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Cholesterol sulfate induces expression of the skin barrier protein filaggrin in normal human epidermal keratinocytes through induction of RORα

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ABSTRACT

Cholesterol sulfate is abundant in the human epidermis and is a putative natural ligand for retinoic acid receptor-related orphan receptor alpha (RORα). Although direct binding of cholesterol sulfate is expected to activate RORα, cholesterol sulfate can also induce RORα expression and increase RORα target gene expression. The purpose of this study was to determine whether cholesterol sulfate induces profilaggrin expression, a precursor of the barrier protein filaggrin in the epidermis, through activation of RORα by directly binding to RORα, or through increased RORα expression. Immunohistochemical and polymerase chain reaction (PCR) analyses showed that RORα was expressed in normal human epidermal keratinocytes (NHEKs) and that its expression increased during keratinocyte differentiation in parallel with that of profilaggrin and cholesterol sulfotransferase, which catalyzes the synthesis of cholesterol sulfate. Exogenous cholesterol sulfate significantly increased both RORα and profilaggrin expression in NHEKs, whereas no effect on profilaggrin expression was observed in cells in which RORα was knocked down with small interfering RNA (siRNA). Additionally, a luciferase reporter gene assay revealed that exogenous RORα dose-dependently increased the activity of the profilaggrin gene promoter even in the absence of cholesterol sulfate, and that this response involves activator protein-1. In conclusion, the results of this study indicate that cholesterol sulfate induces filaggrin expression through increased RORα expression. Further studies are required to fully elucidate the mechanisms involved.

1. Introduction

The skin is an effective physical barrier that protects the body from exogenous pathogens. The outer component of the skin, the epidermis, forms a protective covering, its predominant cell type being the keratinocyte. It consists of four layers: basal, spinous, granular, and cornified layers (in that order from the basement membrane outwards) [1]. Each layer represents a distinct stage of differentiation. In the basal layer, keratinocytes are supplied by their cell division and gradually move towards the skin's surface through a series of differentiation events [2]. When keratinocytes reach the granular layer, their keratin synthesis ceases and they instead begin producing various barrier proteins, including filaggrin [1], which is initially synthesized as the large precursor protein profilaggrin [3]. Profilaggrin is extensively phosphorylated and packaged into keratohyalin granules in cells of the granular layer. While phosphorylated profilaggrin is protected from proteolysis, dephosphorylated profilaggrin is not and its cleavage yields filaggrin monomers [3]. In the cornified layer, filaggrin becomes associated with keratin intermediate filaments, resulting in aggregation of the latter [4]. In the final step of the cell migration process, epidermal keratinocytes and their products are released from the cornified layer in a process known as desquamation [2].

Previous studies have suggested that the expression of profilaggrin in human epidermal keratinocytes is closely linked to cholesterol sulfate and RORα, a nuclear receptor expressed in various tissues [5,6]. Although expression of RORα has not been demon-

Abbreviations: RORα, retinoic acid receptor-related orphan receptor alpha; SULT2B1b, cholesterol sulfotransferase; AP-1, activator protein-1; NHEKs, normal human epidermal keratinocytes; KGM, keratinocyte growth medium; RORE, ROR response element; sg, staggerer.

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strated in human skin [5,7], cholesterol sulfate dose-dependently increases the formation of filaggrin in primary mouse keratinocytes [8]. Additionally, expression of SULT2B1b, the enzyme responsible for cholesterol sulfate synthesis, has been detected in the granular layer of the human epidermis where cholesterol sulfate accumulates [9–11]. Because cholesterol sulfate binds to the ligand-binding domain of RORα with high affinity, and increases its transcriptional activity more strongly than cholesterol or any other cholesterol derivative [12,13], cholesterol sulfate likely increases profilaggrin expression through activation of RORα. However, recent research showed that cholesterol sulfate can induce RORα expression, and that increased RORα expression itself can enhance its target gene expression regardless of the presence of cholesterol sulfate [14].

