGCAAGAGTGGCTGAGATG-3′	5′-GGCAGCAGATAGGTTTGTGG-3′	
LCE1B	5′-TCTGGAGGCTGCTGCTAAAGTG-3′	5′-GGCCTCTGAACTCCAAGACAGAA-3′	
LCE3D	5′-TTGATGCATGAGTTCCCAGATAC-3′	5′-TGACATCCTGGACATCAGACA-3′	
LCE3E	5′-TCCAGATCCTGATGCTGAGACAA-3′	5′-AGCTCAGCCTGTGAAAGTCAGAA-3′	
S100A7	5′-AATTACCTCGCCGATGTCTTTGA-3′	5′-ATGGCTCTGCTTGTGGTAGTCTGT-3′	
S100A15	5′-CTTCAATCCATCGCTACAGTCCAG-3′	5′-TGCCAATTGGACGGAATATTATCAG-3′	
S100A13	5′-CCTGAGGCTCCAGCTCACTCTA-3′	5′-GTCAGTGGTTCTGCTGCCATTA-3′	
TCHHL1	5′-TTTCAACTACAGCCAAGCATCACA-3′	5′-GCACCAGCAGGACTCTCATCA-3′	
HRNR	5′-CCAGCACCAAGAGGAACAAGAAGA-3′	5′-GCCGCGGCCTGAAGACTGATG-3′	
LOR	5′-GGCTGCATCTAGTTCTGCTGTTTA-3′	5′-CAAATTTATTGACTGAGGCACTGG-3′	
IVL	5′-TAACCACCCGCAGTGTCCAG-3′	5′-ACAGATGAGACGGGCCACCTA-3′	
β-Actin	5′-ATTGCCGACAGGATGCAGA-3′	5′-GAGTACTTGCGCTCAGGAGGA-3′	

Culture medium and reagents were replaced every 2 days. The skin samples were fixed in Super Fix (Kurabo, Osaka, Japan) overnight, embedded in paraffin by the conventional method and cut into 3-μm thick sections. Antigen retrieval was performed using Heat Processor Solution pH6 (Nichirei Biosciences, Tokyo, Japan) at 100°C for 40 min, and endogenous peroxidase was blocked by incubating the sections with 3% H_2O_2 (Nichirei Biosciences). The sections were then incubated with anti-filaggrin (1:1000) antibody at 4°C overnight, followed by incubation with secondary antibody, N-Histofine Simple Stain MAX-PO MULTI (Nichirei Biosciences). Immunodetection was conducted with 3,3-diaminobenzidine as chromogen, followed by light counterstaining with hematoxylin. Sections stained without primary antibody served as a negative control.

Transfection with siRNA against AhR

siRNA against AhR (AhR siRNA, s1200), as well as siRNA consisting of a scrambled sequence that would not lead to specific degradation of any cellular message (control siRNA), were purchased from Ambion (Austin, TX, USA). NHEK cultured in 24-well plates were incubated for 48 h in 0.5 mL of culture medium with a mixture containing 5 nmol/L siRNA and 3 μL of HiPerFect Transfection reagent (Qiagen). The siRNA-transfected NHEK were further treated with Gly for 6 h. The siRNA transfection did not affect cell viability, as demonstrated by Trypan blue dye exclusion test (data not shown). The efficiency of siRNA transfection was 91.9 ± 2.1% for AhR and 54.3 ± 5.5% for Nrf2 mRNA expression in NHEK, as determined by qRT–PCR.

Statistical analysis

Unpaired Student's *t*-test or one-way ANOVA was used to analyze the results. A *P*-value of less than 0.05 was considered to indicate a statistically significant difference. All data are presented as mean ± standard error of three independent experiments.

RESULTS

Gly induced nuclear translocation (activation) of AhR with upregulation of CYP1A1 gene expression

We first examined the cytotoxicity of Gly to keratinocytes. Gly did not affect the viability or actin mRNA expression of NHEK in graded concentrations of up to 0.001% of original stock solution. As the concentration of Gly in the commercially available ointment is 0.2%, our experimental concentrations of Gly were considered to be therapeutically meaningful. Under a concentration of 0.001%, Gly actually upregulated CYP1A1 expression in a dose-dependent manner (Fig. 1a). Given these results, we used 0.001% of Gly throughout the following experiments, unless otherwise noted. As Gly upregulated CYP1A1 expression, we next examined whether Gly induces AhR activation by immunofluorescence study. As shown in Fig. 1(b), AhR was distributed mainly in the cytoplasm in control NHEK, whereas it translocated into the nucleus in the presence of Gly (Fig. 1c). In order to confirm the dependence of CYP1A1 upregulation on AhR, we then knocked down AhR expression in NHEK by transfection of AhR siRNA. In the AhR-knockdown NHEK, the CYP1A1 upregulation by Gly was completely

