LXR and PPAR activators stimulate cholesterol sulfotransferase type 2 isoform 1b in human keratinocytes

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Abstract **Liver X receptors (LXRs) and peroxisome proliferator-activated receptors (PPARs) are potent regulators of keratinocyte proliferation, differentiation, and epidermal permeability barrier homeostasis. Cholesterol sulfotransferase type 2B isoform 1b (SULT2B1b) is a key enzyme in the synthesis of cholesterol sulfate (CS), a critical regulator of keratinocyte differentiation and desquamation, as well as a mediator of barrier homeostasis. In this study, we assessed the effect of activators of LXR, PPARα, PPARβ/δ, and PPARγ on SULT2B1b gene expression and enzyme activity in cultured human keratinocytes (CHKs). Our results demonstrate that PPAR and LXR activators increase SULT2B1b mRNA levels, with the most dramatic effect (a 26-fold increase) induced by the PPARγ activator ciglitazone. Ciglitazone upregulates SULT2B1b mRNA in a dose- and time-dependent manner. Moreover, the stimulation of SULT2B1b gene expression by LXR and PPAR activators occurs in both undifferentiated and differentiated CHKs. The upregulation of SULT2B1b mRNA by ciglitazone appears to occur at a transcriptional level, because the degradation of SULT2B1b is not accelerated by ciglitazone. In addition, cycloheximide almost completely blocks the ciglitazone-induced increase in SULT2B1b mRNA, suggesting that the transcription of SULTB1b mRNA is dependent on new protein synthesis. Finally, LXR and PPAR activators also increased the activity of cholesterol sulfotransferase. Thus, LXR and PPAR activators regulate the expression of SULT2B1b, the key enzyme in the synthesis of CS, which is a potent regulator of epidermal differentiation and corneocyte desquamation.**—Jiang, Y. J., P. Kim, P. M. Elias, and K. R. Feingold. **LXR and PPAR activators stimulate cholesterol sulfotransferase type 2 isoform 1b in human keratinocytes. *J. Lipid Res.* 2005.** 46: **2657–2666.**

Supplementary key words cholesterol sulfate • liver X receptor • peroxisome proliferator-activated receptor α/δ/γ

Cholesterol sulfate (CS) plays a number of key roles in regulating epidermal function. It is widely recognized as a critical regulator of several keratinocyte corneocyte functions, most notably corneocyte desquamation (1). It also stimulates keratinocyte differentiation via a number of potential pathways, including the activation of protein kinase C (2), indirectly by binding sphingosine (3), and directly by affecting gene transcription (4). CS also reportedly modulates antimicrobial defense (5) and regulates keratinocyte cholesterol synthesis (6). Moreover, CS is catabolized to cholesterol and sulfate in the stratum corneum (SC), a process catalyzed by the enzyme steroid sulfatase (6), and cholesterol is crucial for the formation of the cutaneous permeability barrier (7, 8). Thus, identification of factors that regulate epidermal CS synthesis could be of potential importance for several keratinocyte functions.

CS is synthesized in the basal and spinous layers of the epidermis by the cytosolic enzyme cholesterol sulfotransferase (CSTase), resulting in peak levels of CS in the granular layer of the epidermis (1, 9, 10). CSTase belongs to a superfamily of cytosolic sulfotransferases (SULTs) that catalyze the sulfoconjugation of hormones and neurotransmitters as well as certain drugs and xenobiotics (11, 12). Five families of SULTs have been identified, with SULT2 primarily engaged in the sulfoconjugation of neutral steroids and sterols (13). Cholesterol sulfotransferase type 2B isoform 1b (SULT2B1b) selectively functions as a CSTase (14), and recent studies have shown that this isozyme catalyzes the synthesis of CS in epidermis/keratinocytes (12). SULT2B1b mRNA and protein levels increase during Ca^{2+}-induced terminal differentiation in parallel with an increase in CSTase activity (15). Despite the great importance of CS

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Abbreviations: CHK, cultured human keratinocyte; CS, cholesterol sulfate; CSTase, cholesterol sulfotransferase; LXR, liver X receptor; 25-OH, 25-hydroxycholesterol; PPAR, peroxisome proliferator-activated receptor; 22R, 22(*R*)-hydroxycholesterol; RAR, retinoic acid receptor; RXR, retinoid X receptor; SC, stratum corneum; SULT2B1b, cholesterol sulfotransferase type 2B isoform 1b.

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synthesis for the epidermis, there is a paucity of information on the factors that regulate SULT2B1b expression in keratinocytes. We have previously shown in rat fetal skin explants that CSTase activity increases in fetal epidermis late in fetal development, in parallel with the formation of a differentiated epidermis (16). Interestingly, CS levels increase dramatically when rabbit tracheal epithelial cells undergo squamous differentiation, owing to a 20- to 30-fold increase in CSTase activity in the absence of retinoic acid, and CS levels decline by >25-fold in the presence of exogenous retinoic acid (17). Similar phenomena occur in cultured human keratinocytes (CHKs) treated with retinoic acid (18, 19). Retinoic acid is a natural ligand of the type 2 nuclear hormone receptor retinoic acid receptor (RAR), and activation of RAR affects many important cellular processes, such as proliferation and differentiation (20).

Recent studies have shown that the nuclear hormone receptors peroxisome proliferator-activated receptor (PPAR) α, δ/β, and γ and liver X receptor (LXR) α and β are expressed in the epidermis/keratinocytes (21–27). These receptors heterodimerize with retinoid X receptor (RXR), after which they can activate the expression of many genes, including those involved in lipid metabolism (20, 28, 29). PPARs are activated by fatty acids, fatty acid metabolites, or various drugs (PPARα-fibrates or WY14643, PPARδ/β-GW501516, PPARγ-ciglitazone or troglitazone), whereas LXRs are activated by certain oxysterols [22(*R*)-hydroxysterol, 24,25-hydroxysterol] (30–32). Activation of PPARs and LXRs stimulates keratinocyte differentiation, accelerates the development of the epidermis in fetal rats, and improves epidermal permeability barrier homeostasis in normal adult mice (21, 26, 27, 33, 34). In rat fetal skin explants, we have shown that treatment with both PPARα and LXR activators increases epidermal steroid sulfatase activity (21). Therefore, we assessed whether PPARs and LXR activators regulate CSTase expression and activity in normal human keratinocytes.

