Tumor Necrosis Factor-Alpha–Nuclear Factor-Kappa B-Signaling Enhances St2b2 Expression during 12-0-Tetradecanoylphorbol-13-acetate-Induced Epidermal Hyperplasia

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The present study shows that TNFα–NF-κB inflammatory signaling pathway, which in turn leads to increased CS concentrations in epidermal cells and hyperplasia.

Key words cholesterol sulfate; epidermal differentiation; phorbol ester; nuclear factor-kappa B; sulfotransferase; tumor necrosis factor-alpha

Skin plays an essential role in protecting the body’s internal environment against environmental insults. Keratinocyte differentiation, including expression of such cornified envelope proteins as involucrin (INV) and loricrin, is essential to maintain the structure and function of the epidermis. Disruption of the expression of these proteins causes epidermal dysplasia and dysfunction. 1–2

Treating skin with 12-O-tetradecanoylphorbol-13-acetate (TPA) causes epidermal hyperplasia through an abnormal increase in cornified envelope protein levels. 3–4 TPA is thought to produce these effects by inducing protein kinase C (PKC) activation, calcium influx, and release of inflammatory cytokines, for example. 4–6 The precise mediators of TPA signaling that cause epidermal hyperplasia, however, remain unclear.

Cholesterol sulfate (CS) is a chemical mediator that maintains epidermal homeostasis. Accumulation of CS—for instance, owing to a deficiency in the catabolic enzyme steroid sulfatase (SSase)—causes dyskeratosis. 7–9 Levels of the CS biosynthetic enzyme cholesterol sulfotransferase (Ch-ST) are also associated with the extent of epidermal differentiation. In particular, St2b2, a member of the cytosolic sulfotransferase family, is the primary Ch-ST in mouse epidermis, 10 where it plays a role in the expression of INV. 11 Ch-ST activity increases together with cornified envelope protein levels after TPA treatment. 12,13 Thus, St2b2 may be linked to TPA-induced epidermal hyperplasia.

Expressed beginning at early cornification, INV is involved in construction of the cornified envelope. 14–15 Overexpression of INV distorts the structure of the epidermis. 1,16 Furthermore, cells that express INV in response to various stimuli are selectively expelled from the basal layer composed of uncornified epidermal cells. 17 Thus, INV expression levels can be used as a marker of the extent of epidermal differentiation.

Application of TPA on mouse skin causes an intense inflammatory response. The associated signaling is mediated by various cytokines and regulatory factors, such as tumor necrosis factor-alpha (TNFα) and interleukins, which are involved in epidermal differentiation and dyskeratosis. 18,19 Indeed, TNFα levels increase when epidermis is exposed to TPA. 20,21 TNFα binding to TNF receptor (TNFR) results in activation of a number of transcription factors, including nuclear factor-kappa B (NF-κB). Moreover, treating keratinocytes with anti-inflammatory drugs suppresses TPA-induced increases in Ch-ST activity, 15,22 suggesting that TNFα–NF-κB signaling mediates TPA-induced enhancement of St2b2 expression.

The present study shows that TNFα–NF-κB signaling contributes to TPA-induced epidermal hyperplasia via increased St2b2 expression.

MATERIALS AND METHODS

Materials Cholesterol, CS, 3′-phosphoadenosine-5′-phospho-
phosphosulfate (PAPS), TPA, and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). [35S]-PAPS and [14C]-cholesterol was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA, U.S.A.). PKC\(\eta\) pseudosubstrate inhibitor was purchased from Merck (Darmstadt, Germany). Recombinant mouse TNF\(\alpha\) and mouse TNF enzyme-linked immunosorbent assay (ELISA) Kit 2 were purchased from Becton Dickinson Co. (San Diego, CA, U.S.A.). TNF\(\alpha\)-small interfering RNA (siRNA), control-siRNA A, siRNA transfection medium, and siRNA transfection reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-mouse TNFR hamster monoclonal antibodies (TNFR-Ab) were purchased from R&D Systems (Minneapolis, MN, U.S.A.). Hamster IgG (LEAF\(^\text{TM}\) purified American hamster IgG isotype control Clone) was purchased from BioLegend (San Diego, CA, U.S.A.). BAY 11-7082 was purchased from BIOMOL Research Labs (Plymouth, PA, U.S.A.). The St2b2-specific small hairpin RNA (St2b2-shRNA)-expressing adenovirus AdSt2b2-shRNA was constructed previously. All other chemicals used were of the highest grade available.