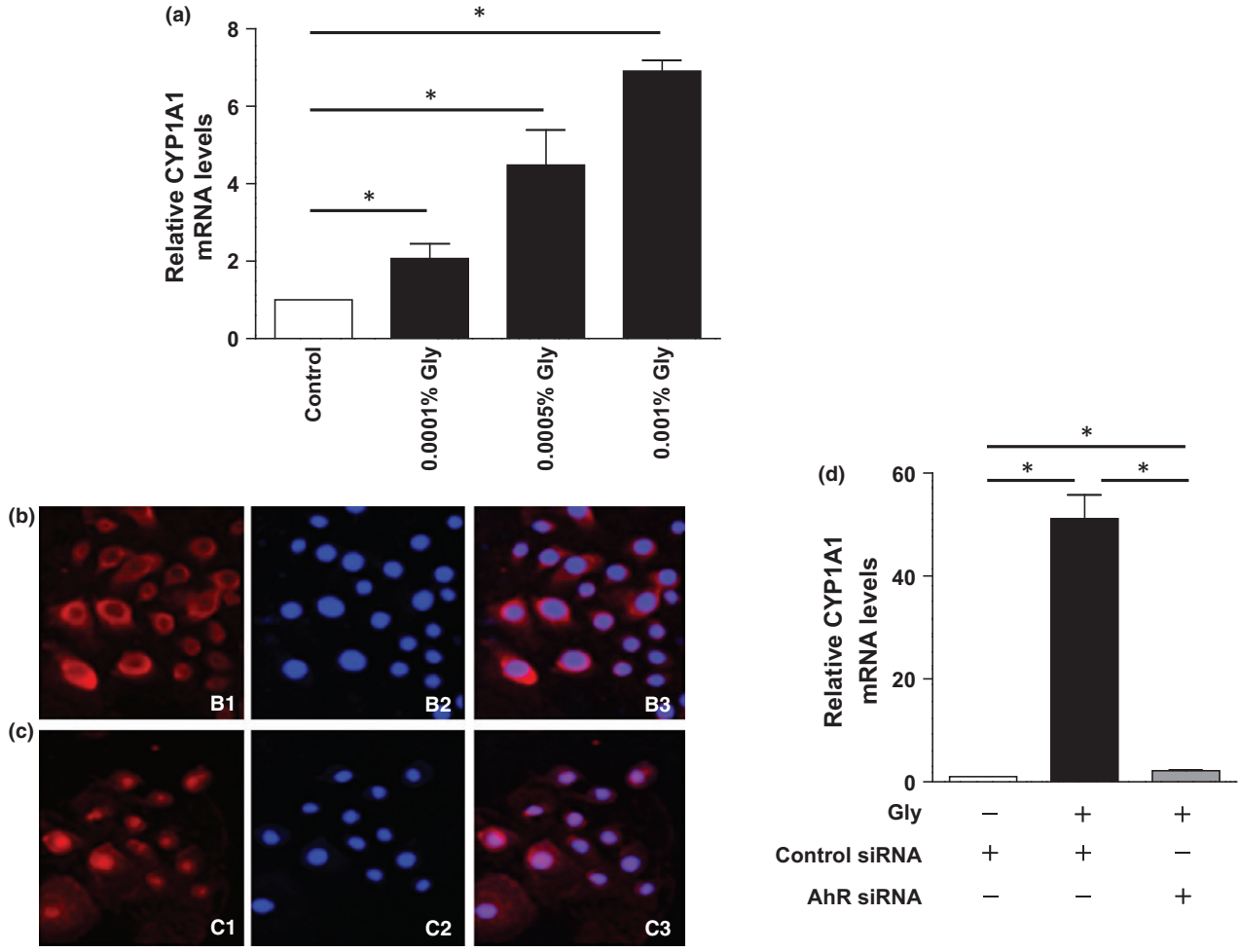

Figure 1. (a) CYP1A1 gene expression was determined in the presence of graded concentrations of Glyteer (Gly). (b) Cytoplasmic localization of aryl hydrocarbon receptor (AhR) in control normal human epidermal keratinocytes (NHEK). B1, AhR (red); B2, nuclear staining by 4′,6-diamidino-2-phenylindole (DAPI) (blue); B3, merge. (c) Nuclear translocation of AhR in NHEK treated with 0.001% Gly. C1, AhR (red); C2, nuclear staining by DAPI (blue); C3, merge. (d) Gly-induced upregulation of CYP1A1 was canceled in the NHEK transfected with AhR siRNA. *$P < 0.05$.

canceled, indicating that the Gly-induced CYP1A1 expression was strongly dependent on AhR (Fig. 1d).