The purpose of the present study was to determine whether cholesterol sulfate induces filaggrin expression through activation of RORα by binding to RORα, or through increased RORα expression.

2. Materials and methods

2.1. Immunohistochemistry

Expression of RORα, SULT2B1b, and filaggrin in normal human skin was examined by immunohistochemistry, as described previously [11]. A scalp biopsy was obtained from a healthy volunteer. Tissue sections were treated with anti-RORα (Santa Cruz Biotechnology, Santa Cruz, CA), anti-SULT2B1b, and anti-filaggrin (Biomedical Technologies, Stoughton, MA). Cell nuclei (SULT2B1b and filaggrin staining) were counterstained with Mayer's hematoxylin (Muto Pure Chemicals, Tokyo, Japan).

2.2. Culture of NHEKs

NHEKs were obtained from individuals who had provided informed consent and all procedures were in adherence with the principles of the Declaration of Helsinki (lot No. 4F1363; Cambrex, East Rutherford, NJ). Cells were seeded in flasks that had been collagen-coated. NHEKs were maintained in KGM-2 medium (Cambrex) containing 0.05 mM $CaCl_2$ and supplements (bovine pituitary extract, epidermal growth factor, insulin, hydrocortisone, transferrin, epinephrine, gentamicin sulfate, and amphotericin B). KGM-2 medium has a total sulfur concentration of 3.653 μM (comprising 2.743 μM $FeSO_4$, 0.0169 μM $CuSO_4$, 0.8916 μM $ZnSO_4$, and 0.001124 μM $MnSO_4$). At the third passage, cells were seeded to 6-well plates (4.0×10^5 cells/well) and 24 h later received fresh medium containing 1.2 mM Ca^{2+} to initiate their differentiation (day 0). The culture medium was replaced every 48 h. On days 1, 3, 5, 7, and 9, cells were harvested and their RNA extracted using an Absolutely RNA RT-PCR Miniprep kit (Stratagene, La Jolla, CA).

2.3. Analysis of RORα isoforms mRNA expression by reverse transcription–polymerase chain reaction (RT–PCR)

Extracted RNA samples were treated with DNase supplied with the SuperScript™ III kit (Invitrogen, Carlsbad, CA), which was subsequently used to reverse transcribe 2.0 μg of total RNA from NHEKs. Aliquots of diluted cDNA (5 μL) were used as templates in PCR reactions. PCR was performed as previously described with minor modifications [15].

2.4. Real-time PCR

Total RNA was prepared from cultured NHEKs and reverse transcribed as described above. Real-time PCR was performed using a fluorescence temperature cycler (7500 Real-Time PCR System) with the dye SYBR Green I (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The data generated were analyzed using 7500 System Sequence Detection Software Version 1.2.3 (Applied Biosystems). The following primers were used: SULT2B1b, 5′-CATCTCGGAAATCAGCCAGAAG-3′ (sense) and 5′-ACCCACAATGGTCTCACACCAG-3′ (antisense); profilaggrin, 5′-GCAGGCTCCTTCAGGCTACATTC-3′ (sense) and 5′-TGTGCTTTCTGTGCTTGTGTCC-3′ (antisense). β-Actin, 5′-CTGGCACCACACCTTCTACAATG-3′ (sense) and 5′-AATGTCACGCACGATTTCCCGC-3′ (antisense). RORα primers were designed using the published sequences of human RORα1, RORα2, RORα3, and RORα4 cDNAs (GenBank ID: U04897, U04898, U04899, and L14611, respectively). The following universal primers were targeted to a sequence common to RORα1–4: 5′-GGCTTCTTTCCCTACTGTTCGTTC-3′ (sense) and 5′-ACCTCCCGCTGCTTGTTTTG-3′ (antisense) (product size 200 bp). The signals for RORα, SULT2B1b, and profilaggrin were normalized to that of β-actin, and expression levels in each test sample were expressed relative to untreated controls. The primers used to quantify profilaggrin mRNA levels were targeted to a region of the profilaggrin mRNA upstream of the region containing multiple tandem filaggrin sequences. Thus, the real-time PCR analysis quantified copies of profilaggrin and not filaggrin.