MATERIALS AND METHODS

Materials

The LXR activators 22(*R*)-hydroxycholesterol (22R) and 25-hydroxycholesterol (25-OH) and the PPARα activators clofibric acid and WY14643 were purchased from Sigma Chemical Co. (St. Louis, MO). Actinomycin D and cycloheximide were also purchased from Sigma. The PPARγ activator ciglitazone was purchased from Cayman Chemical Co. (Ann Arbor, MI); troglitazone was obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). GI262570 was kindly provided by Dr. Tim Willson (GlaxoSmithKline, Pittsburgh, PA). The PPARδ activator GW501516 was purchased from Calbiochem, Inc. (San Diego, CA). Molecular grade chemicals such as TRI reagent were obtained from either Sigma or Fisher Scientific (Fairlawn, NJ). [α-^{32}P]dCTP (3,000 Ci/mmol) was purchased from New England Nuclear (Boston, MA). [^{32}S]phosphoadenosine phosphosulfate was purchased from Perkin-Elmer (Boston, MA). The Multiprime Labeling System was purchased from Amersham (Amersham, UK). Mini-spin columns (G-50) were purchased from Worthington (Freehold, NJ). Oligo(dT)-cellulose, type 77F, was purchased from Pharmacia (Uppsala, Sweden). Nytran Plus membranes were purchased from Schleicher and Schuell (Keene, NH). Spin-X centrifuge filters were purchased from Corning Costar (Cambridge, MA).

Keratinocyte culture

Human foreskin keratinocytes, second passage, were seeded and maintained in 0.07 mM Ca^{2+} serum-free keratinocyte growth medium (Clonetics, San Diego, CA). Once the cells attached, the culture medium was changed to either 0.03 mM (low) or 1.2 mM (high) Ca^{2+}. In the presence of 1.2 mM Ca^{2+}, keratinocytes were induced to differentiate. In a typical experiment, cells were harvested at 80–100% confluence. Cells were treated with each activator at their optimized concentration at preconfluence (60–70%) in either low (0.03 mM) or high (1.2 mM) calcium conditions. Alternatively, cells were coincubated with individual activator and 10 μg/ml cycloheximide, a classical protein synthesis inhibi-

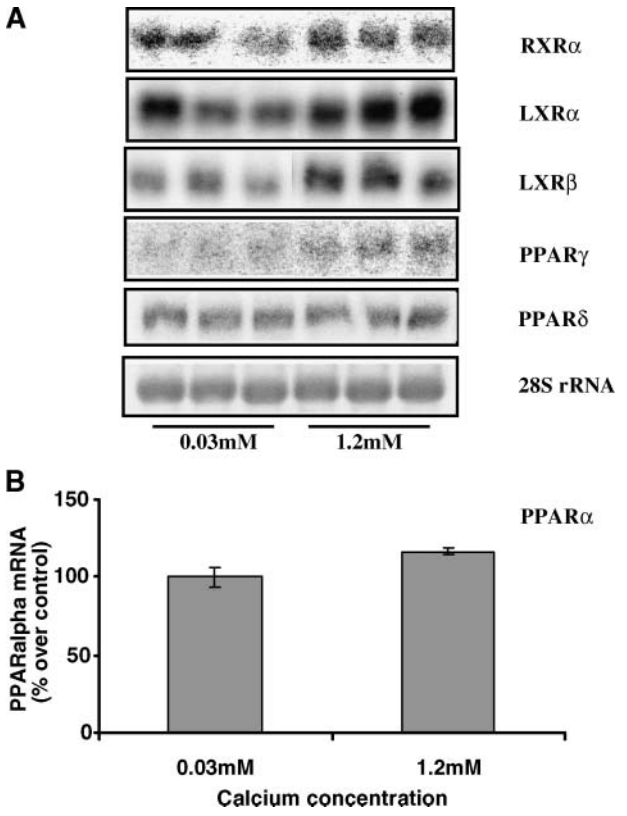

Fig. 1. Basal levels of retinoid X receptor α (RXRα), liver X receptor (LXR), and peroxisome proliferator-activated receptor (PPAR) mRNAs in undifferentiated versus differentiated cultured human keratinocytes (CHKs). Cells at second passage were plated in normal medium containing 0.07 mM Ca^{2+} overnight and then switched to either low-Ca^{2+} (0.03 mM) or high-Ca^{2+} (1.2 mM) medium for 24 h. Total RNA was isolated and subjected to Northern blot analyses. RXRα, LXRα, LXRβ, PPARδ, and PPARγ mRNAs were examined by Northern blot as described in Materials and Methods. PPARα and internal control cyclophilin mRNAs were examined by real-time PCR. Equal loading, the integrity of RNA, and the densitometry value were verified and normalized by ethidium bromide staining of 28S rRNA. The mRNA level of PPARα was normalized by cyclophilin. Data are expressed as percentages of vehicle control and presented as means ± SEM (n = 3) from at least two independent experiments. A representative blot is shown (A) and a plot is shown for PPARα (B).

tor, or they were coincubated with 22R and ciglitazone. Control keratinocytes were treated with vehicle (0.05% ethanol).

Total RNA isolation, cDNA probes, and Northern blotting

Total RNA was isolated using TRI reagent according to the manufacturer's protocol, and Northern blot analysis was performed to measure SULT2B1b mRNA. Briefly, Northern blots were prepared, and equal loading, to test the integrity of RNA, was verified by ethidium bromide staining of 28S rRNA before transfer to Nytran membranes. Blots were then hybridized with ^{32}P-labeled probes of SULT2B1b overnight at 65°C and washed. Alternatively, poly(A$^+$) RNA was isolated using oligo(dT) cellulose, and Northern blot analysis was performed to measure mRNA levels of RXRα, LXRα, LXRβ, PPARδ, and PPARγ. Human Sult2B1b probe was prepared using PCR, and the PCR primer pairs used were: SULT2B1b, upper (5′-TCT CGG AAA TCA GCC AGA AGTT-3′) and lower (5′-ATA GTG GAT GCT CCT CGA CGT-3′). Human RXRα cDNA was a gift from Dr. D. D. Bikle (University of California San Francisco). LXRα and LXRβ were kindly provided by Dr. D. J. Mangelsdorf (University of Texas Southwestern Medical Center, Dallas, TX). PPARδ and PPARγ cDNAs were gifts from Dr. A. Bass (University of California San Francisco). Subsequently, the blots were exposed to X-ray films for various durations to ensure that measurements were performed on the linear portion of the curve, and the target bands were quantitated by densitometry (Bio-Rad Laboratories, Hercules, CA). Some blots were probed with cyclophilin as an internal control signal. The densitometry quantitation of the target gene was adjusted for either cyclophilin or 28S level and expressed as a percentage of control, with the control as 100%.

