Fetal Epidermal Differentiation and Barrier Development *In Vivo* is Accelerated by Nuclear Hormone Receptor Activators[1]

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Nuclear receptors which interact with the retinoid X receptor are involved in the regulation of epidermal differentiation and development. We have recently shown that activators of the peroxisome proliferator-activated receptor and of the farnesoid X-activated receptor accelerate epidermal barrier maturation in fetal rat skin *in vitro*. In this study we asked whether cutaneous development *in utero* was affected by peroxisome proliferator-activated receptor or farnesoid X-activated receptor activators, or by an activator of another retinoid X receptor partner, liver X receptor. Activators of the peroxisome proliferator-activated receptor (clofibrate or linoleic acid), farnesoid X-activated receptor (farnesol or juvenile hormone III), or liver X receptor (22R-hydroxycholesterol), were injected into the amniotic fluid of fetal rats on gestational day 17. Fetal epidermal barrier function and morphology was assessed on day 19. Whereas vehicle-treated fetal rats displayed no measurable barrier (transepidermal water loss > 10 mg per cm^2 per h), a measurable barrier was induced by the intra-amniotic administration of all activators tested (transepidermal water loss range 4.0–8.5 mg per cm^2 per h). By light microscopy, control pups lacked a well-defined stratum corneum, whereas a distinct stratum corneum and a thickened stratum granulosum were present in treated pups. By electron microscopy, the extracellular spaces of the stratum corneum in control pups revealed a paucity of mature lamellar unit structures, whereas these structures filled the stratum corneum interstices in treated pups. Additionally, protein and mRNA levels of loricrin and filaggrin, two structural proteins of stratum corneum, were increased in treated epidermis, as were the activities of two lipid catabolic enzymes critical to stratum corneum function, β-glucocerebrosidase and steroid sulfatase. Finally, peroxisome proliferator-activated receptor-α and -δ and liver X receptor-α and -β mRNAs were detected in fetal epidermis by reverse transcriptase–polymerase chain reaction and northern analyses. The presence of these receptors and the ability of their activators to stimulate epidermal barrier and stratum corneum development suggest a physiologic role for peroxisome proliferator-activated receptor and liver X receptor and their endogenous ligands in the regulation of cutaneous development. ***Key words: farnesoid X-activated receptor/liver X receptor/peroxisome proliferator activated receptor. J Invest Dermatol 113:788–795, 1999***

The outermost layer of the epidermis, the stratum corneum (SC), provides the skin with a barrier to water loss, necessary for terrestrial life. Lipids in the extracellular spaces of the SC, predominantly fatty acids, ceramides, and cholesterol, arranged into extracellular lamellar membranes, contribute to the water-tight properties of the skin, whereas corneocyte proteins provide structural rigidity and cohesiveness, and provide a scaffold for membrane lipids (reviewed in Elias and Menon, 1991; Downing, 1992; Roop, 1995; Kalia *et al*, 1998). In mammals, a mature epidermis and competent barrier are normally present prior to birth, whereas very premature infants lack an adequate barrier, increasing their susceptibility to hypothermia, fluid and electrolyte imbalance, and transcutaneously acquired infections (Hammarlund and Sedin, 1979; Harpin and Rutter, 1983; Cartlidge and Rutter, 1992).

Epidermal barrier development in the fetal rat occurs over a short time period late in their 22 d gestation. Pups of gestational age day 19 lack a measurable barrier to transepidermal water loss (TEWL), whereas a competent barrier is present by day 21 (Aszterbaum *et al*, 1992). The emergence of a functionally mature barrier correlates with the appearance of a multilayered SC and with the formation of mature lamellar membranes in the SC interstices (Aszterbaum *et al*, 1992). Furthermore, the expression

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Abbreviations: FXR, farnesoid X-activated receptor; JH III, juvenile hormone III; LXR, liver X receptor; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; SC, stratum corneum; TEWL, transepidermal water loss; 22R-OH Ch, 22R-hydroxycholesterol.

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of structural proteins, such as loricrin, filaggrin, and involucrin, necessary for corneocyte formation, increase during barrier maturation (Bickenbach *et al*, 1995; Kömüves *et al*, 1998). Finally, the activities of lipid enzymes important for normal SC function, such as β-glucocerebrosidase, which converts glucosylceramide to ceramide, and steroid sulfatase, which catalyzes the desulfation of cholesterol sulfate, also increase during barrier maturation (Hanley *et al*, 1997a, b).

Hormones such as glucocorticoids, estrogens, and androgens, which exert effects on transcriptional events in a wide variety of tissues via their respective nuclear steroid hormone receptors, regulate fetal SC formation *in vitro* and barrier development *in vivo* when administered to the mother (Aszterbaum *et al*, 1993; Hanley *et al*, 1996a). Additionally, fetal mice made deficient in either glucocorticoids or thyroid hormone exhibit delayed epidermal and SC maturation (Hanley *et al*, 1997c, 1998c). These hormones do not appear to be essential, however, because: (i) a mature SC is present at birth in glucocorticoid-deficient or hypothyroid mice (Hanley *et al*, 1997c, 1998c), and (ii) the timetable of epidermal development proceeds normally in skin explants taken from fetal rats of gestational day 17 and incubated in hormone- and serum-free media (Hanley *et al*, 1996b). Taken together, these findings suggest that endogenous skin-derived factors, rather than factors delivered systemically, may be important mediators of epidermal and SC development.