2.5. RNA interference

Double-stranded 21-nucleotide small interfering RNAs (siRNAs) specific for human RORα [5′-GGAAAGAGUUUAUGUUCUAUU-3′ (No. D-003440-01)] and scrambled control siRNA (No. D-001210-02) (Dharmacon, Lafayette, CO) were diluted and stored according to the manufacturer's instructions. Twenty-four hours after seeding, passage 3 NHEKs were transfected with siRNA [5 nM in medium containing 0.1% DharmaFECT 2 transfection reagent (Dharmacon)] and cultured for up to 4 days in medium containing various concentrations of cholesterol sulfate (Sigma–Aldrich, St. Louis, MO) or cholesterol (negative control). Then their RNA was extracted as described above. Cytotoxicity arising from siRNA treatment was assessed 48 h post-transfection in a viability assay performed using alamarBlue reagent (BioSource International, Camarillo, CA).

2.6. Luciferase reporter gene assay

The 5′-upstream region of the human profilaggrin gene (Human Genome Resources at NCBI) was amplified by PCR, which was performed with appropriate sense and antisense primers flanked by SacI and HindIII restriction sites and using human genomic DNA (Clontech) as the template. The 2460-bp PCR product (PD0) comprises nucleotides −2430 to +30 relative to the exon 1 initiation codon. This sequence contains both an RORE (AGGTCA) at positions −2363 to −2358, and an activator protein 1 (AP-1)-binding motif (ATGAATCA) at −77 to −70. The PCR product was gel-purified (Qiagen, Hilden, Germany) and subcloned into the pCR2.1 TA cloning vector (Invitrogen). After nested PCR with PfuUltra DNA polymerase (Stratagene, La Jolla, CA), the PCR products were double-digested, subcloned into the pGL3 firefly luciferase expression vector (Promega) at the SacI and HindIII restriction sites, and sequenced.

We generated six profilaggrin promoter constructs (PD1-6) through serial deletions, using PD0 as the template, and subcloned them into the pGL3 firefly luciferase expression vector at the SacI and HindIII restriction sites. PD1-5 lacked the RORE, but retained the AP-1-binding motif. The sequences of PD1-5 were as follows: PD1, −1189/+30; PD2, −759/+30; PD3, −566/+30; PD4, −293/+30; and PD5, −132/+30. PD6 (−2430/+30AP-1del), which lacked the AP-1-binding motif, was generated by site-directed mutagene-

sis, performed using a QuickChange II XL site-directed mutagenesis kit (Stratagene). RORα expression vectors were generated by subcloning cDNAs for human RORα1 (Origene; cat. No. SC123126) and RORα4 (Ultimate ORF clone; Invitrogen) into the expression vectors pCMV-XL5 and pcDNA3.1(+).

A luciferase reporter gene assay was performed as follows. HeLa cells were seeded to 6-well plates at a density of 2.5×10^5 cells/well. The next day, the cells were transfected with one of the seven constructs (PD0-6) and an RORα expression vector using Lipofectamine 2000 reagent (Invitrogen) in the presence or absence of a Renilla luciferase plasmid (Promega). Forty-eight hours later, the cells were harvested and firefly luciferase and Renilla luciferase activities were measured using a Dual-Luciferase Reporter Assay kit (Promega). In some experiments, the culture medium was replaced with serum-free medium (UltraCHO Medium, Lonza, NJ) 12 h before gene transfection to ensure that no cholesterol sulfate was present in the medium. All experiments were performed in triplicate and repeated twice.

2.7. Statistical analyses

Results are expressed as the mean ± SE. Mann-Whitney *U* tests were used to identify statistically significant changes. A *p*-value of <0.05 was deemed to indicate statistical significance.