Quantitative real-time PCR

Total RNA was extracted from keratinocytes using TRIZOL reagent (Sigma). First-strand cDNA for PCR was synthesized using an Advantage RT-for-PCR kit according to the manufacturer's protocol. Briefly, cDNA was synthesized from 1 μg of total RNA using Moloney murine leukemia virus reverse transcriptase with random hexamer primer at 42°C for 60 min. Relative mRNA levels of the target gene (PPARα) and an invariant transcript, cyclophilin, were determined using the Mx3000P™ Real-Time PCR

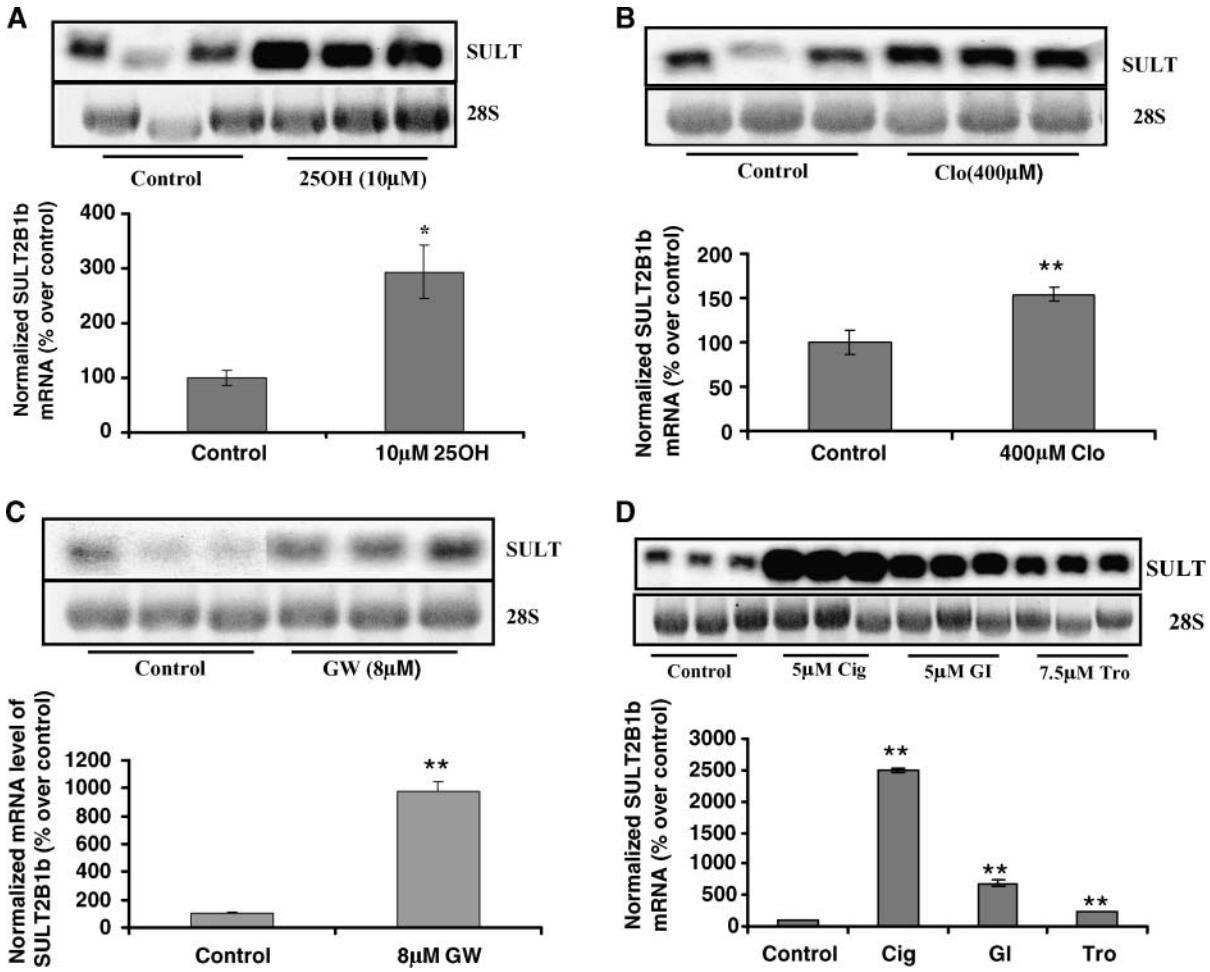

Fig. 2. LXR and PPAR activators increase cholesterol sulfotransferase type 2B isoform 1b (SULT2B1b) mRNA levels in undifferentiated keratinocytes. Cells were incubated in low-Ca^{2+} (0.03 mM) medium in the presence or absence of each LXR/PPAR activator alone for 24 h: 10 μM 25-hydroxycholesterol (25OH) (A); 400 μM clofibrate (Clo) (B); 8 μM GW501516 (GW) (C); or 10 μM troglitazone (Tro), 5 μM GI262570 (GI), or 5 μM ciglitazone (Cig) (D). Total RNA was isolated and subjected to Northern blot analyses. SULT2B1b mRNA was examined as described in Materials and Methods. Equal loading, the integrity of RNA, and the densitometry value were verified and normalized by ethidium bromide staining of 28S rRNA. Data are expressed as percentages of vehicle control and presented as means ± SEM (n = 3) from at least two independent experiments. A representative blot is shown above each plot for the corresponding activator. * $P < 0.05$, ** $P < 0.01$.

System (110 V) with a Notebook Computer (Stratagene Corp., La Jolla, CA). This system uses SYBR Green chemistry for highly accurate quantification of mRNA levels. Individual PCRs were carried out in a mixture of 20 μl containing 20 ng of cDNA, 450 nM forward or reverse primer, and 10 μl of 2× SYBR Green Q-PCR Master Mix (Applied Biosystems, Foster City, CA). The sequences for human PAARα primers were 5′-ATA TCT CCC TTT TTG TGG CTG CTA-3′ (sense) and 5′-TCC GAC TCC GTC TTC TTG ATG A-3′ (antisense). The reaction was performed at 50°C for 2 min, 95°C for 10 min, and then 40 cycles of amplification with melting at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s on Mx3000P 96-well plates (Stratagene). PCR was performed in triplicate and replicated in three independent experiments. Gel electrophoresis and melting curve analyses were performed to confirm correct PCR product sizes and the absence of nonspecific bands. The expression level of PPARα was normalized against cyclophilin using the comparative C_T method according to the manufacturer's protocols.

mRNA stability

Cells were seeded at 60% confluence in low-calcium medium overnight and then incubated with the medium containing 5 μM ciglitazone or vehicle alone (ethanol) for 24 h. Cells were then treated with 2 μg/ml actinomycin D for various periods of time (0, 2, 4, 6, 8, and 10 h). Cells were harvested at each time point and subjected to Northern blot analysis, and the SULT2B1b mRNA levels were determined. The interpolation, calculation, and graphing of mRNA values were performed using SigmaPlot 8.0 software (Systat Software, Inc., Point Richmond, CA).

Enzyme source preparation

After treatment, cells were washed twice with ice-cold PBS and harvested. Cell pellets were subsequently homogenized in a buffer consisting of 10 mM Tris(hydoxymethyl)aminomethane, pH 7.5, 0.15 M sucrose, and 2 mM ethylenediaminetetraacetic acid. Crude homogenates were first centrifuged at 10,000 *g* for 10 min, followed by 100,000 *g* for 60 min, both at 4°C. The CSTase activity was measured in the cytosolic fraction (supernatant after 100,000 *g* spin). Protein concentration was assayed by the method of Bradford (Bio-Rad Laboratories).