**Animal Treatments** Six-week-old female CD-1 mice (Charles River Japan, Atsugi, Japan) were housed in an air-conditioned room (22—23 °C) with a 12-h light period from 6 a.m. to 6 p.m. Food and water were available ad libitum. We used female mice in this study because epidermal St2b2 levels and cholesterol sulfation are slightly higher in females than in males. Animal experiments were conducted in accordance with the guidelines of the Ministry of Education, Culture, Sports, Science and Technology of Japan. A dorsal area (2 cm\(^2\)) of each 6-week-old mouse was shaved using an electric shaver 24 h before the first treatment.

TPA treatment: TPA (16 nmol) dissolved in 200 \(\mu\)l of acetone (vehicle) was painted onto a shaved dorsal area of each mouse using a pipette. The skin was removed 40 h after treatment.

AdSt2b2-shRNA and TPA treatment: A previous report showed that a target gene could be introduced into epidermal cells by painting a mouse’s skin with a suspension containing gene-encoding adenovirus. Therefore, the shaved area of the skin was removed 40 h after TPA treatment. Treatment with 30 pg/ml TNF\(\alpha\) was performed as described above.

NF-\(\kappa\)B Inhibitor and TNF\(\alpha\) Treatment: The NF-\(\kappa\)B inhibitor BAY 11-7082 was dissolved to 1, 3, or 10 nm in DMSO (vehicle). NME cells were incubated with 0, 1, 3, or 10 \(\mu\)M BAY 11-7082 for 1 h. TNF\(\alpha\) (30 pg/ml) treatment was performed as described above.

**Preparation of Cytosolic, Microsomal, and Membrane Fractions** Skin was removed and the dermis was excised from the skin by scraping with a surgical razor. The epidermis was homogenized in homogenizing buffer containing 75 mM potassium phosphate (pH 7.4), 75 mM KCl, and 1 mM dithiothreitol using a Polytron PT-10 homogenizer at 4 °C. The homogenate was filtered through gauze and centrifuged for 20 min at 4 °C and 9000x\(g\). The resultant pellet was resuspended in homogenizing buffer and used as the membrane fraction. The supernatant (S-9) was further centrifuged for 60 min at 4 °C and 105000x\(g\). The resultant supernatant was used as the cytosolic fraction, and the pellet was resuspended in homogenizing buffer and used as the microsomal fraction. NME cells were harvested and homogenized using homogenizing buffer. The homogenate was fractionated using the same method described for fractionation of epidermis. Protein concentrations were determined using the Bradford method.

**Immunoblotting** Immunoblotting for St2b2 and INV was performed as described previously. Cytosolic samples (20—40 \(\mu\)g of protein/lane) and membrane fractions (10—20 \(\mu\)g of protein/lane) were loaded.

**Determination of Enzymatic Activities** Cholesterol-sulfating activities were determined using a previously reported method. The assay for CS desulfation was performed as described previously with some modifications. SSase activity was determined based on desulfo-conjugation...
of \([^{14}\text{C}]\)-CS, which was prepared using \([^{14}\text{C}]\)-cholesterol as a substrate, PAPS as a sulfate donor, and recombinant St2b2 protein, and purified using thin layer chromatography (TLC aluminum plate silica gel 60, Merck, Darmstadt, Germany). The desulfation assay was performed in 30 mM Tris–HCl buffer (pH 7.2) containing 0.25 M sucrose, 20 mM KCl, 5 mM guanidinium thiocyanate–phenol–chloroform method. mRNA prepared from epidermis tissue or NME cells using the acid guanidinium thiocyanate–phenol–chloroform method. mRNA levels were measured using RT-PCRs. The reaction conditions included 30 cycles of a three-phase PCR (denaturation at 95 °C for 15 s; annealing at 55 °C for 30 s; extension at 72 °C for 30 s). PCR products were analyzed on 2% (w/v) agarose gels containing ethidium bromide. The intensities of stained bands were measured using NIH image software (version 1.59, Bethesda, MD, U.S.A.). The specific primers of TNF and stained with methyl green-pyronin.