Gly upregulated Nqo1 expression with nuclear translocation of Nrf2

Because some ligands for AhR activate the Nrf2 antioxidant pathway by upregulating a downstream antioxidant enzyme, Nqo1,[5,15] we next addressed whether Gly activates Nrf2 and enhances Nqo1 gene expression. Nrf2 was localized in the cytoplasm in the control unstimulated NHEK (Fig. 2a). However, Gly activated Nrf2 and induced its nuclear translocation (Fig. 2b). Although Gly did not significantly upregulate Nrf2 gene transcription (Fig. 2c), it did induce dose-dependent upregulation of that of Nqo1 (Fig. 2d). In order to confirm the AhR or Nrf2 dependence of Nqo1 expression, we used AhR- or Nrf2-knockdown NHEK. In NHEK treated with control siRNA, Gly upregulated Nqo1 gene expression (Fig. 2e). However, its expression was partially but significantly downregulated in the AhR-knockdown NHEK (Fig. 2e). Considering a complete inhibition of CYP1A1 expression in the AhR-knockdown NHEK (Fig. 1d), a partial inhibition of Nqo1 may mean that both AhR-dependent and AhR-independent components in Gly contributed to the upregulation of Nqo1 expression. In accordance with previous reports,[15,27] induction of Nqo1 was apparently Nrf2-dependent because Gly-induced Nqo1 upregulation was completely abrogated in NHEK transfected with Nrf2 siRNA relative to that in NHEK transfected with control siRNA (Fig. 2e).

Gly inhibited TNF-α- and BaP-induced ROS production in NHEK

As previous studies demonstrated that Nrf2-Nqo1 activation potently suppressed ROS production in keratinocytes stimulated by TNF-α or BaP,[4,15] we next examined whether Gly is a feasible option to inhibit the ROS production of NHEK treated