In searching for putative locally generated regulators of SC development, we have considered the retinoid X receptor (RXR) subfamily of nuclear receptors. RXR forms heterodimers with other ligand-activated receptors, such as RAR, the vitamin D receptor, and the thyroid hormone receptor, whose ligands are known to exert potent effects on growth and differentiation of skin and other tissues (Mangelsdorf and Evans, 1995; Kang *et al*, 1996). Other members of the RXR-heterodimerizing family include: (i) peroxisome proliferator-activated receptor (PPAR), involved in fatty acid metabolism in tissues such as liver and kidney, for which an array of fibrates, eicosanoids, and fatty acids have recently been identified as ligands (Keller *et al*, 1993; Gottlicher *et al*, 1992; Yu *et al*, 1995; Schoonjans *et al*, 1996); (ii) farnesoid X-activated receptor (FXR), a relative of the ecdysone receptor, which regulates development in insects, is activated by juvenile hormone III (JH III) and farnesol, an intermediate in the cholesterol biosynthetic pathway (Forman *et al*, 1995); and (iii) liver X receptor (LXR), which regulates bile acid synthesis in the liver and is activated by oxidized sterols (Willy *et al*, 1995; Lehmann *et al*, 1997). We have shown previously that PPAR and FXR activators accelerate barrier formation in fetal skin explants (Hanley *et al*, 1998a). Furthermore, we have shown that PPAR activators induce keratinocyte differentiation *in vitro* (Hanley *et al*, 1998b). Thus, putative products of epidermal metabolism, including some free fatty acids, an intermediate in the cholesterol biosynthetic pathway, and oxidative products of cholesterol, which activate nuclear receptors in the RXR family, may regulate epidermal differentiation and development.

In this study we sought to determine whether activators of PPAR, FXR, and LXR regulate epidermal development *in vivo*. We show here that intra-amniotic administration of PPAR activators, clofibrate and linoleic acid, FXR activators, farnesol and JH III, and 22R-hydroxycholesterol (22R-OH Ch), an LXR activator, accelerates the maturation of the SC and epidermal barrier in fetal rats.

MATERIALS AND METHODS

Animal treatments Timed-pregnant Sprague–Dawley rats (plug date = day 0) were obtained from Simonsen Laboratories (Gilroy, CA). On day 17 of gestation, maternal animals were anesthetized with forane and a ventral midline incision was made. The uterine horns were exposed and kept moist with warm saline, and 50 μl of either saline, clofibrate (2-(p-chlorophenoxy)-2-methyl-propionic acid ethyl ester) (1.0 mg), linoleic acid (1.0 mg), farnesol (0.75 mg), juvenile hormone III (JH III) (1.0 mg), 22R-OH Ch (0.75 mg), or vehicle [dimethylsulfoxide (DMSO)], all from Sigma (St. Louis, MO), was injected into the amniotic fluid space of individual amniotic sacs. Pregnant animals were immediately closed, and the pregnancy was allowed to proceed for an additional 48 h. Separate maternal animals were used for each treatment group to avoid possible neighbor effects, or effects on littermates in the opposite uterine horn. Fetal animals were removed prematurely on day 19 by cesarean section. For sham-operated controls, maternal animals were anesthetized and surgically opened as above, but were immediately closed, with no injections made.

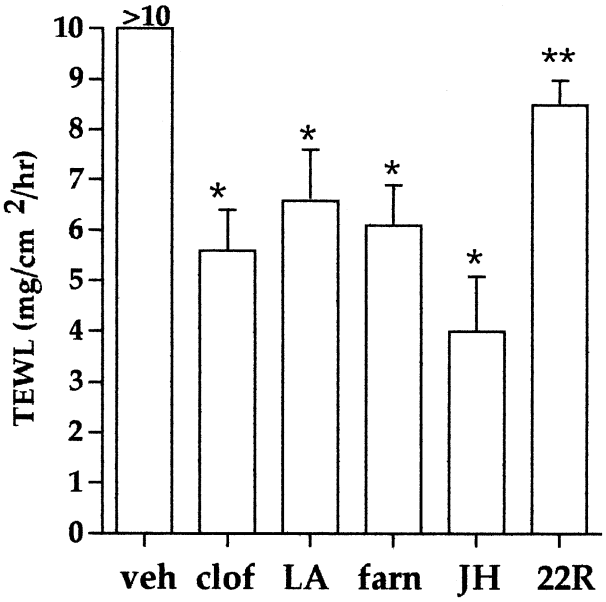

Figure 1. Fetal epidermal barrier function is improved by treatment with activators of PPAR, FXR, or LXR. Vehicle (veh) (DMSO), clofibrate (clof), linoleic acid (LA), farnesol (farn), juvenile hormone III (JH), or 22R-OH Ch (22R) were injected intra-amniotically on gestational day 17, and TEWL measured in fetal rat epidermis on gestational day 19, as described in *Materials and Methods*. Results are expressed as the mean ± SEM, n = 8; *$p < 0.01$; **$p < 0.05$. Each data point represents the average of two experiments (eight fetuses) for each treatment.

TEWL This was measured in fetal animals on day 19 using a Meeco electrolytic water analyzer as described previously (Aszterbaum *et al*, 1992).

Light and electron microscopy Samples of fetal skin from the lower anterior abdominal wall were fixed in modified Karnovsky's fixative, Epon-embedded and 0.5 μm sections were stained with hematoxylin/eosin for light microscopy, as described previously (Hanley *et al*, 1996b). Parallel samples for electron microscopy were minced to 1 mm^3 fragments, fixed in modified Karnovsky's fixative and postfixed in reduced 1% osmium or buffered 0.2% ruthenium tetroxide with 0.5% ferrocyanide (Aszterbaum *et al*, 1992). Samples were then dehydrated in graded ethanol, embedded in Epon, and sectioned. Samples were examined in a Zeiss electron microscope at 60 kV. Pictures shown for both light and electron microscopy are representative of the changes observed in samples obtained from at least two different litters for each treatment.