3. Results

3.1. RORα expression in human skin

RORα, SULT2B1b, and filaggrin were detected in the outer parts of the suprabasal region of the normal human epidermis in immunohistochemical analyses performed using specific antibodies. Both RORα and SULT2B1b were weakly detected in the spinous layer and stained more intensely in the granular layer (Fig. 1). Filaggrin was expressed both in the granular layer and the lower part of the cornified layer. Moreover, while RORα localized exclusively to cell nuclei, SULT2B1b and filaggrin were detected in extranuclear regions. RT–PCR confirmed the expression of only the α1 and α4 isoforms of RORα in human skin. Differentiated NHEKs expressed the α4 isoform of RORα, but not the α1 isoform (data not shown).

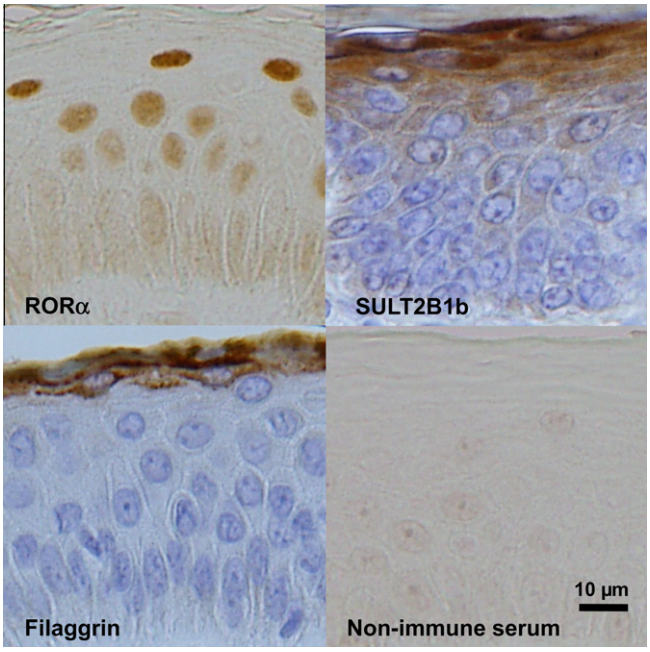

Fig. 1. Immunohistochemical detection of RORα, SULT2B1b, and filaggrin in normal human scalp skin. The skin specimen was fixed in paraformaldehyde and embedded in paraffin. Sections (6 μm thick) were stained with antibodies specific for RORα, SULT2B1b, and filaggrin, or non-immune serum.

3.2. RORα expression during the differentiation of NHEKs

Expression of RORα, SULT2B1b, and profilaggrin mRNAs in NHEKs increased significantly and progressively above that in cells treated with 0.05 mM Ca^{2+} during 1.2 mM Ca^{2+}-induced differentiation (Fig. 2). Furthermore, the patterns of expression of these mRNAs were similar to one another.

3.3. Effect of exogenous cholesterol sulfate on RORα expression in NHEKs

To investigate whether the effect of cholesterol sulfate could be attributable to a change in the RORα expression level, we examined the expression of RORα in NHEKs treated with exogenous cholesterol sulfate. Expectedly, exogenous cholesterol sulfate dose-dependently increased the expression level of RORα mRNA; at 40 μM cholesterol sulfate, the expression of RORα mRNA was 2.3-fold the expression in the vehicle control ($p < 0.05$, Fig. 3A).

3.4. Effect of RORα knockdown on NHEKs profilaggrin expression

To examine the role of RORα in the regulation of profilaggrin expression, we performed knockdown experiments. Transfection of NHEKs with siRNA targeted to RORα, which produced $a > 96\%$ reduction in RORα mRNA expression and comparable suppression of RORα protein expression (data are not shown). As expected, exogenous cholesterol sulfate dose-dependently increased profi-