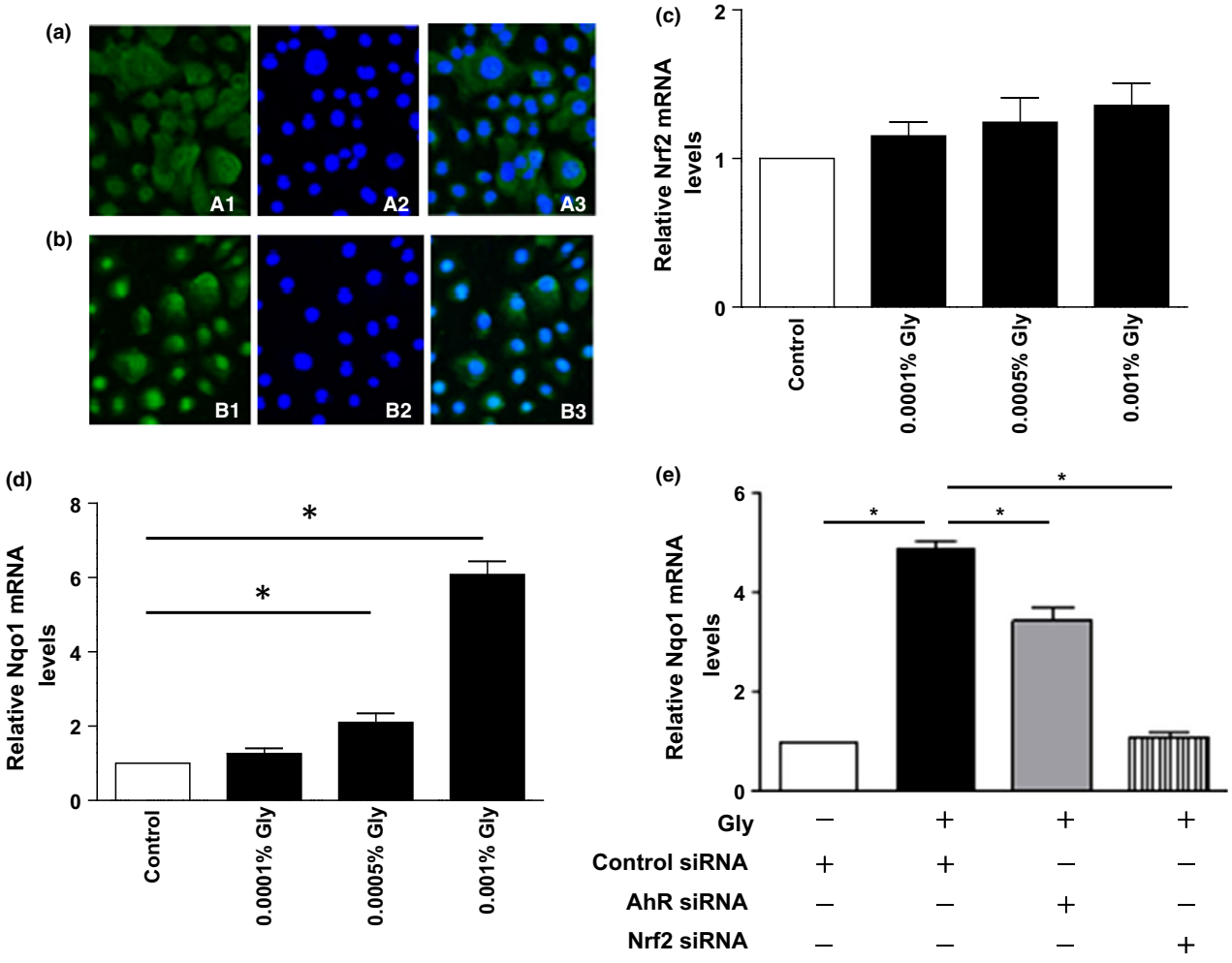

Figure 2. (a) Nrf2 was mainly localized in the cytoplasm of control normal human epidermal keratinocytes (NHEK). A1, Nrf2 (green); A2, nuclear staining by 4′,6-diamidino-2-phenylindole (DAPI) (blue); A3, merge. (b) Gly-induced nuclear translocation of Nrf2. B1, Nrf2 (green); B2, nuclear staining by DAPI (blue); B3, merge. (c) Glyteer (Gly) did not significantly upregulate Nrf2 gene transcription. (d) Transcription of Nqo1 was significantly and dose-dependently enhanced by Gly. (e) The mRNA expression of Nqo1 was partially downregulated in AhR-knockdown NHEK, whereas it was completely abrogated in Nrf2-knockdown NHEK. *$P < 0.05$.

with TNF-α or BaP. Compared with that in the control NHEK, Gly itself did not induce ROS generation (Fig. 3a). In contrast, TNF-α induced robust ROS production, which was markedly inhibited by the simultaneous presence of Gly (Fig. 3a). The antioxidant activity of Gly was also involved in inhibiting the BaP-induced ROS production (Fig. 3b). The BaP-induced ROS production was potently inhibited by simultaneous treatment with Gly (Fig. 3b).

Gly upregulated the expression of filaggrin in an AhR-dependent manner

Because the filaggrin gene is one of the genes involved downstream of AhR activation,[11,12,20,21] we examined whether Gly upregulates the expression of filaggrin. As expected, Gly dose-dependently upregulated filaggrin gene expression (Fig. 4a). The enhancing effect of Gly on the expression of filaggrin was again canceled in the AhR-knockdown NHEK (Fig. 4b). As the expression of filaggrin is spatiotemporally regulated in air-exposed culture conditions,[12,28] we next confirmed the effects of Gly on filaggrin expression using a human 3-D epidermal equivalent by immunohistochemistry. In the control epidermal equivalent, filaggrin expression was confined to the region from the upper living layer to the upper cornified layer (Fig. 4c). However, in the presence of 0.0001% Gly, acceleration of the appearance of filaggrin expression from the suprabasal layer occurred (Fig. 4d), which was further augmented in the epidermal equivalent treated with 0.001% Gly (Fig. 4e).

Gly restored the Th2 cytokine-mediated downregulation of filaggrin

It is known that a Th2 milieu downregulates the expression of filaggrin,[12,29] which was the case in our study. As shown in