***In situ* hybridization** Digoxigenin-labeled RNA probes to detect loricrin and filaggrin were made from linearized cDNA sequences as templates, using reagents purchased from Boehringer-Mannheim (Indianapolis, IN), as described previously (Kömüves *et al*, 1998). *In situ* hybridization was performed on sections of paraffin-embedded tissue as described (Stelnicki *et al*, 1998), with minor modifications. Briefly, probes were applied to the sections and hybridized at 55°C for loricrin or 40°C for filaggrin/profilaggrin. Washing and blocking was performed as described (Stelnicki *et al*, 1998).

Protein detection by immunohistochemistry Immunohistochemistry was performed as described previously (Kömüves *et al*, 1998). Loricrin was detected with a rabbit anti-peptide antibody, specific for mouse loricrin, and a rabbit antibody specific for rat filaggrin was used to localize filaggrin/profilaggrin antibody (both antibodies were from BabCo, Berkeley, CA).

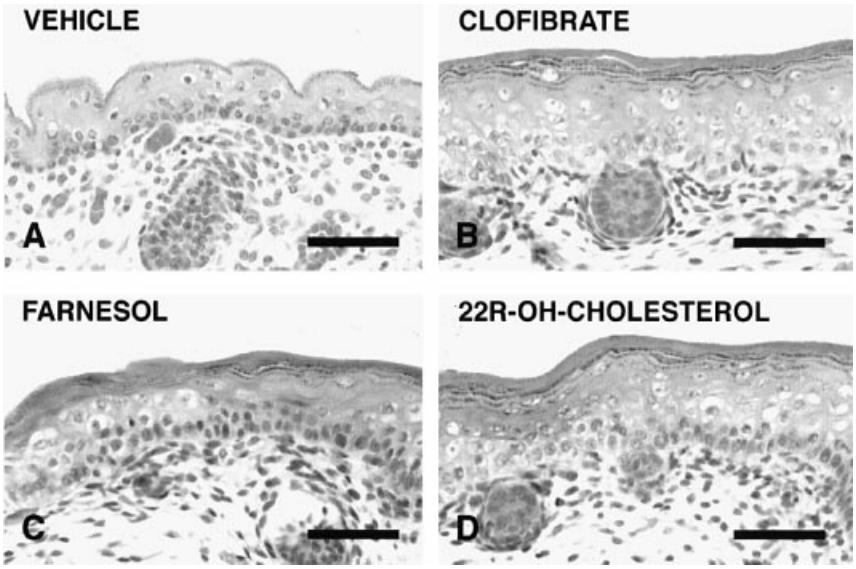

Figure 2. Epidermal development is accelerated by PPAR, FXR, and LXR activators. Animals were treated as described in the legend to **Fig 1** and epidermal samples from gestational age day 19 fetal rats were immediately processed as described in *Materials and Methods*. Paraffin-embedded sections were stained with hematoxylin–eosin. (*A*) DMSO vehicle; (*B*) clofibrate; (*C*) farnesol; (*D*) 22R-OH Ch. *Scale bar*: 10 μm.

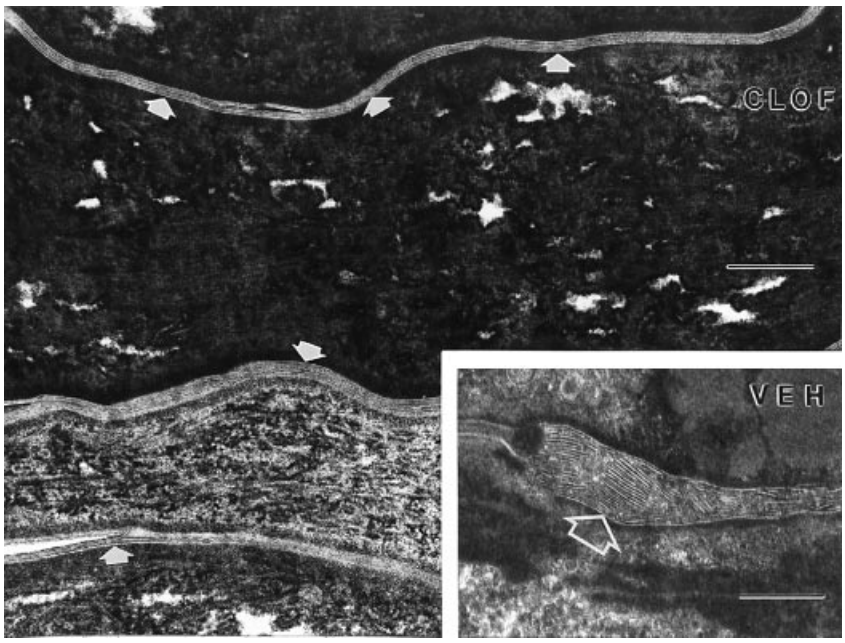

Figure 3. The appearance of mature lamellar membranes in the SC is accelerated by PPAR activators. Parallel samples were taken from animals as described in **Fig 2**, and postfixed in ruthenium tetroxide. The extracellular spaces of the SC in epidermis from clofibrate (CLOF) -treated pups exhibit mature lamellar membranes (*closed arrows*). *Inset*: the SC interstices in DMSO vehicle (VEH)-treated epidermis are filled predominantly with material not yet fully processed (*open arrow*). *Scale bar*: 0.2 μm.

Enzyme assays Epidermis was isolated from whole skin and β-glucocerebrosidase activity was measured as described previously (Hanley *et al*, 1997a). Briefly, epidermis from day 19 fetal rats was homogenized in phosphate-buffered saline containing 0.1 mM phenylmethylsulfonyl fluoride and 0.1% Tween 20, incubated 30 min on ice, and centrifuged at 10 000 × *g* for 10 min (4°C). Fifty microliters of the resultant supernatant was added to an equal volume of reaction buffer (5 mM sodium taurocholate with 0.5 mM 4-methylumbelliferyl-β-D-glucopyranoside in citrate phosphate buffer, pH 5.6) and incubated 60 min at 37°C. The reaction was terminated with 2 ml of pH 10.5 carbonate-bicarbonate buffer, and fluorescence was measured at 360 λ (emission) and 450 λ (excitation).