Immunohistochemistry Dorsal skin was frozen in OCT Compound (Tissue-Tek, Sakura Finetechnical Co., Ltd., (version 1.59, Bethesda, MD, U.S.A.). The specific primers of TNF and stained with methyl green-pyronin.

Detection of Intracellular and Extracellular TNFα Concentrations TNFα levels in the cells (S-9 fraction) and media were quantified using Mouse TNF ELISA Kit 2 in accordance with the manufacturer’s instructions.

Statistical Analysis All data are shown as the means ± S.D. Statistical differences between groups were assessed by one-way ANOVA followed by Tukey’s multiple comparison. Probability values less than 0.05 were considered statistically significant.

RESULTS

Effects of TPA Treatment on the Activities of Ch-ST and SSase in Mouse Skin To clarify the contributions of Ch-ST and SSase to the increased CS concentrations after TPA treatment, CS levels in skin, the specific activities of Ch-ST and SSase to the increased CS concentrations after TPA treatment, CS levels in skin, the specific activities of Ch-ST and SSase, and St2b2, St2a4/9, and SSase mRNA levels were determined. Skin INV protein levels were used as a marker of epidermal differentiation. After TPA treatment, CS levels in mouse epidermis were 50% higher than those in control (Fig. 1A). TPA treatment also enhanced INV expression (120% increase; Fig. 1B) and skin Ch-ST activity (40% increase; Table 2). St2b2 mRNA and protein levels in skin increased by 120% and 50%, respectively (Fig. 2). Neither the activity nor mRNA levels of SSase changed significantly, however (Fig. 2, Table 2). In addition, mRNA encoding St2a4 or St2a9, which mediate cholesterol sulfation in mice, was not detected in normal mouse epidermal cells, as reported previously, or in TPA-treated skin (Fig. 2).

Effects of AdSt2b2-shRNA on TPA-Induced Epidermal Differentiation in NME Cells St2b2-shRNA was then administered to NME cells and the levels of CS and INV protein were examined (Fig. 3). St2b2 protein levels increased by 40% after TPA treatment in control AdSt2b2-shRNA(−) cells, whereas their MOI-dependent decreased in response to AdSt2b2-shRNA. St2b2 expression decreased to 80% of that observed in control TPA(−) cells (Fig. 3A). Consistent with this finding, CS levels also decreased to 60% of those observed in control cells following infection with AdSt2b2-shRNA (MOI, 6; Fig. 3B). A correlation was observed between the levels of CS and St2b2 protein (Fig. 3D). The TPA-mediated increase in INV expression levels was attenuated to 90% of levels detected in control cells following infection with AdSt2b2-shRNA (MOI, 6; Fig. 3C). A correlation was observed between expression levels of INV and St2b2 (Fig. 3E). SSase mRNA levels were not

Table 1. Primer Sequences for RT-PCRs

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<th>Gene</th>
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<th>Antisense</th>
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<td>St2b2</td>
<td>5'-TGGGAGGCTCGTGAGAAAATGTT-3'</td>
<td>5'-TGGAAGCGCTTAGATTGCTCCGC-3'</td>
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<td>St2a4/9</td>
<td>5'-TGTAGTCAGACTATAATGGTGGTAAAGGC-3'</td>
<td>5'-GATTAGTCAGTGCTGTTCTTATTGG-3'</td>
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<tr>
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<td>5'-CTCCAGTGTGTTGCGCTTCC-3'</td>
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<td>GAPDH</td>
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<td>5'-GTCCACCCACCTGTGGCTTAG-3'</td>
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Fig. 1. Expression of CS and INV Protein in TPA-Treated Mouse Epidermis