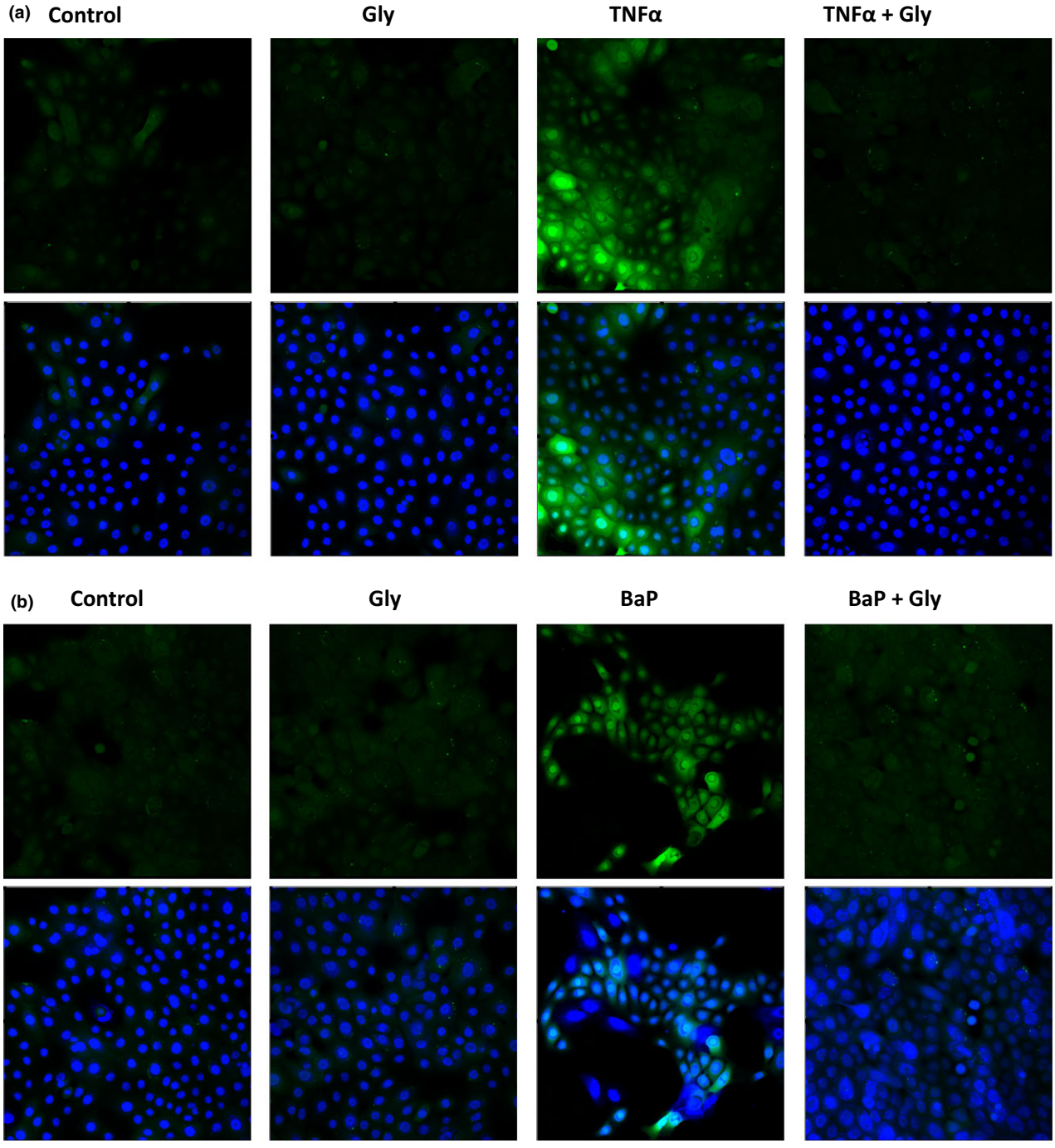

Figure 3. Glyteer (Gly) inhibited the reactive oxygen species (ROS) production (green) of normal human epidermal keratinocytes (NHEK) treated with (a) tumor necrosis factor (TNF)-α or (b) benzo[α]pyrene (BaP). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue) in the lower panels of (a) and (b). *$P < 0.05$.

Figure 5(a), IL-4 or IL-13 significantly inhibited the expression of filaggrin compared with that in the control. Simultaneous addition of Gly markedly restored the IL-4- or IL-13-induced inhibitory activity on filaggrin gene expression (Fig. 5a). Immunohistochemical study confirmed these results in the 3-D epidermal equivalent. When compared with the control epidermal equivalent (Fig. 5b), Th2 cytokines (IL-4 and IL-13) apparently decreased filaggrin expression from the upper living