CSTase activity assay

CSTase activity was measured as described previously (16, 35). Briefly, cholesterol (100 μg in benzene) was evaporated to dryness and suspended by sonication in 0.05 ml of homogenization buffer. [^{32}S]phosphoadenosine phosphosulfate (50,000 cpm/tube in 0.025 ml) was then added to an equal volume of the enzyme preparation (50–100 μg). Reactions were carried out at 37°C for 1 h and were terminated by the addition of 2.0 ml of chloroform-methanol (2:1, v/v) and 1.0 ml of chloroform-methanol-water-KCl (15 ml:240 ml:235 ml:35.5 g), forming the lower and upper phases, respectively, to separate the substrate from sulfated product. The upper aqueous phase was saved in a separate tube, and the lower phase was extracted once more with 1.0 ml of upper phase. The upper phase was discarded, and an aliquot of the lower organic phase containing the sulfated product was then dried and counted by scintillation spectrophotometry.

Statistics

All data are expressed as means ± SEM. Comparison between two groups was undertaken using two-tailed and unpaired *t*-tests. In the multigroup experiments, data were compared by one-way ANOVA and Tukey's test. Differences in values were considered significant at $P < 0.05$.

RESULTS

The steady-state expression levels of LXRs and PPARs in undifferentiated versus differentiated CHKs

To compare the basal levels of LXR and PPAR expression in undifferentiated versus differentiated CHKs, we measured the mRNA levels of RXRα, LXRα, LXRβ, PPARδ, and PPARγ by Northern blot (**Fig. 1A**) and PPARα by real-time PCR analysis (Fig. 1B). As reported by others, RXRα and PPARγ mRNA levels increase with keratinocyte differentiation (23, 36, 37). In addition, both LXRα and LXRβ mRNAs are expressed in CHKs (34), and in this study we show that their levels increase with calcium-induced keratinocyte differentiation (Fig. 1A). In contrast, PPARδ and PPARα mRNA levels do not significantly increase with short-term (24 h) differentiation (Fig. 1A, B). These results demonstrate that these receptors are expressed in CHKs and in some instances are regulated by keratinocyte differentiation.

LXR and PPAR activators increase SULT2B1b mRNA levels in undifferentiated keratinocytes

We next measured the changes in mRNA levels of SULT2B1b by Northern blot analysis in the second passage of undifferentiated CHK treated with LXR and PPAR activators. Before these experiments, a set of preliminary studies was conducted to optimize the dose for each activator. As depicted in **Fig. 2**, activators of LXR, PPARα, PPARβ/δ, and PPARγ all upregulate SULT2B1b mRNA significantly. For LXR activators, we used two natural ligands, 22R and 25-OH. 25-OH (10 μM) increased SULT2B1b mRNA

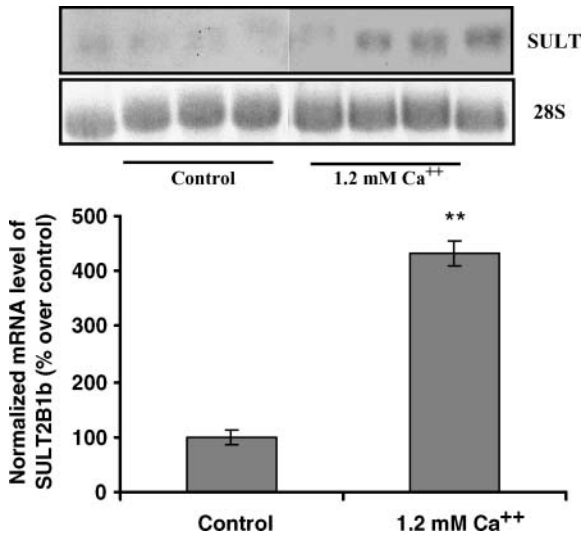

Fig. 3. Increase in SULT2B1b mRNA levels by high Ca^{2+}. Cells were incubated with either low-Ca^{2+} (0.03 mM) or high-Ca^{2+} (1.2 mM) medium for 24 h. Total RNA was isolated and subjected to Northern blot analyses. SULT2B1b mRNA was examined as described. Equal loading, the integrity of RNA, and the densitometry value were verified and normalized by ethidium bromide staining of 28S rRNA. Data are expressed as percentages of low-Ca^{2+} control and presented as means ± SEM (n = 3) from two independent experiments. A representative blot is shown above the plot. ** $P <$ 0.01.

levels by 2.9-fold (Fig. 2A), and 22R increased them by 7.1-fold (data not shown). Similarly, an activator of PPARα, clofibric acid, also modestly increased SULT2B1b mRNA levels (39.9%) at 400 μM (Fig. 2B), but the change induced by a lower concentration (200 μM) did not archive statistical significance (data not shown). In contrast, a 9.8-fold increase in SULT2B1b mRNA occurred with the PPARδ synthetic activator GW501516 (Fig. 2C). Finally, whereas all three PPARγ activators stimulated SULT2B1b expression (Fig. 2D), their potencies differed: ciglitazone increased expression by 25.1-fold, troglitazone increased it by 2.4-fold, and GI262570 increased it by 6.8-fold (Fig. 2D). Thus, LXR and PPAR activators increase SULT2B1b expression in undifferentiated CHKs.

LXR and PPAR activators increase SULT2B1b expression in differentiated keratinocytes

As shown in **Fig. 3**, SULT2B1b mRNA levels increased 3.3-fold when CHKs were grown in 1.2 mM Ca^{2+}, confirming previous findings that calcium induces SULT2B1b mRNA in CHKs (15). Next, we assessed whether LXR and PPAR activators would induce a further increase in SULT2B1b mRNA even in differentiated CHKs. The LXR activator 22R increased SULT2B1b mRNA by 3.2-fold (**Fig. 4A**), and 25-OH increased it by 2.2-fold (data not shown). Moreover, the PPARα activator clofibric acid, the PPARδ activator GW501516, and the PPARγ activators ciglitazone, troglitazone, and GI262570 also all further increased SULT2B1b mRNA levels (~2- to 7-fold increase over high Ca^{2+} alone) (Fig. 4B–D). Thus, LXR and PPAR activators stimulate SULT2B1b expression not only in undifferentiated CHKs but in differentiated CHKs as well.