TPA (16 nmol) dissolved in acetone (vehicle) was painted on shaved dorsal skin in mice. The skin was removed 40 h after treatment, and the dermis was excised. (A) The amount of CS in the epidermis was measured using thin layer chromatography. (B) INV levels in the membrane fraction from the epidermis were determined by immunoblotting. Expression levels are shown as ratios to levels observed in control TPA(−) epidermis. Data are shown as the means ± S.D. (n=3). *p < 0.05.

Table 2. Effects of TPA Treatment on Ch-ST and SSase Activities in Mouse Epidermis

<table>
<thead>
<tr>
<th>Activity</th>
<th>Vehicle</th>
<th>TPA</th>
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<tr>
<td>Ch-ST (pmol/mg protein/min)</td>
<td>2.64±0.02</td>
<td>3.82±0.51*</td>
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<tr>
<td>SSase (pmol/mg protein/min)</td>
<td>0.69±0.11</td>
<td>0.78±0.08</td>
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Cytosolic protein and microsomal protein were examined from the epidermis used in Fig. 1. Methods used to determine the activities of Ch-ST in the cytosol and SSase in the microsomal fraction are provided in the Materials and Methods. Each value is shown as the mean ± S.D. (n=3). *p < 0.05.
affected by infection with AdSt2b2-shRNA (data not shown).

Effects of a PKC Inhibitor on TPA-Induced Increases in St2b2 and INV Expression in NME Cells The effects of a PKC inhibitor on St2b2 and INV protein levels were then examined during TPA-induced epidermal differentiation in NME cells. After TPA treatment, St2b2 protein levels increased to 160% of those observed in control cells (PKC inhibitor(−) and TPA(−)); the PKC inhibitor (0.5 or 1.5 μM) did not significantly affect St2b2 levels (Fig. 4A). Expression levels of INV protein also increased to 160% of those observed in control cells after TPA treatment, whereas, in response to 1.5 μM PKC inhibitor, the level decreased to 70% of that detected in control cells (Fig. 4B).

Knocking Down St2b2 Gene Expression during TPA-Induced Epidermal Hyperplasia in Mouse Skin The effects of knocking down St2b2 gene expression were then examined during epidermal hyperplasia. As shown in Fig. 5, the thickness of the epidermis increased after TPA treatment, but not when AdSt2b2-shRNA was also introduced together with TPA. We also examined Ch-ST activity and expression.
of St2b2 and INV. St2b2 protein levels decreased to 60% of those in control skin following infection with AdSt2b2-shRNA (Fig. 6A). St2b2 proteins level increased by 30% after TPA treatment in control skin, but were not significantly affected by TPA treatment in AdSt2b2-shRNA–infected skin (Fig. 6A). Ch-ST activity also increased by 50% after TPA treatment in control skin, but not in AdSt2b2-shRNA–infected skin (Fig. 6B). Protein levels increased by 40% after TPA treatment in control skin, but not in AdSt2b2-shRNA–infected skin (Fig. 6C).

Time-Dependent Changes in St2b2, INV, and TNFα Expression after TPA Treatment in NME Cells To explore the potential involvement of TNFα in TPA-induced enhancement of St2b2 and INV expression, time-dependent changes in the expression profiles of these proteins were examined after TPA treatment in NME cells. Significant increases in St2b2 expression were observed after 24 h (Fig. 7A). INV expression also increased after 24 h (Fig. 7B). Cellular levels of TNFα protein peaked within 3 h of TPA treatment (Fig. 7C), whereas extracellular levels reached a maximum at 24 h (Fig. 7D).