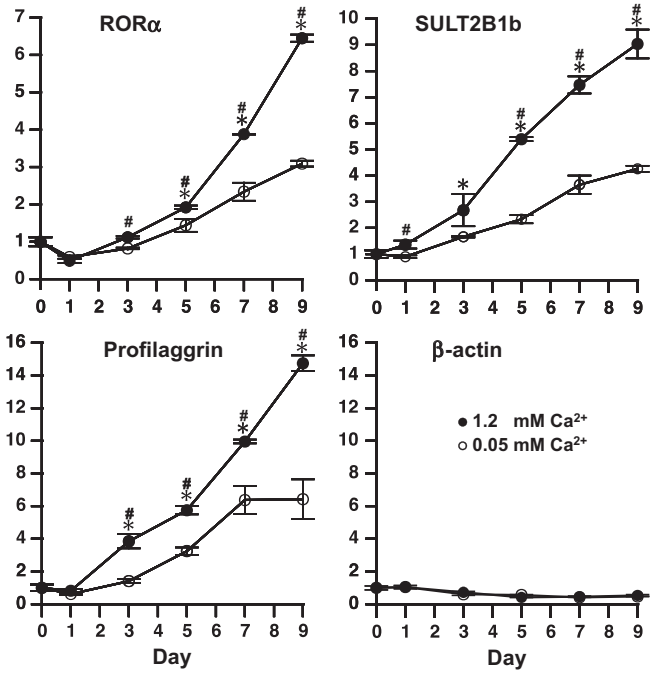

Fig. 2. Expression of RORα, SULT2B1b, profilaggrin, and β-actin mRNAs during Ca^{2+}-induced differentiation of NHEKs. NHEKs were incubated with medium containing 0.05 mM Ca^{2+} or 1.2 mM Ca^{2+} for up to 9 days and harvested at the indicated times. mRNAs were analyzed by real-time PCR. Data represent the mean ± SE ($n = 3$) of fold changes in mRNA copy number, normalized to β-actin and quantified relative to the value at day 0. $^{*}p < 0.05$ vs. day 0 and $^{\#}p < 0.05$ vs. 0.05 mM at the same time point (Mann–Whitney *U*-test).

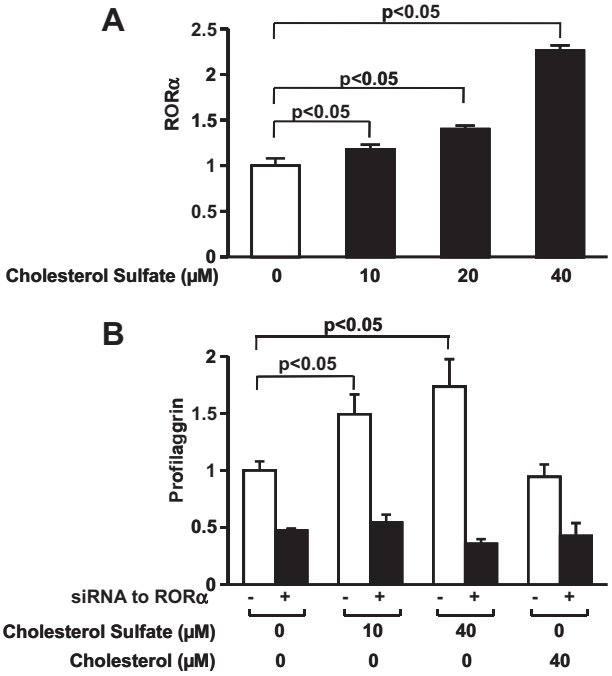

Fig. 3. (A) Effect of exogenous cholesterol sulfate on RORα mRNA expression in NHEKs. Cholesterol sulfate was added to the culture medium at the indicated concentrations. After 4 days, cells were harvested and RORα mRNA levels were quantified by real-time PCR. Data represent the mean ± SE ($n = 3$) of the fold changes in mRNA copy number, normalized to β-actin and quantified relative to a vehicle control (0.4% methanol). The *p*-values (vs. control) were calculated using the Mann–Whitney *U*-test. (B) Effect of exogenous cholesterol sulfate and cholesterol on profilaggrin mRNA expression in RORα-knockdown cells. NHEKs were transfected with siRNA specific for RORα (5 nM) and cultured for 4 days in medium containing cholesterol sulfate or cholesterol at the indicated concentrations. Cells were then harvested and profilaggrin mRNA levels quantified by real-time PCR. Data represent the mean ± SE ($n = 3$) of fold-changes in mRNA copy number, normalized to β-actin and quantified relative to a vehicle control (0.4% methanol). The *p* values (vs. control) were calculated using the Mann–Whitney *U* test.