The PPARγ activator ciglitazone induces SULT2B1b mRNA in a dose- and time-dependent manner

In the experiments described above, the PPARγ activator ciglitazone increased SULT2B1b mRNA levels to a greater extent than any of the other tested LXR and PPAR agonists. Hence, we next examined the effects of various doses and incubation times on the ability of ciglitazone to increase SULT2B1b mRNA levels. As shown in **Fig. 5**, SULT2B1b mRNA levels began to increase at doses of 2.5

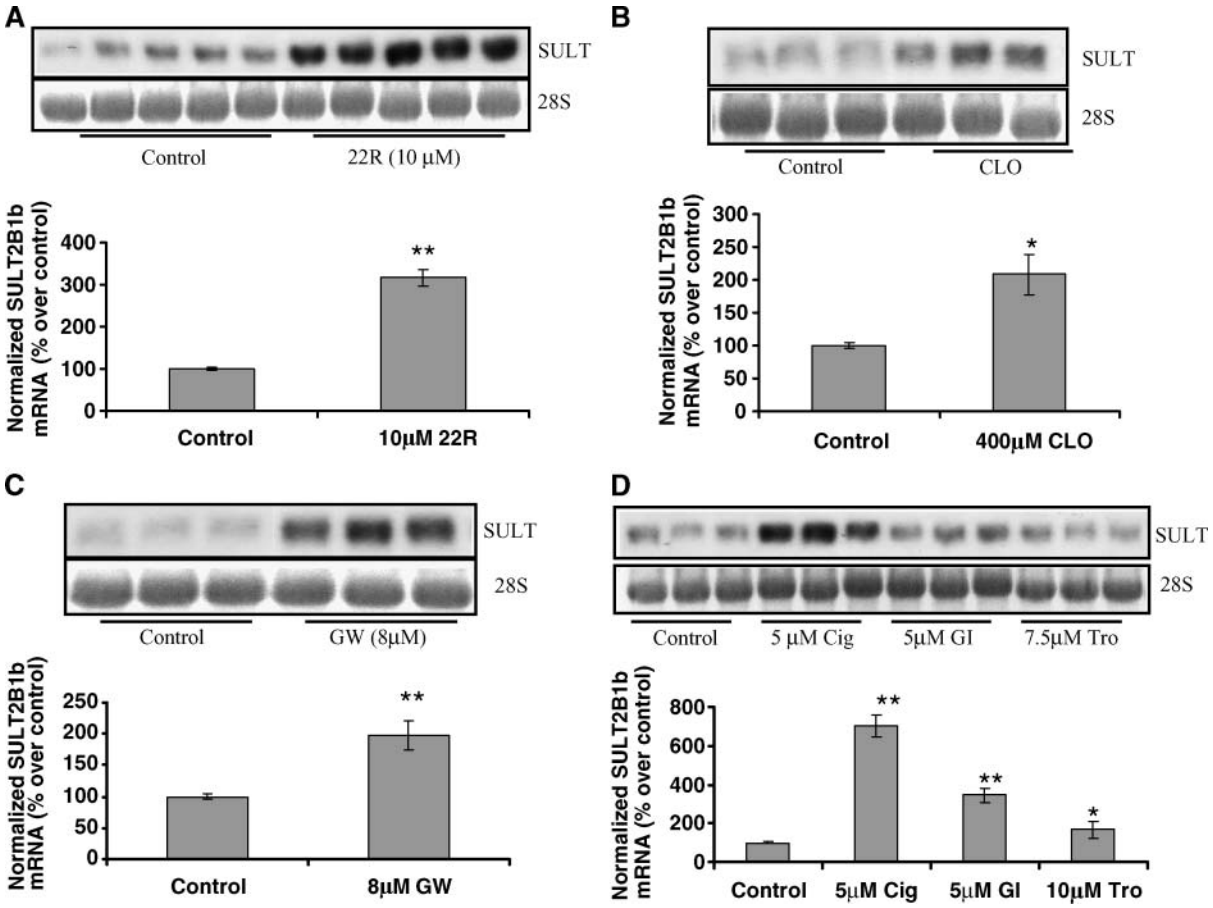

Fig. 4. LXR and PPAR activators increase SULT2B1b mRNA levels in differentiated keratinocytes. Cells were incubated with each activator alone for 24 h in 1.2 mM Ca^{2+} medium: LXR, 10 μM 22(*R*)-hydroxycholesterol (22R) (A); PPARα, 400 μM clofibrate (CLO) (B); PPARδ, 8 μM GW501516 (GW) (C); PPARγ, 10 μM troglitazone (Tro), 5 μM GI262570 (GI), or 5 μM ciglitazone (Cig) (D). Total RNA was isolated and subjected to Northern blot analyses. Blots were hybridized with human SULT2B1b cDNA. Equal loading, the integrity of RNA, and the densitometry value were verified and normalized by ethidium bromide staining of 28S rRNA. Data are expressed as percentages of vehicle control and are presented as means ± SEM from two independent experiments (n = 3). A representative blot is shown above each plot. * $P < 0.05$, ** $P < 0.01$.

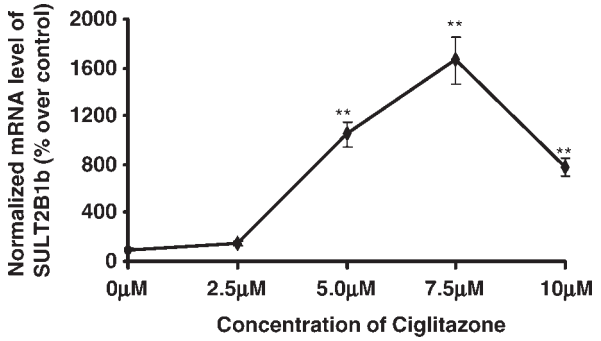

Fig. 5. Dose-dependent regulation of SULT2B1b mRNA by the PPARγ activator ciglitazone. Cells were incubated with various doses of ciglitazone (0, 2.5, 5.0, 7.5, and 10 μM) in low-Ca^{2+} (0.03 mM) medium for 24 h. SULT2B1b mRNA levels were examined by Northern blot analysis as described. Equal loading, the integrity of RNA, and the densitometry value were verified and normalized by ethidium bromide staining of 28S rRNA. Data are expressed as percentages of vehicle control and are presented as means ± SEM from two independent experiments (n = 4). ** $P < 0.01$.

μM ciglitazone (1.5-fold), peaked at 7.5 μM (10.5-fold), and decreased slightly at 10 μM (7.8-fold). We next determined the time course of the effect of 5 μM ciglitazone on SULT2B1b mRNA levels (**Fig. 6**). As early as 3 h, SULT2B1b mRNA levels increased by 25% compared with vehicle controls ($P < 0.05$) (Fig. 6, inset), and levels continued to increase, reaching a peak at 48 h (~30-fold increase over control). Thus, the PPARγ-induced increase in SULT2B1b mRNA levels occurs rapidly and is sustained over an extended period of time.