Steroid sulfatase was assayed in epidermal microsomal preparations as previously described (Hanley *et al*, 1997b). Assays were performed in 0.1 M Tris–HCl, pH 7.4, with 5.6 mM glucose and 15 μM (5 μCi) [^{3}H]dehydroepiandrosterone sulfate for 2 h at 37°C. Following extraction with benzene, product ([^{3}H]dehydroepiandrosterone) was quantitated by scintillation spectroscopy.

Reverse transcriptase–polymerase chain reaction (PCR) Full-thickness skin was excised from day 17 fetal rats and floated dermis side down on 10 mM ethylenediamine tetraacetic acid in calcium- and magnesium-free phosphate-buffered saline, pH 7.4, at 37°C for 45 min. Epidermis was isolated as a sheet, snap-frozen, and stored at −70°C. Total RNA was isolated using Trizol (Sigma) following manufacturer's instructions. Total RNA (0.8 μg) was reverse transcribed with 20 ng random hexamer (reverse transcriptase-for-PCR kit, Clontech, San Diego, CA) at 42°C for 2 h. As a control experiment, cDNA synthesis reactions were carried out without reverse transcriptase for every RNA sample to evaluate DNA contamination. The PCR mixture (50 μl) contained 0.4 μM final concentration of each (forward and reverse) primer, 0.2 mM of each deoxynucleotide triphosphate, and 1 × Taq DNA polymerase in PCR buffer (all from Clontech). FXR-specific cDNA products were amplified by PCR with the following oligonucleotides: forward primer: 5′ cgt gac ttg cgn caa gtg acc 3′, reverse: 5′ cca nga cat cag cat ctc agc g 3′ (primer set 1), designed to yield a 683 bp product; forward: 5′ ctg acc caa aac aat cc 3′, reverse: 5′ ccc ctt tta ttc ttc cc 3′ (primer set 2), for a product size of 448 bp. Samples were heated to 97°C for 5 min, followed by 35 cycles of denaturation at 97°C for 1 min, annealing at 68°C (primer set 1) or 56°C (primer set 2) for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 5 min, using a Perkin-Elmer Thermal Cycler (Model 480). PCR products were separated by electrophoresis on a 1.5% agarose gel and visualized with ethidium bromide staining. A 1Kb DNA ladder (Promega, Madison, WI) was used for molecular weight markers. The identity of the amplified PCR product was confirmed as an FXR cDNA by southern analysis using a [^{32}P]-labeled FXR cDNA (a gift from Dr S. Jackson, UCLA).

PPAR-specific cDNA products were amplified by PCR using oligonucleotides derived from published cDNA sequences. For rat PPARα the down primer was 5′-tct gta gat ctc ttg caa cag-3′ and the up primer was 5′-gcc agt gca tgt ccg tgg aga-3′, spanning 568 bp in the ligand-binding

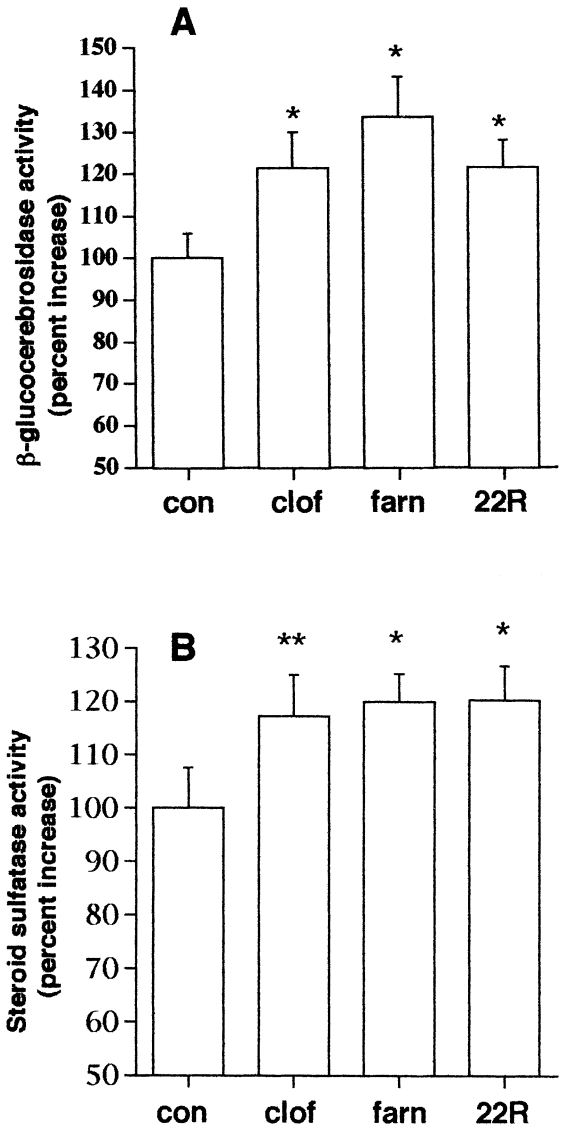

Figure 4. Activities of β-glucocerebrosidase (*A*) and steroid sulfatase (*B*) are increased in epidermis from clofibrate (clof), farnesol (farn), or 22R-OH Ch (22R) -treated pups compared with vehicle (DMSO) -treated controls (con). Enzyme activity was measured in epidermis taken from fetal rats of gestational age day 19 as described in *Materials and Methods*. Results are expressed as the mean ± SEM *$p < 0.01$, **$p < 0.05$; $n = 5$.

domain (Goettlicher *et al*, 1992). For rat PPARδ, down primer 5′-cat cac agc cca tct gca gct ggt-3′, and up primer 5′-gtc atg gaa cag cca cag gag gag-3′ were used to span a 191 bp sequence within the A/B domain (Xing *et al*, 1995). For PPARγ, primers derived from the mouse PPARγ cDNA sequence were used as described (Braissant *et al*, 1996): down 5′-tat cat aaa taa gct tca atc gga tgg ttc-3′; up: 5′-gag atg cca ttc tgg ccc acc aac ttc gg-3′, spanning 420 bp in the A/B and C domains. Samples were heated to 95°C for 5 min, followed by 32 cycles at 95°C for 1 min, 59°C for 1 min, and 72°C for 1.5 min with a final extension step at 72°C for 10 min. PPAR PCR products were separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide, using a 100 bp DNA ladder (Gibco, Grand Island, NY) to determine the size of the PCR products.