laggrin mRNA expression in NHEKs (Fig. 3B, white bars). In contrast, exogenous cholesterol sulfate had no effect on profilaggrin mRNA expression in NHEKs in which RORα mRNA expression had been knocked down (Fig. 3B, black bars).

Under the conditions used in the knockdown experiments described above, the rates of cell death caused by siRNA transfection, as assessed at 48 h post-transfection using alamarBlue reagent, were less than 5% in all cases.

3.5. Luciferase reporter gene assay

The luciferase assay showed that RORα expression increased profilaggrin promoter activity in a dose-dependent manner in the absence of cholesterol sulfate (Fig. 4A). RORα1 and RORα4 strongly promoted transcription of the profilaggrin gene to a similar extent. In the control cells, neither empty pCMV-XL5 nor pcDNA3.1(+) had any effect on the transcription of the profilaggrin gene.

3.6. Profilaggrin promoter characterization

We examined the role of the profilaggrin promoter in detail using various constructs. The promoter activity of PD1, the longest profilaggrin gene construct lacking the RORE, was 30% lower than that of the full-length construct (PD0). In shorter constructs lacking the RORE (PD2-5), the promoter activity declined in parallel with a reduction in the length of the 5′-upstream region (Fig. 4B). Notably, the construct that retained the RORE but lacked the AP-1-binding site (PD6) exhibited almost complete loss of luciferase activity.

To examine the effect of exogenous cholesterol sulfate on the profilaggrin promoter, we added cholesterol sulfate to the culture medium and performed the same luciferase activity assay as described above. Exogenous cholesterol sulfate (up to 40 μM) did not result in any additional increase in profilaggrin promoter activity, even in the PD0 construct, which retained both RORE and AP-1 sites. To ensure that no cholesterol sulfate was present in the medium, we repeated the same experiments using serum-free medium. However, the addition of cholesterol sulfate had no effect on profilaggrin expression (data not shown).

4. Discussion

In the present study, we indicated that cholesterol sulfate induces filaggrin expression through increased RORα expression. We found that exogenous cholesterol sulfate significantly increased RORα mRNA expression in NHEKs (Fig. 3A). Moreover, exogenous cholesterol sulfate significantly increased profilaggrin mRNA expression in NHEKs, whereas no effect on profilaggrin was observed in cells in which RORα was knocked down with siRNA (Fig. 3B). In addition, a luciferase reporter gene assay revealed that RORα dose-dependently increased the transcriptional activity of a profilaggrin gene promoter construct even in the absence of cholesterol sulfate and that cholesterol sulfate could not increase it (Fig. 4A).

RORα was identified as an orphan nuclear receptor. Extensive research has shown that it modulates the expression of various genes linked to tissue differentiation, atherosclerosis, inflammation, and bone metabolism. The sg mutation, a spontaneous 122-bp deletion in the RORα gene that produces a shift in the translational reading frame, results in the expression of an inactive form of RORα. sg/sg mice display not only ataxia and cerebellar degeneration, but also suffer from a range of aging-associated diseases, including atherosclerosis, immunodeficiency, and osteoporosis [16,17]. Because the mouse genes encoding apoA-I (major apolipoprotein of high-density lipoprotein) and apoC-III (apolipoprotein inhibiting lipoprotein lipase activity) contain ROREs in their promoters [18,19], atherosclerosis in sg/sg mice may partly stem from impaired regulation of reverse cholesterol transport and/or triglyceride-rich lipoprotein metabolism due to the loss of RORα activity. Furthermore, RORα exerts anti-inflammatory effects by inducing the expression of I-κBα, an inhibitor of the transcription factor NF-κB, thereby reducing both NF-κB activity and inflammatory cytokine production [16].