LXR and PPARγ activators additively induce SULT2B1b mRNA

Activation of LXR and PPARγ has been shown to synergistically induce several genes in macrophages (38, 39). To examine whether this is the case for SULT2B1b in CHKs, we next measured SULT2B1b mRNA levels after cells were treated with both 22R and ciglitazone. As shown in **Fig. 7**, each activator alone induces a significant increase in SULT2B1b (46% and 85%, respectively; $P < 0.05$) at a lower dose (5 μM 22R or 2.5 μM ciglitazone). When coincubated with both activators (5 μM 22R + 2.5 μM ciglitazone), there is an additive effect (1.3-fold increase in SULT2B1b; $P < 0.01$).

The PPARγ activator ciglitazone alters SULT2B1b mRNA transcription, rather than stability

The higher level of SULT2B1b mRNA can result from increased transcription of this particular gene or by decreased degradation (stability). We next examined the effect of ciglitazone on SULT2B1b mRNA stability by incubating cells with actinomycin D, a classical RNA synthesis inhibitor. Keratinocytes were treated with ciglitazone or vehicle for 24 h and then incubated with 2 μg/ml actinomycin D for 0–10 h. As shown in **Fig. 8**, ciglitazone treatment caused a marked increase in SULT2B1b mRNA level without affecting the stability of mRNA (Fig. 8A), as indicated by slightly higher degradation rates in the ciglitazone-treated group compared with vehicle controls (Fig. 8B). Together, these results suggest that the PPARγ activator ciglitazone stimulates SULT2B1b gene expression.

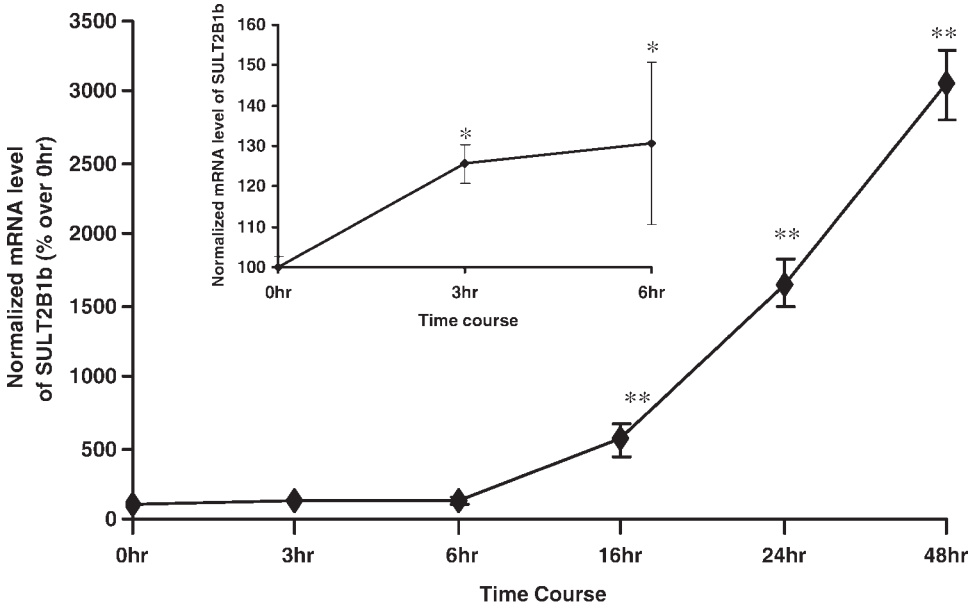

Fig. 6. Upregulation of SULT2B1b mRNA by the PPARγ activator ciglitazone is time-dependent. Cells were incubated with 7.5 μM ciglitazone for various periods of time (0, 3, 6, 16, 24, and 48 h) in 0.03 mM Ca^{2+} medium. SULT2B1b mRNA levels were examined by Northern blot analysis as described. Equal loading, the integrity of RNA, and the densitometry value were verified and normalized by ethidium bromide staining of 28S rRNA The inset shows the increase of SULT2B1B mRNA levels after 3 and 6 h of treatment comparing with the 0 h control. Data are expressed as percentages of the 0 h control and are presented as means ± SEM (n = 4). * $P < 0.05$, ** $P < 0.01$.

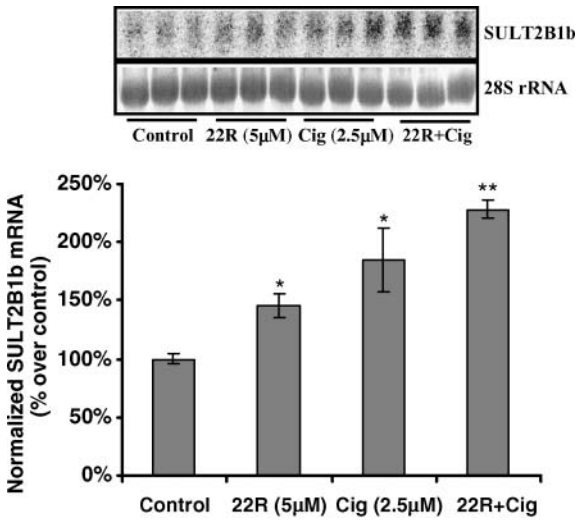

Fig. 7. LXR and PPARγ activators additively induce SULT2B1b mRNA. Cells were coincubated with or without 22R and ciglitazone (Cig) (5 μM 22R + 2.5 μM ciglitazone) in 0.03 mM Ca^{2+} medium for 24 h. SULT2B1b mRNA levels were examined by Northern blot analysis as described. Equal loading, the integrity of RNA, and the densitometry value were verified and normalized by ethidium bromide staining of 28S rRNA. Data are expressed as percentages of control and are presented as means ± SEM (n = 3). * $P < 0.05$, ** $P < 0.01$.

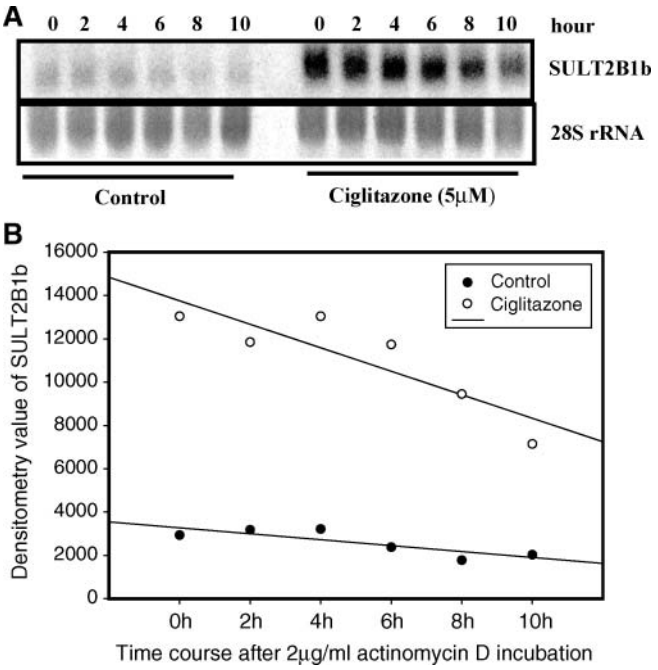

Fig. 8. Effect of actinomycin D on SULT2B1b mRNA stability. Cells were incubated with 5 μM ciglitazone or vehicle for 24 h and subsequently challenged with 2 μg/ml actinomycin D for the indicated periods of time (0, 2, 4, 6, 8, and 10 h). SULT2B1b mRNA levels were examined by Northern blot analysis as described. Equal loading, the integrity of RNA, and the densitometry value were verified and normalized by ethidium bromide staining of 28S rRNA. Similar results were obtained from two independent experiments (n = 2). A representative blot is shown above the leaner regression plot.