Northern blotting Poly(A)$^+$ mRNA was isolated from day 17 rat epidermis as previously described (Hanley *et al*, 1997a, b). Eight micrograms per sample was size fractionated through a 1% agarose gel containing 2.2 M formaldehyde, as described previously (Hanley *et al*, 1997a, b). RNA integrity was visualized following Acridine orange staining of the electrophoresed gel. The RNA was transferred to a nylon membrane which was subsequently baked at 80°C for 2 h. Blots were hybridized with [^{32}P]-labeled LXRα and LXRβ cDNAs (kindly provided by Dr D. Mangelsdorf (SW University, Dallas, TX) or FXR cDNA overnight at 65°C. Washes were then performed in a solution containing 0.1% sodium citrate/chloride buffer and 0.1% sodium dodecyl sulfate for 30 min at room temperature, followed by a 30 min wash at 65°C. Autoradiography was performed at –70°C. Molecular biology-grade reagents were obtained from Sigma or from Pharmacia Biotech (Uppsala, Sweden).

Statistics Statistical analysis was performed using a Student's t test.

RESULTS AND DISCUSSION

Surgical and vehicle controls Surgery accelerates fetal lung development in several species, perhaps due to an increase in cortisol levels (Hitchcock, 1979). We have shown previously that glucocorticoids accelerate epidermal barrier development in rats (Aszterbaum *et al*, 1993); therefore, we first determined if surgical stress alone affected the maturation of the epidermal barrier. TEWL was measured on gestational day 19 in animals from mothers who had undergone sham surgical procedures on day 17 but were otherwise untreated, and in control untreated day 19 fetal animals. TEWL was $>$ 10 mg per cm^2 per h (off-scale) in both groups of animals, as previously reported for 19 d fetal rat pups (Aszterbaum *et al*, 1992). The morphology of the epidermis, examined by light and electron microscopy, was also unaffected by sham surgery (data not shown). We also compared epidermal function and morphology in sham-operated, noninjected control animals to animals injected intra-amniotically with 50 μl of saline or DMSO. We found no effect of either vehicle on epidermal barrier function or morphology (data not shown). Thus, neither surgery alone, nor the intra-amniotic administration of saline or DMSO, alters epidermal development.

Nuclear hormone receptor activators accelerate barrier formation To determine whether treatment with activators of PPAR, FXR, or LXR accelerates epidermal barrier formation, we initially measured epidermal barrier function in animals injected intra-amniotically with either clofibrate, linoleic acid, farnesol, JH III, 22R-OH Ch, or DMSO (vehicle). As shown in **Fig 1**, vehicle-treated fetal rats on day 19 exhibit no measurable barrier to water loss ($>$ 10 mg per cm^2 per h, off-scale). For statistical purposes, off-scale readings were recorded as 10.00 mg per cm^2 per h. In contrast, intra-amniotic administration of the PPAR activators, clofibrate or linoleic acid, on day 17, resulted in decreased rates of TEWL on day 19. TEWL was reduced by approximately 50% in treated pups compared with vehicle-treated animals: clofibrate 5.60 ± 0.80 mg per cm^2 per h; linoleic acid 6.60 ± 1.0 mg per cm^2 per h, $n = 8$ (two litters each treatment). Administration of FXR or LXR activators into the amniotic fluid also accelerated the maturation of epidermal barrier function (**Fig 1**): farnesol-treated 6.10 ± 0.80 mg per cm^2 per h; JH III-treated 4.00 ± 1.10 mg per cm^2 per h; 22R-OH Ch-treated 8.5 ± 0.4 mg per cm^2 per h, $n = 8$ (resulting from two litters each treatment). These data indicate that activators of PPAR, FXR, or LXR decrease trans-epidermal water movement in intact animals.

We next determined whether the appearance of morphologic correlates of barrier maturity were also accelerated by clofibrate, farnesol, or 22R-OH Ch. By light microscopy, control pups displayed a thin, distinct SC but lacked a well-defined SC (**Fig 2*A***). The epidermis from pups treated with clofibrate, farnesol, or 22R-OH Ch exhibited a thickened SC and a distinct, multilayered SC (**Fig 2*B–D***, respectively). Electron microscopy revealed that the majority of the lipid material in the SC interstices of control animals (**Fig 3**, VEH) was not yet organized into mature lamellar unit structures; localized areas of lamellar unit structures were present in the extracellular spaces of the SC in some of the control animals (data not shown) whereas others completely lacked mature lamellae (**Fig 3**, VEH). In contrast, the SC interstices in epidermis from clofibrate-treated fetal rats (**Fig 3**, CLOF) were filled with mature lamellar unit structures. Similar results were observed following treatment with linoleic acid, JH III, farnesol, and 22R-OHCh (data not shown). Thus, intra-amniotic administration of PPAR, FXR, or LXR activators accelerates both the functional and the morphologic maturation of the fetal epidermal permeability barrier.