Recent research has shown that RORα does not always require ligand binding for increasing the transcriptional activities of target genes. Cholesterol sulfate treatment significantly induced the mRNA expression of RORα in human endometrial cells [14]. Additionally, RORα significantly activated the promoter of human NR1D1, one of the genes regulated by RORα, regardless of the presence of cholesterol sulfate [14]. Their observation is very similar to ours (Figs. 3 and 4). The luciferase assay with HeLa cells clearly showed that cholesterol sulfate neither activated RORα nor directly increased profilaggrin expression. In our previous study, we exposed cultured NHEKs to 1.2 mM Ca^{2+} to induce differentiation and found that SULT2B1b expression and activity markedly increased [11]. This is consistent with the concentration of cholesterol sulfate being highest (about 5% of the total lipid content) in the granular layer of the epidermis [20]. It is likely that cholesterol sulfate accumulates during differentiation [10] and increases RORα expression. Instead of being activated by cholesterol sulfate, RORα increases target gene expression on its own. Our hypothesis can explain the fact that SULT2B1b co-localized with

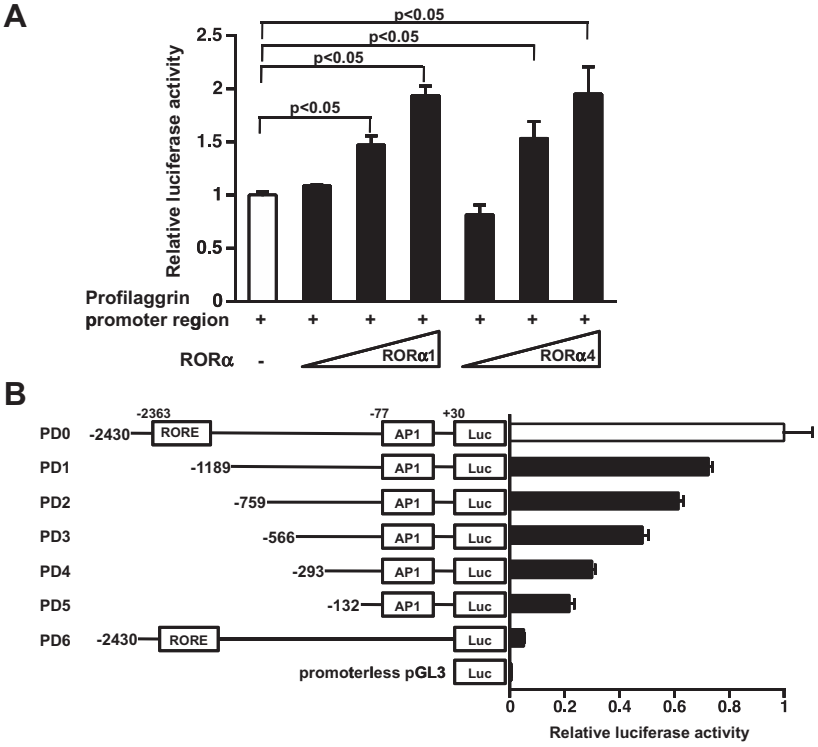

Fig. 4. (A) Luciferase reporter gene assay. A construct comprising the 5′-upstream region of the profilaggrin gene (nucleotides −2430 to +30) was fused to a firefly luciferase reporter gene. The resulting vector (0.8 μg) was co-transfected along with 0.8 μg of the RORα1 or RORα4 expression vector into HeLa cells. Firefly luciferase activity was normalized to Renilla luciferase activity. The luciferase activity was calculated relative to that of cells transfected with the empty expression vector lacking RORα sequences. Data represent the mean ± SE of multiple replicates. The RORα expression vector DNA was serially diluted (3-fold) prior to transfection, as indicated. (B) Profilaggrin promoter characterization. We generated six constructs (PD1–PD6) carrying sequences from the 5′-upstream region of the profilaggrin gene through serial deletions of sequences from the full-length construct PD0, which comprises nucleotides −2430 to +30. Both PD0 and PD6 retained the RORE, while only PD6 lacked the AP-1 site. The truncated constructs PD1–PD5 retained the AP-1 site but lacked the RORE. The constructs were fused to the firefly luciferase reporter gene, and the resulting vectors (0.8 μg) were co-transfected along with 0.8 μg of the RORα4 expression vector into HeLa cells. Firefly luciferase activity was normalized to Renilla luciferase activity. Luciferase activity was calculated relative to that of cells transfected with the vector carrying PD0. Data represent the mean ± SE of multiple replicates.

RORα and filaggrin (Fig. 1), and that SULT2B1b mRNA levels increased in parallel with those of RORα and profilaggrin during Ca^{2+}-induced differentiation of NHEKs (Fig. 2). Exogenous cholesterol sulfate induced profilaggrin mRNA expression dose-dependently and far more potently than exogenous cholesterol (Fig. 3B). Knockdown of RORα reduced profilaggrin mRNA expression. Exogenous cholesterol sulfate had no effect on profilaggrin mRNA expression in RORα-knockdown cells (Fig. 3B).