Ciglitazone-induced increase in SULT2B1b mRNA is dependent on protein synthesis

To examine whether the ciglitazone-induced increase in SULT2B1b mRNA is dependent on protein synthesis, we next assessed the effect of cycloheximide. Whereas 5 μM ciglitazone alone induced a robust increase in SULT2B1b mRNA, coincubation of cells with 10 μg/ml cycloheximide for 16 h largely abrogated the expected increase in SULT2B1b mRNA (**Fig. 9**). These results indicate that the ciglitazone-induced increase in SULT2B1b mRNA is dependent on new protein synthesis. In contrast, cyclophilin (a housekeeping protein) mRNA expression was unaffected by either ciglitazone or cycloheximide (Fig. 9).

LXR and PPAR activators increase CSTase activity

We next examined the effect of PPAR and LXR activators on CSTase activity in undifferentiated CHKs. As shown in **Fig. 10**, activators of LXR, PPARα, PPARβ/δ, and PPARγ all significantly increased CSTase activity. In accordance with the changes in SULT2B1b mRNA levels, those activators that increase mRNA levels maximally likewise increase enzyme activity to the greatest extent. Thus, the increase in SULT2B1b mRNA levels, induced by PPAR and LXR activators, correlated with an increase in CSTase enzyme activity.

DISCUSSION

The SC is composed of corneocytes (bricks) embedded in an extracellular lipid-rich matrix (mortar). CS is an important lipid in the extracellular lipid matrix, where it plays a major role in regulating corneocyte desquamation (40). In patients with X-linked ichthyosis, mutations in the steroid sulfatase gene, the enzyme responsible for the catabolism of CS in the SC, lead to an increase in CS levels (~10-fold increase) in the SC, in parallel with a reduction in rates of corneocyte desquamation (1, 41–43). The apparent mechanism that accounts for the CS-induced inhibition of corneocyte desquamation is likely an inhibition of serine protease activity in the SC (44, 45), a general characteristic of CS. In addition to its key role in regulating corneocyte desquamation, CS also has been shown to stimulate keratinocyte differentiation (4), to play a role in antimicrobial defense (46), and to regulate cholesterol synthesis in keratinocytes (6, 46). Finally, although CS degradation to cholesterol contributes to the extracellular pool of cholesterol, this source of sterol is not required for the maintenance of permeability homeostasis (7).

CS is synthesized in the nucleated epidermis by CSTase. Of the three enzymes with CSTase activity, SULT2B1b is the only isozyme detected in either CHKs or epidermis (12). Because of the multiple roles of CS in epidermal physiology, the factors that regulate SULT2B1b could be of considerable importance for epidermal homeostasis, but these factors have not yet been completely described. Induction of keratinocyte differentiation by calcium increases SULT2B1b expression and CSTase activity in kera-

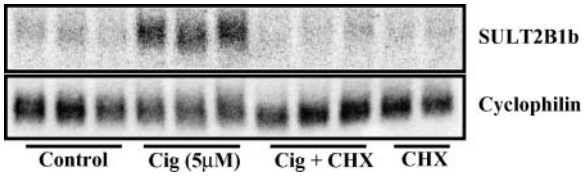

Fig. 9. Effect of cycloheximide on SULT2B1b mRNA. Cells were incubated with 5 μM ciglitazone (Cig) alone, or 5 μM ciglitazone plus 10 μg/ml cycloheximide (CHX), or 10 μg/ml cycloheximide alone, or vehicle in 0.03 mM Ca^{2+} medium for 16 h. SULT2B1b and cyclophilin mRNA levels were examined by Northern blot analysis as described. Equal loading and the integrity of RNA were verified by ethidium bromide staining of 28S rRNA. The densitometry value was further verified by cyclophilin. Similar results were obtained from two independent experiments (n = 3). A representative blot is shown.

tinocytes (15), which we again show here in CHKs grown in high Ca^{2+}. We further demonstrate that activators of LXR, PPARα, PPARβ/δ, and PPARγ all increase mRNA levels of SULT2B1b in both a dose- and a time-dependent manner. Although PPARα activators increased mRNA levels to a modest degree (50% increase), activation of LXR, PPARβ/δ, or PPARγ resulted in a robust increase (5- to 25-fold increase). The increase in SULT2B1b expression induced by the activation of LXR, PPARα, PPARβ/δ, and PPARγ occurs in both proliferating and differentiated keratinocytes (i.e., it is independent of, and in addition to, the effects of high calcium). Furthermore, associated with the LXR, PPARα, PPARβ/δ, and PPARγ activator-induced increases in SULT2B1b mRNA levels, there was also an increase in CSTase enzyme activity that correlated with the extent of the increase in mRNA levels. Of note is the fact that the absolute magnitude of induction of SULT2B1b mRNA by each activator is greater than the induction in its enzyme activity (compare Figs. 2, 10). The disparity between the increase in SULT2B1b mRNA and the increase in its activity level may be attributable to the tight regulation of SULT2B1b at both transcriptional (by nuclear hormone receptors and other transcriptional factors) and posttranscriptional (by other unknown factors) levels. Posttranscriptional control of gene expression appears to be an important mechanism involved in the regulation of many enzymes involved in lipid metabolism in response to various physiological stimuli (47). It will be interesting in future studies to further explore the posttranscriptional regulation of SULT2B1b protein. Thus, our study shows that both SULT2B1b mRNA levels and CSTase activity are increased by the activation of LXR, PPARα, PPARβ/δ, and PPARγ.