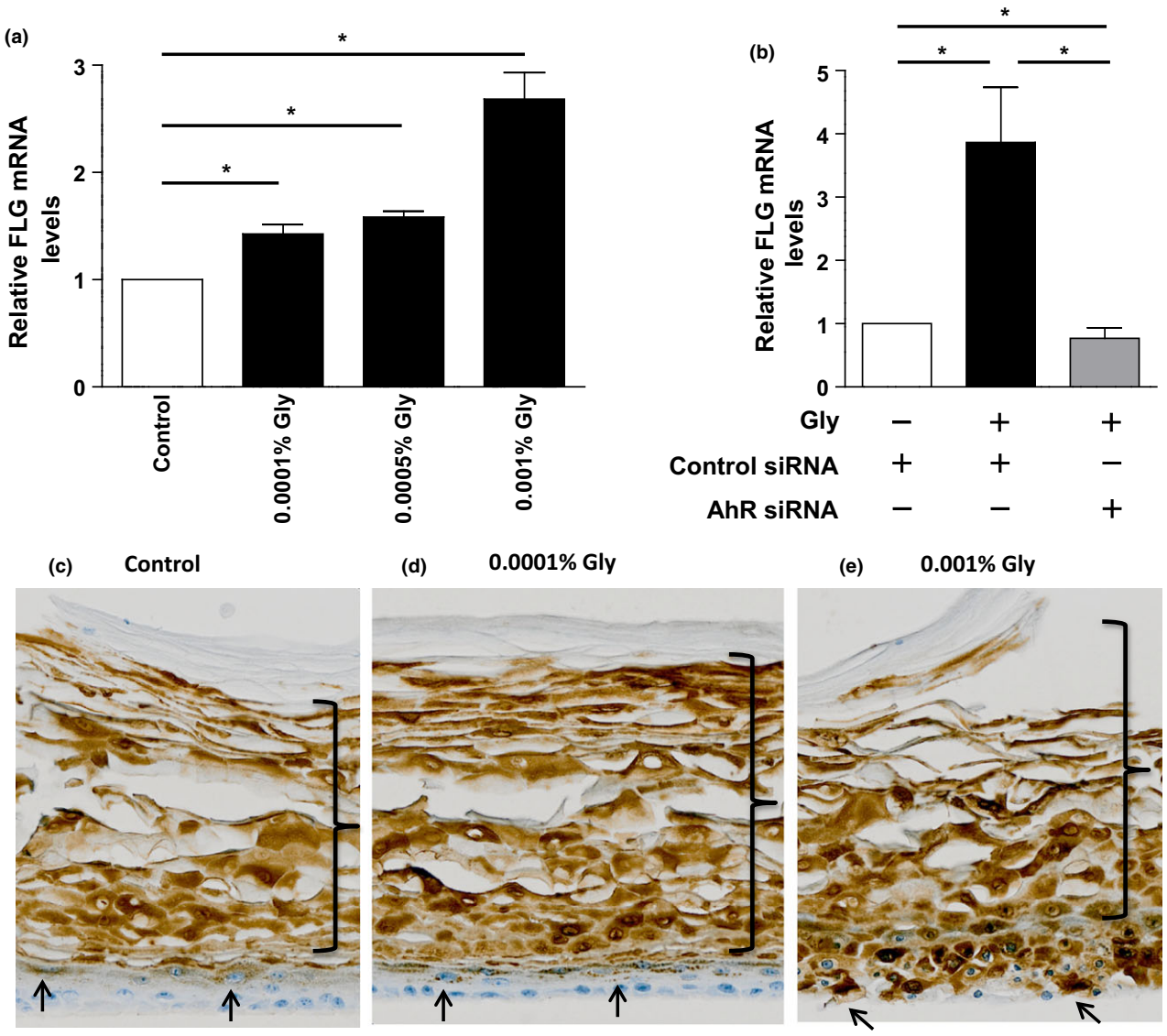

Figure 4. (a) Glyteer (Gly) upregulated the mRNA expression of filaggrin in normal human epidermal keratinocytes (NHEK) in a dose-dependent manner. (b) The enhancing effect of Gly on the expression of filaggrin was canceled in the aryl hydrocarbon receptor (AhR)-knockdown normal human epidermal keratinocytes (NHEK). (c) Immunohistochemical detection of filaggrin expression in 3-D epidermal equivalents treated with or without 0.0001% or 0.001% Gly. In control epidermal equivalent, the expression of filaggrin was observed in the upper living layer (arrow). In the presence of 0.0001% of Gly, the expression of filaggrin was enhanced and noted even in the suprabasal cells of living layer (arrow). The expression of filaggrin was markedly augmented in the epidermal equivalent treated with 0.001% Gly. Some basal cells also expressed the filaggrin (arrow). }, cornified layer. *$P < 0.05$.

layer to the lower cornified layer (Fig. 5c). As shown in Figure 5(d), Gly restored the Th2 cytokine-mediated decrease of filaggrin.

Gly coordinately upregulated a series of epidermal differentiation molecules

In addition to filaggrin, previous studies have demonstrated that AhR activation by TCDD upregulates various epidermal barrier proteins, such as loricrin, involucrin, hornerin, small proline-rich proteins (SPRR), late cornified envelope proteins (LCE) and S100A proteins.[11,20,21] These barrier proteins are called the "epidermal differentiation complex" because they are mainly encoded on chromosome 1q21.[30] We thus examined the effects of Gly on these epidermal differentiation complex proteins. Interestingly, Gly coordinately upregulated the gene expression of all of the examined barrier proteins, including SPRR1A, SPRR1B, SPRR2A, SPRR2B, SPRR2D, SPRR2F, SPRR3, SPRR4, LCE1A, LCE1B, LCE3D, LCE3E, S100A7, S100A15, trichohyalin-like 1 protein, hornerin, loricrin and involucrin (Fig. 6). However, the upregulation of SPRR2A, SPRR2B,