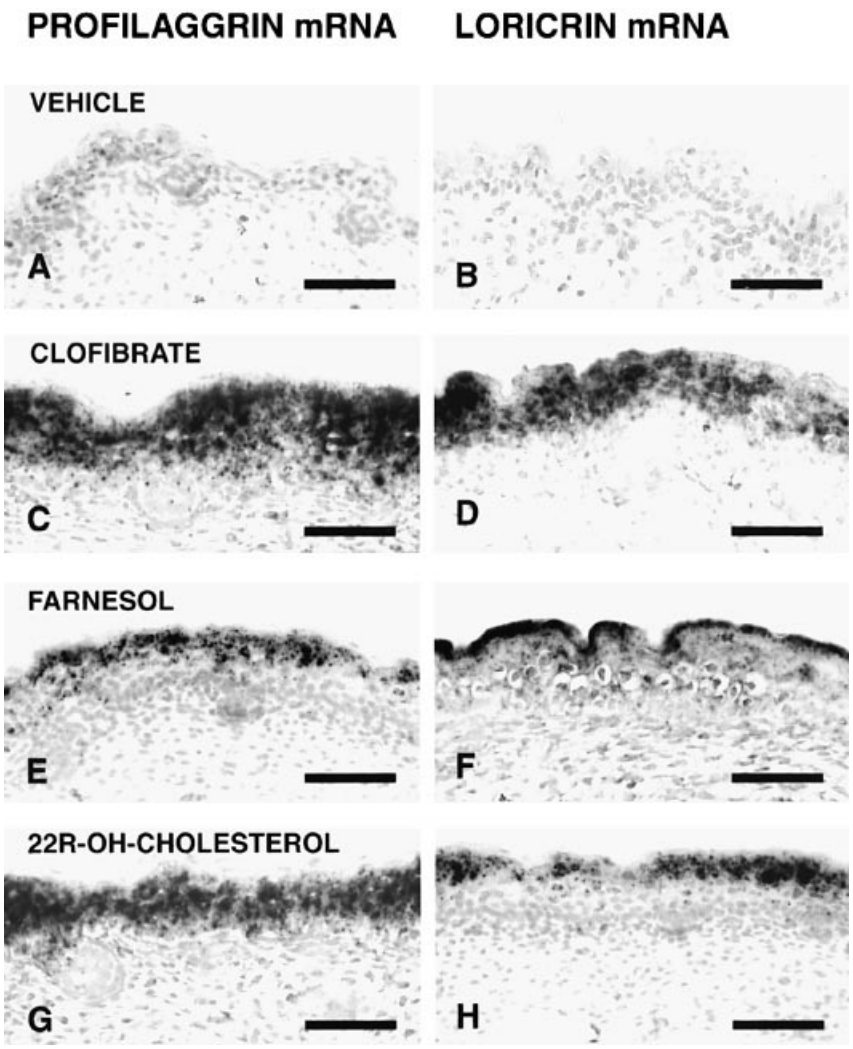

Figure 5. mRNA levels of profilaggrin and loricrin are increased in epidermis from treated fetal rats. Loricrin and profilaggrin mRNA was detected in fetal rat epidermis by *in situ* hybridization, as described in *Materials and Methods*. Increased staining is evident in the epidermis taken from treated pups (*C*, *D*, clofibrate; *E*, *F*, farnesol; and *G*, *H*, 22R-OH Ch) compared to vehicle (DMSO) -treated (*A*, *B*). *Scale bar*: 0.5 μm.

Increased SC lipid enzyme activity and expression of structural proteins by nuclear receptor activators Previous studies have shown that the lipid metabolizing enzymes β-glucocerebrosidase and steroid sulfatase increase during SC and epidermal barrier development (Hanley *et al*, 1997a, b). β-glucocerebrosidase catalyzes the conversion of glucosylceramides to ceramides in the SC interstices. Ceramides are required for the formation of normal lamellar membranes and for normal barrier function, and inhibition of β-glucocerebrosidase activity results in an abnormal permeability barrier (Holleran *et al*, 1993). Steroid sulfatase catalyzes the conversion of cholesterol sulfate to cholesterol, a reaction essential for normal SC desquamation (Epstein *et al*, 1981). Additionally, recent studies have shown that the impaired conversion of cholesterol sulfate to cholesterol also results in an abnormal permeability barrier (Zettersten *et al*, 1998). As shown in **Fig 4**(***A***, ***B***), the activity of β-glucocerebrosidase and steroid sulfatase are modestly but significantly increased in pups treated with PPAR, FXR, or LXR activators. These data indicate that nuclear hormone receptor activators stimulate the activity of key lipid catabolizing enzymes that are required for normal SC function.

Profilaggrin and loricrin are structural protein markers of late epidermal development. Both are key constituents of keratohyalin granules (Dale *et al*, 1985; Bickenbach *et al*, 1995). Subsequently, loricrin is cross-linked into the cornified envelope, whereas filaggrin participates in keratin filament assembly and is required for corneocyte hydration (Fuchs, 1990; Steven *et al*, 1990). Here we examined the expression of profilaggrin and loricrin mRNA by *in situ* hybridization. In comparison with vehicle-treated animals, animals treated with nuclear hormone receptor activators displayed striking increases in the expression of profilaggrin and loricrin mRNA in the upper epidermis [**Fig 5**: controls (***A***, ***B***); PPAR activators (***C***, ***D***); FXR activators (***E***, ***F***); LXR activators (***G***, ***H***)]. Moreover, as shown in **Fig 6**, protein levels of profilaggrin/filaggrin and loricrin are also increased by treatment with clofibrate, farnesol, or 22R-OH Ch. Thus, the expression of proteins required for the formation of the cornified envelope and keratohyalin granules are also stimulated by nuclear hormone receptor activators, resulting in accelerated but apparently normal epidermal development, and suggesting that there is a co-ordinate regulation of both the lipid and protein components required for epidermal and SC development.