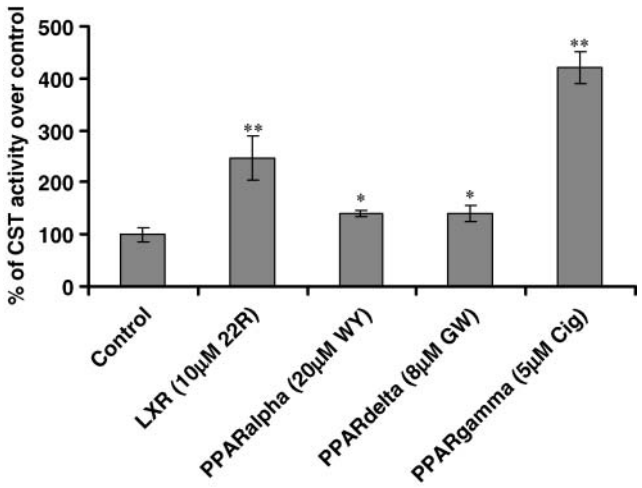

Fig. 10. LXR and PPAR activators increase cholesterol sulfotransferase [CSTase (CST)] activity. Cells were incubated in low-Ca^{2+} medium in the presence or absence of one of these activators: 10 μM 22R, 400 μM WY14643 (WY), 8 μM GW501516 (GW), or 5 μM ciglitazone (Cig). At the end of the incubation, the cytosolic fraction from cell homogenates was collected and CSTase activity was measured as described in Materials and Methods. Enzyme activities are expressed as percentages of vehicle control and presented as means ± SEM (n = 3) from two separate experiments. * $P < 0.05$, ** $P < 0.01$.

Previous studies have shown that activation of LXR, PPARα, PPARβ/δ, and PPARγ stimulates the expression of the cornified envelope proteins involucrin and loricrin as well as transglutaminase 1, which cross-links these cornified envelope proteins (21–27). Additionally, PPARα and LXR activators increase profilaggrin, which is converted to filaggrin, which plays a key role in SC water-holding capacity (33, 34, 48, 49). Of note, CS has also been shown to stimulate the expression of involucrin and transglutaminase 1 (4, 44). Hence, CS has been proposed to regulate keratinocyte differentiation by directly stimulating the expression of involucrin and transglutaminase 1 (4) and indirectly regulating cholesterol and fatty acid bioavailability (6). Whether the stimulation of CS synthesis by LXR, PPARα, PPARβ/δ, and PPARγ activators contributes to the increased formation of the cornified envelope induced by these nuclear hormone receptors is unknown. Additionally, previous studies have shown that activation of LXR, PPARα, PPARβ/δ, and PPARγ improves permeability barrier homeostasis (21, 26, 27, 33, 34). In the present study, we demonstrate that activation of LXR, PPARα, PPARβ/δ, and PPARγ plays an important regulatory role in the synthesis of CS. As discussed above, CS facilitates corneocyte cohesion, and the regulated breakdown of CS allows for desquamation. CS can be thought of as the "glue" that helps hold together the SC. It is thus apparent that activation of LXR, PPARα, PPARβ/δ, and PPARγ results in the formation of many of the key components of the SC, including cornified envelope proteins, filaggrin, the lipid matrix required for barrier function, and the CS required for normal desquamation, that are essential for the formation of a competent SC.

The protein (corneocytes) and lipid (extracellular lipid matrix) arms of SC formation are traditionally viewed as concurrent but independent processes. However, PPARs and LXRs are activated by lipids (PPARs are activated by fatty acids and their products, whereas LXRs are activated by oxysterols), which suggests that there may be cross-talk between these two arms of SC formation. One can speculate that as lipid precursors accumulate in stratum granulosum (SG) cells, these lipids or their metabolites could

serve as endogenous activators of these liposensor receptors, which, in turn, could stimulate the formation of the various components required for SC formation, including, as shown here, CS.

The mechanism by which activation of LXR, PPARα, PPARβ/δ, and PPARγ increases SULT2B1b expression is unknown. Our study demonstrates that the upregulation of SULT2B1b mRNA by PPARγ activator occurs at the level of transcription, because ciglitazone treatment fails to stabilize SULT2B1b mRNA (Fig. 8). Our data also show that the ciglitazone-induced SULT2B1b mRNA increase depends on protein synthesis, because coincubation with cycloheximide, a classical protein synthesis inhibitor, results in a complete blockage of the increase in SULT2B1b expression in these cells (Fig. 9). This finding suggests that, rather than directly activating a response element in the promoter region of SULT2B1b, ciglitazone activation of PPARγ instead may stimulate the production of other transcription factors, which then activate response elements in the promoter of SULT2B1b. Alternatively, this result could indicate that *1*) a labile cofactor is required, or *2*) an autoinduction of PPAR/LXR is required for the effects to occur. In searching the promoter sequence of SULT2B1b (GenBank accession number NM_94315) using the Transcription Element Searching System, we did not find either a LXR or a PPAR response element at the 5′ proximal promoter region (−1 to −1,951 bp), further supporting our hypothesis that upregulation of SULT2B1b mRNA by ciglitazone and other activators occurs indirectly. In previous studies, we have shown that LXR and PPARα activators increase the expression of involucrin and that this increase is mediated at an AP-1 site in the distal portion of the involucrin promoter (−2,117 to −2,111) (4). Subsequent studies have shown that LXR activators increase the levels of AP-1 factors, particularly Fra-1, but also Jun D and c-FOS, which leads to increased binding of AP-1 factors to AP-1 response elements, thereby increasing the expression of AP-1-regulated genes (50). Whether similar indirect effects of the activation of LXR, PPARα, PPARβ/δ, and PPARγ on AP-1 or other transcription factors account for the increase in SULT2B1b expression remains to be determined.

Previous studies have demonstrated that retinoids inhibit CS production as well as the expected increase in CSTase activity that accompanies keratinocyte differentiation (18, 19). This is not surprising because it is well recognized that activation of RAR inhibits keratinocyte differentiation (51). Depending upon the specific retinoid used, one can activate RAR and/or RXR (many retinoids activate both receptors). In many respects, activation of RAR results in effects that are opposite the effects induced by activation of PPARs or LXR (RAR activation inhibits differentiation and adversely affects permeability barrier function, whereas PPAR and LXR activation stimulates differentiation and improves permeability barrier homeostasis). However, retinoids that predominantly activate RXR also decrease CSTase activity (51). It is well known that RXR ligands activate PPAR/RXR and LXR/RXR heterodimers, so one might expect that RXR ligands would increase rather than decrease CSTase activity. There are a number of potential explanations for this paradox. First, RXR ligands activate RXR/RXR homodimers, which can directly regulate gene expression. Second, RXR forms heterodimers with a large number of nuclear hormone receptors, and it is possible that under certain circumstances RXR ligands could activate these heterodimers, thereby inhibiting CSTase activity (e.g., activation of RAR/RXR heterodimers). Lastly, retinoids could affect CSTase activity independently of their effects on nuclear hormone receptors. At present, the precise molecular mechanisms by which retinoids inhibit and activators of PPARs and LXR increase CSTase activity are unknown.

In conclusion, this study demonstrates that activation of LXR, PPARα, PPARβ/δ, and PPARγ stimulates the expression of SULT2B1b and increases CSTase activity in keratinocytes. The resulting increase in CS would provide a key factor required for the formation of a normal SC.

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