To determine whether cholesterol sulfate increases profilaggrin expression through activation of RORα, increased RORα expression, or both, we used HeLa cells instead of NHEKs for the luciferase assay. Cholesterol sulfate could increase profilaggrin expression in NHEKs by indistinguishable mechanisms, that is, (1) the activation of RORα, or (2) induction of RORα expression, or (3) both. NHEKs express RORα intrinsically, and exogenous cholesterol clearly increased RORα expression in these cells (Fig. 3A). On the other hand, HeLa cells do not express RORα intrinsically, and we do not have to concern about any interference by an endogenous RORα in the luciferase assay. In the present study, the RORα gene was transfected into HeLa cells, and driven by the human cytomegalovirus promoter. Unlike RORα gene of keratinocytes, this transfected gene is not regulated by cholesterol sulfate, and express RORα transiently but stably. In fact, cholesterol sulfate did not increase RORα expression in our preliminary experiment for Fig. 4A. In this assay system, profilaggrin expression does not increase after addition of cholesterol sulfate if an increase in RORα is crucial for the induction of profilaggrin expression.

RORα likely binds to the profilaggrin gene promoter and increases profilaggrin expression by interacting with AP-1. Although promoter constructs lacking the RORE exhibited lower luciferase activity than the full-length promoter construct (Fig. 4B, PD0-5), they retained significant luciferase activity as long as the AP-1-binding site was preserved. Both keratinocytes and HeLa cells express AP-1 [21,22]. RORα may associate with AP-1 and enhance profilaggrin gene expression. Similar crosstalk between AP-1 and another nuclear receptor, retinoic acid receptor (RAR), has been described in murine parietal endoderm cells [23].

Despite our extensive experiments on profilaggrin expression, some questions remain. Although induction of RORα by cholesterol sulfate significantly increased expression of the profilaggrin gene, this gene is also known to be regulated by other factors. In cultured human epidermal cells, for example, profilaggrin promoter activity has been suggested to be regulated, at least in part, by retinoic acid and glucocorticoids [24]. The extent to which cholesterol sulfate-induced RORα contributes to the regulation of profilaggrin gene expression should be clarified in future studies.

The results of the present study indicate that RORα is expressed in NHEKs, and that cholesterol sulfate induces filaggrin expression through increased RORα expression. Because cholesterol sulfate and RORα have been co-detected in other tissues, cholesterol sulfate may regulate the local expression of other genes. Further studies are required to more fully determine the physiological actions of cholesterol sulfate.

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