Effects of TNFα on St2b2 and INV Expression in NME Cells To examine the role of TNFα in TPA-induced enhancement of St2b2 expression, NME cells were treated with TNFα. St2b2 protein levels increased by up to 260% after TNFα treatment (Fig. 8A). INV protein levels also similarly increased (by a maximum of 210%; Fig. 8B). Maximum effects for both proteins were observed with 30 pg/ml TNFα.

Effects of Knocking Down St2b2 Expression on TNFα-Induced INV Expression in NME Cells AdSt2b2-shRNA was then used to examine the roles of St2b2 on enhanced INV expression in NME cells. St2b2 protein expression levels in TNFα-treated cells decreased from 410 to 130% of those observed in control cells after AdSt2b2-shRNA infection (Fig. 9A). AdSt2b2-shRNA infection also decreased INV protein expression in TNFα-treated cells from 330 to 130% of control levels (Fig. 9B).

Effects of Inhibiting TNFα Signaling on TPA-Induced Enhancement of St2b2 and INV Expression in NME Cells To verify that TNFα signaling mediates the TPA-induced enhancement of St2b2 and INV expression, TNFα-siRNA was introduced into NME cells. TNFα protein levels in the...
medium increased to 45 pg/ml after TPA treatment (control cells produced 21 pg/ml). On the other hand, TNFα protein levels did not increase after TPA treatment in the presence of TNFα-siRNA; TPA-treated cells that were also exposed to 90 nM TNFα-siRNA produced 22 pg/ml TNFα (Fig. 10A). St2b2 protein levels in TPA-treated cells (190% of levels detected in control cells) decreased to 100% of those observed in control cells after treatment with 90 nM TNFα-siRNA (Fig. 10B). TP A-induced enhancement of INV expression (150% of levels in control cells) also decreased to 100% of that in control cells after treatment (Fig. 10C).

To confirm further the involvement of TNFα in TPA-induced enhancement of St2b2 and INV expression, St2b2 and INV protein levels were examined in NME cells treated with TNFR-Ab and TPA. TNFα (8 μg/ml) reduced TPA-induced enhancement of St2b2 expression (170% of that in control TNFR-Ab(-) and TPA(-) cells) to 70% of that observed in the control cells (Fig. 11A). The TPA-induced enhancement of INV expression (160% of that in control cells) was also reduced to 110% of that detected in control cells after treatment (Fig. 11B). No marked difference was observed in extracellular TNFα protein levels before and after treatment (data not shown).

Effects of NF-κB Inhibition on TNFα-Induced Enhancement of St2b2 and INV Expression in NME Cells

NF-κB, which is activated through TNFα–TNFR signaling,
plays a critical role in cell differentiation. To examine NF-κB during TPA-induced enhancement of St2b2 and INV expression, an NF-κB inhibitor, BAY 11-7082, was administered to NME cells. BAY 11-7082 (10 μM) decreased TNFα-induced enhancement of St2b2 expression (140% of that in control BAY 11-7082 (−) and TNFα (−) cells) to 100% of that in control cells (Fig. 12A). TNFα-induced enhancement of INV expression (140% of levels observed in control cells) decreased to 80% of levels in control cells after treatment (Fig. 12B).

**DISCUSSION**

CS is involved in epidermal differentiation and, at high concentrations, causes epidermal hyperplasia. Skin CS levels are believed to be maintained by both Ch-ST and SSase. In the present study, TPA-mediated CS accumulation in the epidermis occurred in parallel with increased Ch-ST activity, without changes in SSase activity (Table 2). Expression of St2b2 mRNA and protein in mouse skin increased after TPA treatment (Fig. 2). These results were consistent with the idea that TPA-mediated CS production mainly results from enhanced St2b2 expression.

To assess St2b2 functions during TPA-induced epidermal hyperplasia and differentiation, St2b2 gene expression was knocked down using shRNA in mice. TPA-induced epidermal hyperplasia was clearly blocked by St2b2-shRNA (Fig. 5). Decreases in INV and St2b2 expression were also observed in epidermis tissue infected with AdSt2b2-shRNA (Fig. 6). These results suggested that an increase in St2b2 levels is a prerequisite for TPA-induced epidermal hyperplasia.