During normal epidermal development, co-ordinate regulation of the lipid and protein components of the SC has been demonstrated both *in vivo* and *in vitro* (Bickenbach *et al*, 1996; Kömüves *et al*, 1998). Similarly, in this study, nuclear hormone receptor activators coordinately stimulated both lipid and protein elements essential for normal epidermal and SC development. Coordinate regulation is not invariably observed, however. In intrauterine growth-retarded fetal rats, the expression of cornification markers is decreased whereas the formation of the permeability barrier proceeds normally (Hoath *et al*, 1990; Williams *et al*, 1993). Moreover, in mice overexpressing a retinoic acid receptor dominant negative mutant in the suprabasal epidermis, corneocyte formation proceeds normally whereas the development of the extracellular lipid lamellae in the SC is impaired (Imakado *et al*, 1995).

Expression of nuclear hormone receptors in fetal epidermis To determine whether PPAR, LXR, or FXR activators could be

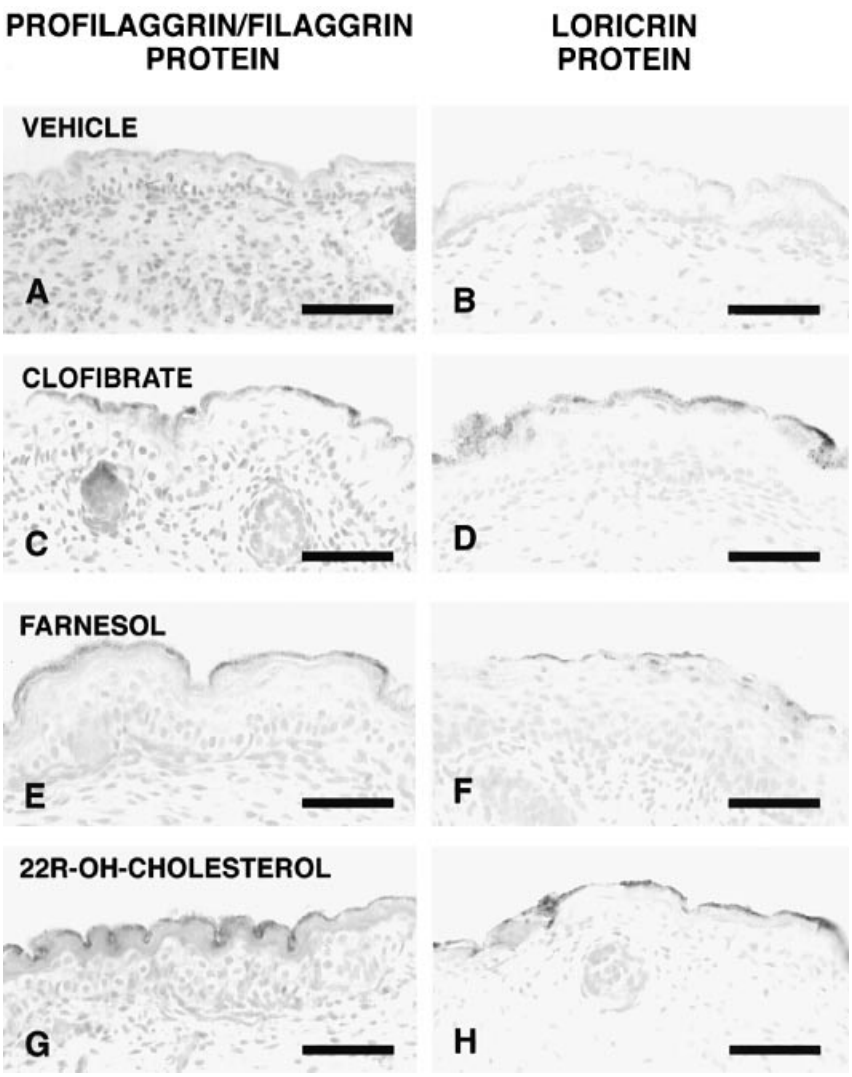

Figure 6. Protein levels of profilaggrin/filaggrin and loricrin are increased in epidermis from treated fetal rats. Filaggrin and loricrin proteins were detected in fetal rat epidermis by immunocytochemistry as described in *Materials and Methods*. Increased staining is evident in the epidermis taken from treated pups (*C*, *D*, clofibrate; *E*, *F*, farnesol; and *G*, *H*, 22R-OH Ch) compared to vehicle (DMSO) -treated (*A*, *B*). *Scale bar*: 0.5 μm.

acting via their respective receptors, we next sought evidence that these receptors are expressed in fetal epidermis. Similar to recent findings by others (Braissant and Wahli, 1998), PPAR isoforms α and δ, but not γ, were detected by reverse transcriptase–PCR in epidermis from rats of gestational age day 17 (**Fig 7*A***). The presence of PPARα was confirmed by northern analysis (data not shown). LXRα and LXRβ were detected in day 17 epidermis by northern analysis as transcripts of approximately 1.9 and 2.0 kb, respectively (**Fig 7*B***). In contrast, FXR mRNA was not detected in day 17 epidermis by northern analysis (data not shown) or by reverse transcriptase–PCR (**Fig 7*C***, *lanes 1* and *2*) (see *Materials and Methods*, primer set 1) (liver and kidney, **Fig 7*C***, *lanes 4* and *5*, are shown as positive controls) or using an alternate set of primers (see *Materials and Methods*, primer set 2) (not shown). Southern blot analysis using an FXR cDNA probe confirmed the PCR product from rat liver and kidney as FXR mRNA; no signal was detected in lanes containing epidermis (not shown). Similarly, FXR mRNA was not detected in full-thickness skin from day 17 rats (**Fig 7*C***, *lane 3*). These data indicate that PPARα and PPARδ and LXRα and LXRβ mRNAs are present in fetal epidermis, but that FXR is not present in epidermis or dermis on gestational day 17. These data are consistent with the hypothesis that clofibrate and linoleic acid could be stimulating epidermal and SC development by activating PPARα or PPARδ, or both; and that 22R-OH Ch could be stimulating development by activating LXRα or LXRβ or both. In contrast, farnesol and JH III cannot be stimulating barrier ontogenesis via FXR, because FXR could not be demonstrated using a variety of techniques. It remains possible that there is an unrecognized nuclear hormone receptor closely related to FXR that is present in the skin and activated by farnesol and JH III. Alternatively, it is possible that farnesol and JH III activate PPAR. Indeed, recent studies by our laboratory have shown that farnesol and JH III increase the expression of a PPRE transfected into keratinocytes (unpublished observations). Similar observations have been reported by O'Brien *et al* (1996) who demonstrated that farnesol and farnesoic acid increase PPRE expression in CV-1 cells. Thus, it is possible that farnesol and JH III stimulate epidermal and SC development by activating PPARα or PPARδ. Finally, these compounds could exert their effects via non-nuclear receptor mediated pathways.