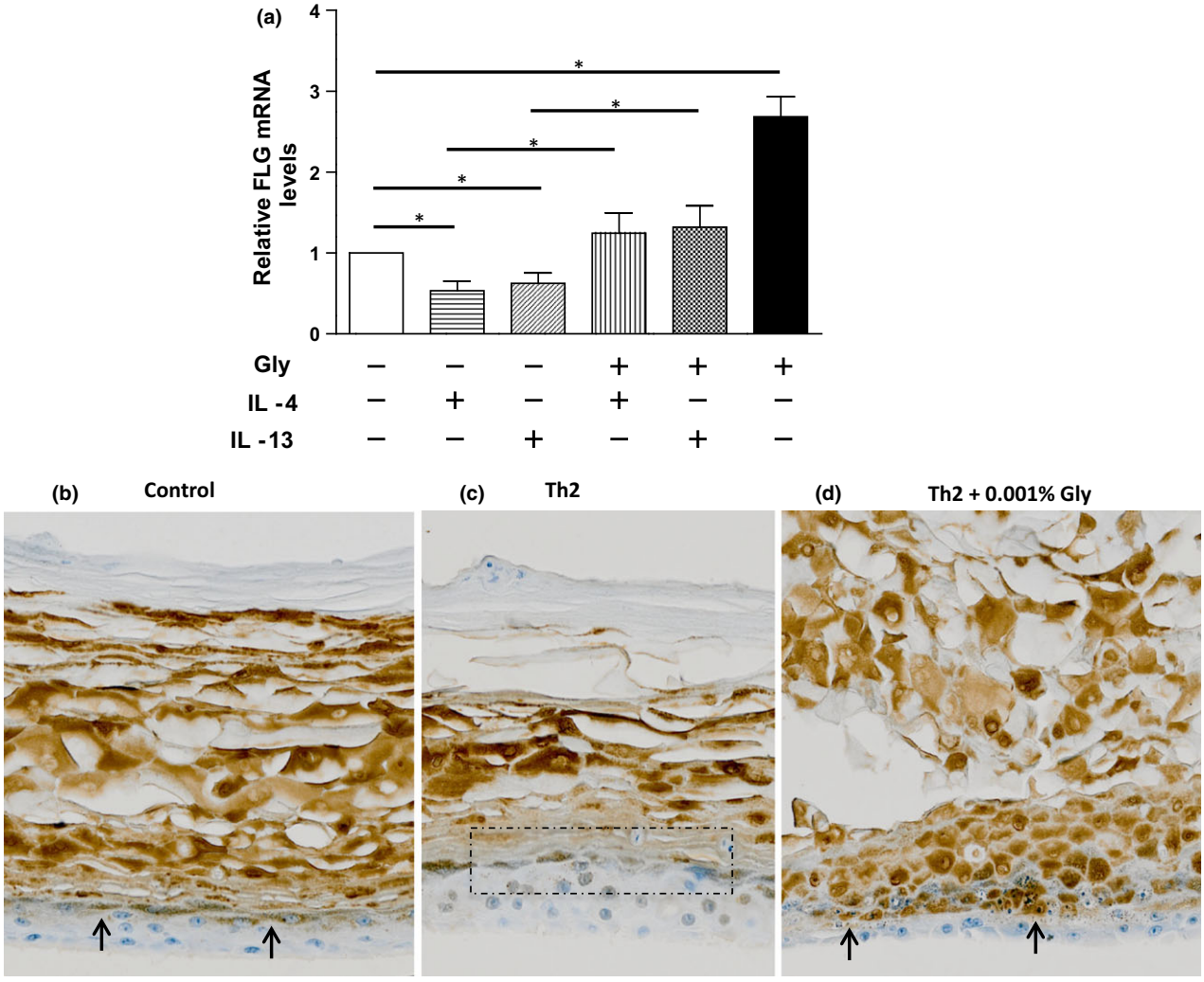

Figure 5. (a) Interleukin (IL)-4 or IL-13 significantly downregulated filaggrin gene expression. Simultaneous presence of Glyteer (Gly) restored the inhibitory action of IL-4 or IL-13. (b) The expression of filaggrin was detected in the upper living layer of 3-D epidermal equivalent (arrow) (left panel), whereas T-helper (Th)2 cytokines (IL-4 and IL-13) appeared to downregulate filaggrin expression (dotted area) (center panel). The inhibitory activity of Th2 cytokines was restored in the presence of 0.001% Gly and the expression of filaggrin was again detected in the upper living layer (arrow) (right panel). *$P < 0.05$.

SPRR2F, LCE1B and S100A7 expression did not reach statistical significance. S100A13 expression was not affected by AhR ligation with Gly, as pointed out for TCDD by Kennedy *et al.*[11]

DISCUSSION

The skin is the largest organ of the human body, providing the primary anatomical barrier between internal and external environments. The outermost epidermis, mainly composed of keratinocytes, needs to sense an array of external stimuli. AhR is a small molecule sensor for various halogenated and non-halogenated polycyclic aromatic hydrocarbons, phytochemicals and tryptophan photoproducts with a wide range of affinities.[2] Coal tar has been used for the treatment of inflammatory skin diseases for a long time.[23] Because coal tar is thought to consist of various polycyclic aromatic hydrocarbons,[23] van den Bogaard *et al.*[12] investigated its therapeutic mechanism with special reference to AhR. Using organotypic skin models with primary keratinocytes from atopic dermatitis patients and controls, they demonstrated that coal tar activates AhR, resulting in the induction of epidermal differentiation (i.e. upregulation of filaggrin, loricrin and hornerin expression), and thickens the cornified layer.[12] Furthermore, AhR knockdown by siRNA completely abrogates these effects. In atopic dermatitis patients, coal tar completely restores the expression of major skin barrier proteins including filaggrin. Coal tar also diminishes spongiosis and apoptosis in organotypic skin stimulated by the Th2 cytokines IL-4 and IL-13.[12]

Soybean tar, Gly, has been widely used in Japan for 90 years as an alternative to coal tar because it is less viscous