PKC activation is involved in the differentiation of many cell lines, including epidermal cells, and is a key process during TPA-induced epidermal differentiation. TPA was reported to activate PKC via direct binding to PKC; this study suggested that direct binding triggers TPA-induced epidermal differentiation, but the mechanism for PKC activation during TPA-induced epidermal differentiation has not been elucidated. In the present study, TPA-induced epidermal differentiation and hyperplasia were suppressed by inhibition of increases in St2b2 expression, the primary cause of increased CS concentrations after TPA treatment (Figs. 5, 6). CS may be involved in epidermal differentiation through the activation of PKC. Combined with our present results obtained with a PKC inhibitor (Fig. 4), TPA-induced PKC activation appears to result from increased St2b2 expression and consequent elevations in CS concentrations.

Our results suggest that TPA-induced increases in CS concentrations cause hyperplasia (Figs. 3, 5, 6). TPA stimulates proliferation and differentiation in skin epidermis via activation of several forms of PKC. Knocking down St2b2 expression, and consequent inhibition of CS biosynthesis, likely inhibited epidermal differentiation and blocked hyperplasia. Previous reports showed that successive doses of CS inhibited the tumor-promoting effects of TPA in mouse skin. Chida et al. also reported that a single large dose of CS (400 μg) caused desquamation but not proliferation. Together, these results suggest that repeated high doses of CS inhibit TPA-induced tumorigenesis.

The anti-inflammatory agent indomethacin decreased TPA-mediated increases in Ch-ST activity and epidermal hyperplasia. This suggested that inflammatory cytokines mediate the enhanced St2b2 expression after TPA treatment. In fact, TNFα did not promote INV expression in the absence of increased St2b2 expression (Fig. 9). In addition, TPA-induced St2b2 expression diminished when TNFα signaling was suppressed (Fig. 10). Inhibiting TNFα binding to TNFR also reduced St2b2 expression (Fig. 11). These results indicated that TNFα triggers St2b2 expression in TPA-induced epidermal hyperplasia and differentiation.

NF-κB is known to participate in TPA-induced epidermal hyperplasia. In the present study, an NF-κB inhibitor suppressed TNFα-induced expression of cornified envelope proteins and St2b2 (Fig. 12). These results suggest that TNFα-NF-κB signaling regulates St2b2 expression during TPA-induced epidermal hyperplasia. In fact, a putative NF-κB binding sequence was found in the 5′-flanking region of
the St2b2 gene (DNASIS software version 3.0, Hitachi Software Engineering Co., Tokyo, Japan). Therefore, St2b2 expression may increase owing to NF-κB activation of gene transcription. We also identified putative NF-κB binding sequences in the 5′-flanking region of the human CH-ST ST2B1. In addition, ST2B1 expression increased when the LS174T human epithelial colon carcinoma cell line was treated with TPA or TNFα (unpublished results, Matsuda et al.). Thus, NF-κB may be involved in TPA-induced enhancement of ST2B expression in humans as well. 

TNFα-siRNA decreased TNFα levels in the medium but not the expression of St2b2 or INV protein in the group not treated with TPA (Fig. 10). Neither TNFR-Ab nor BAY 11-7082 significantly inhibited basal expression levels of St2b2 and INV (Figs. 11, 12). These results suggested that TNFα–NF-κB signaling does not drive constitutive expression of St2b2 and INV, although the mechanisms underlying basal expression of these proteins remain unknown.

In conclusion, St2b2 is required for TPA-induced epidermal hyperplasia, a relationship that involves TNFα–NF-κB signaling. Our hypothesis for the mechanism of TPA-induced epidermal differentiation is shown in Fig. 13. TPA increases TNFα levels to activate NF-κB following binding to TNFR. Then, NF-κB activates St2b2 gene transcription, leading to an increase in the levels of St2b2 and epidermal CS. Finally, increased epidermal CS concentrations enhance the expression of cornified envelope proteins, such as INV, through PKC activation.

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REFERENCES