Previous studies have shown that fetal epidermis is an active site of both cholesterol and fatty acid synthesis, with synthetic rates high on gestational day 17 and declining with epidermal differentiation and the maturation of the SC (Hurt *et al*, 1995). Thus, endogenous fatty acids, intermediates in the cholesterol synthetic pathway, and oxidation products of cholesterol, are all likely to be present in fetal epidermis early in development, and could serve as activators of nuclear hormone receptors, thereby locally regulating epidermal and SC development. We have previously shown that epidermal maturation and SC formation proceeds normally in skin explants incubated in hormone- and serum-free media, suggesting that locally derived factors may be important mediators of epidermal and SC development (Hanley *et al*, 1996b). Endogenous activation of nuclear hormone receptors

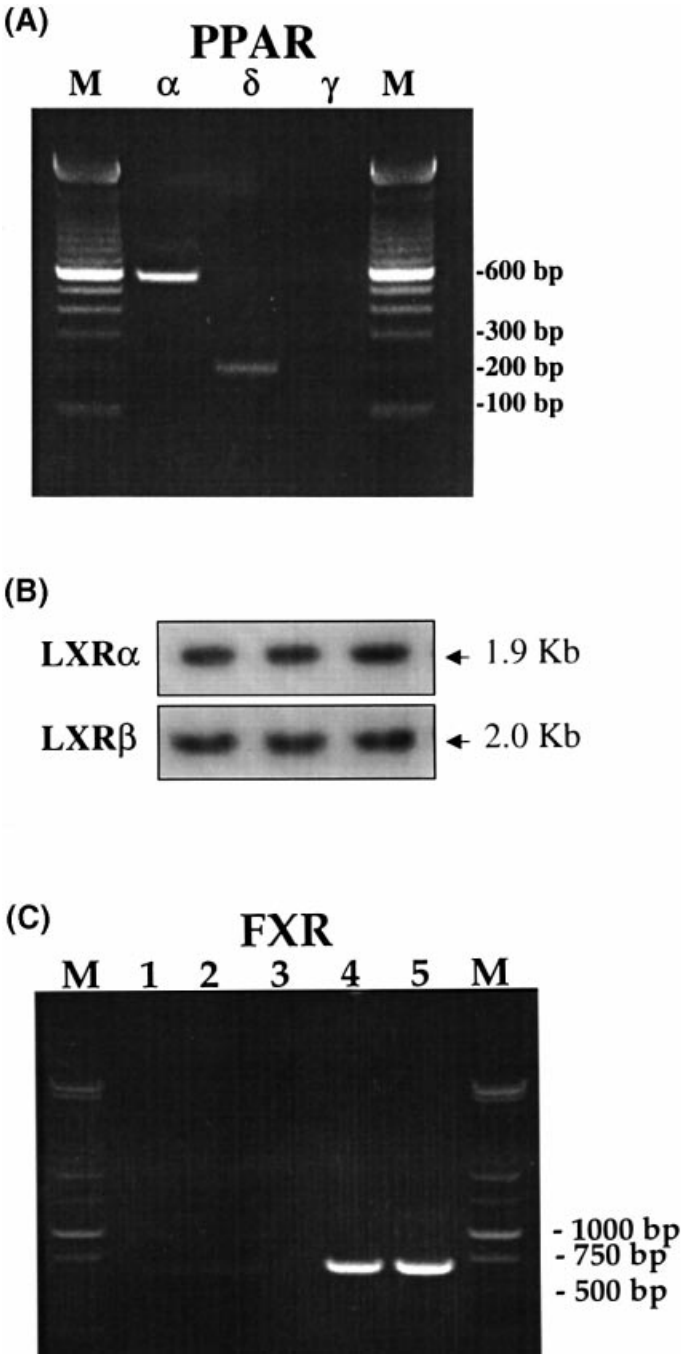

Figure 7. PPARα and PPARδ and LXRα and LXRβ mRNAs in day 17 fetal rat epidermis. (*A*) Total RNA was isolated, reverse transcribed, and amplified by PCR using PPAR-specific primers as described in *Materials and Methods*. Predicted size of PPARα, 568 bp; PPARδ, 191 bp; PPARγ, 420 bp. M = molecular weight markers. (*B*) Poly(A)$^+$ mRNA was isolated as described in *Materials and Methods*. Eight micrograms was loaded per lane, northern analysis performed, and blots hybridized with either an LXRα or LXRβ cDNA as described in *Materials and Methods*. The *arrows* at right represent the approximate size of LXRα- and LXRβ-specific transcripts. (*C*) Reverse transcriptase–PCR using FXR-specific primers (set 1) was performed as described in *Materials and Methods*. M, molecular weight markers; *lanes 1* and *2*, day 17 epidermis; *lane 3*, day 17 full-thickness skin; *lane 4*, kidney (rat); *lane 5*, liver (rat).

may be one pathway by which locally derived factors stimulate epidermal development, suggesting that these receptors may be targets for interventions to reduce morbidity and mortality resulting from epidermal barrier immaturity in very premature infants.

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