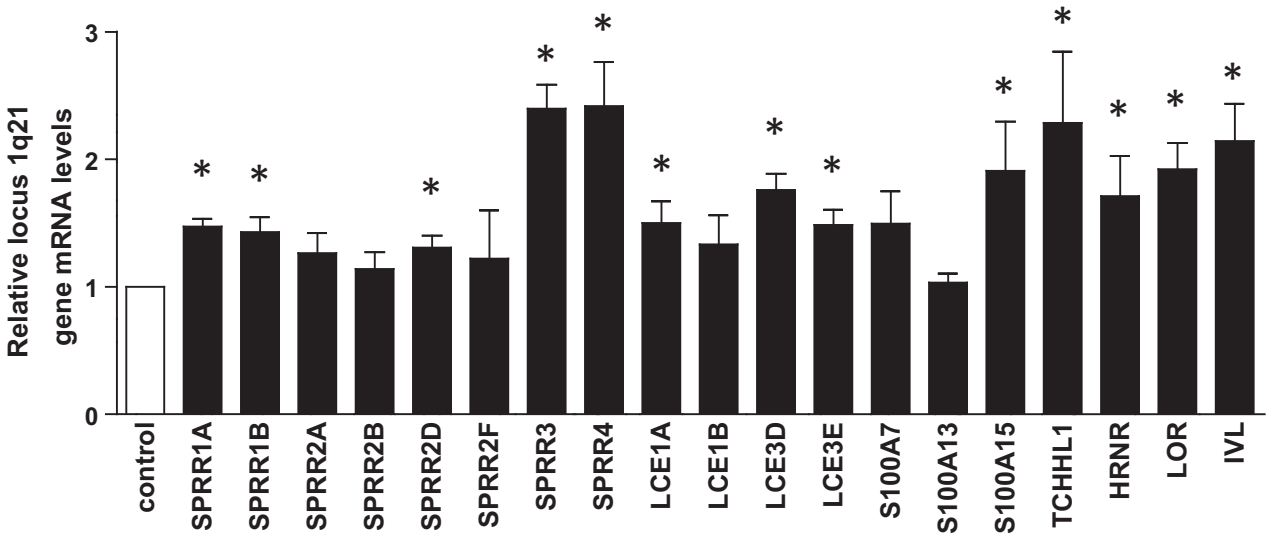

Figure 6. Glyteer (Gly) coordinately upregulated the expression of various epidermal differentiation complex genes located on chromosome 1q21. HRNR, hornerin; INV, involucrin; LCE, late cornified envelope protein; LOR, loricrin; SPRR, small proline-rich protein; TCHHL, trichohyalin-like 1 protein. *$P < 0.05$.

and does not have as strong a smell. Although its beneficial effects on inflammatory skin diseases are known empirically, the fundamental mechanisms have remained unclear throughout the time it has been on the market. In accordance with the results for coal tar, Gly did indeed activate AhR and induce the nuclear translocation of AhR in keratinocytes. The AhR ligation by Gly was biologically active in inducing significant and dose-dependent upregulation of CYP1A1 expression, which is a specific marker for AhR activation.[3,4] Gly also upregulated the expression of filaggrin in an AhR-dependent manner because its enhancing effect was completely abrogated in AhR-knockdown keratinocytes. In the present study, we confirmed the inhibitory action of Th2 cytokines on filaggrin expression, which was also restored by Gly, similar to the effect of coal tar.[12] Moreover, Gly coordinately upregulated a series of epidermal differentiation complex genes including involucrin, loricrin, hornerin, SPRR, LCE and S100A. These results also match those of a previous study by Kennedy *et al.*,[11] who demonstrated that AhR ligation by TCDD upregulates the expression of 40% of the genes encoding proteins included in the epidermal differentiation complex. However, the present study demonstrated some discrepancy between TCDD and Gly in terms of upregulating SPRR2A, SPRR2B, LCE1A, A100A7, trichohyalin-like protein 1, loricrin and involucrin expression.[11]

In addition to the capacity to enhance the expression of barrier proteins, Gly exhibited potent antioxidant activity. This antioxidant activity was paralleled by activation of the Nrf2–Nqo1 pathway, which was again canceled in the AhR-knockdown keratinocytes. Previous studies have shown that the AhR system acts as a master switch for up- and downregulating oxidative stress.[2] Dioxins and other dioxin-related compounds induce robust ROS production via AhR.[4,31] In contrast, the ligation of AhR by phytochemicals and ketoconazole induces antioxidant activity via the AhR–Nrf2–Nqo1 signaling pathway.[15,32,33] The present study reveals that Gly per se did not evoke the ROS generation; instead, it potently inhibited TNF-α- and Bap-induced ROS production. In this context, the precise mechanisms by which AhR discriminates oxidative and antioxidative ligands remain largely unknown and open for further investigation.[2] With regards to TNF-α and filaggrin, Kim *et al.*[34] have recently described that TNF-α is a potent inhibitor of the filaggrin expression. It is intriguing to elucidate whether Gly potentially prevents the TNF-α-induced inhibition on filaggrin expression. There are several limitations in this study. Like coal tar, Gly may contain a lot of chemical substances. Because precise chemical analysis had never been performed previously, we could not know the ingredients of Gly. However, it is confirmed that Gly does not contain harmful BaP. In addition, there is no information available about the percutaneous absorption of Gly. Although a therapeutic dose of coal tar does not increase the risk of skin and non-skin cancer in patients with atopic dermatitis and psoriasis, there still remains a chance of carcinogenesis.[35,36] Careful observation is mandatory to overcome the risk.

Taking these findings together, antioxidant Gly rescues the inhibited expression of filaggrin (and plausibly other barrier proteins) in a Th2-skewed milieu via AhR activation, which may partly explain its empirically identified anti-inflammatory therapeutic effects.

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