The Effect of LXR Activators on AP-1 Proteins in Keratinocytes

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Oxysterols, via activation of liver X receptor (LXR), regulate keratinocyte differentiation by stimulating transglutaminase cross-linking of several constituent proteins leading to the formation of the cornified envelope. We previously reported that oxysterols increase the expression of one of these cross-linked proteins, involucrin, and that this effect can be abolished by mutations of the distal activator protein (AP)-1 response element in the involucrin promoter. Furthermore, oxysterols increase AP-1 binding in an electrophoretic gel mobility shift assay and increase the expression of an AP-1 reporter. In this study, we describe the individual components of the AP-1 complex that are involved in the oxysterol-mediated AP-1 activation and stimulation of keratinocyte differentiation. We identified Fra-1 within the AP-1 DNA binding complex by supershift analysis of nuclear extracts from oxysterol-treated, cultured keratinocytes and confirmed that oxysterol treatment increased the levels of Fra-1 by western blot analysis. Additionally, on Western and Northern analysis, oxysterol treatment increased two other AP-1 proteins, Jun-D and c-Fos, whereas Fra-2, Jun-B, and c-Jun were not changed. Similar alterations in AP-1 proteins occurred when 25-OH-cholesterol or non-steroidal LXR agonists (GW3965, TO-901317) were used. These results indicate that oxysterols induce specific AP-1 proteins, thereby activating involucrin, one of the genes required for epidermal differentiation.

Key words: cornified envelope/differentiation/involucrin/nuclear hormone receptor/transcription factor
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The epidermis is a self-renewing epithelium that undergoes a highly regulated differentiation program culminating in the formation of corneocytes in the outermost layers of the epidermis (Yuspa *et al*, 1989; Fuchs, 1990; Younus and Gilchrest, 1992; Jackson *et al*, 1993; Eckert *et al*, 1997). Along with the lipid-enriched extracellular matrix, these rigid cells form a remarkably durable shield, the stratum corneum (SC), which provides an important protective function to the skin. The SC consists of a protein component, the corneocyte (Kalinin *et al*, 2002), which provides both resistance to external physical forces and a platform for the extracellular matrix, which consists of multi-layers of lipids that mediate several key SC functions, particularly the cutaneous permeability barrier (Jackson *et al*, 1993). The regulation of the underlying differentiation program that leads to this two-compartment structure is incompletely understood. Increased extracellular calcium, subsequently resulting in elevated intracellular calcium, is the most widely recognized signal for the induction of normal keratinocyte differentiation, both *in vitro* and *in vivo* (Gibbons *et al*, 1996; Bikle *et al*, 2001; Elias *et al*, 2002). Among other activities, calcium stimulates the protein kinase C (PKC) pathway, which is known to influence gene expression through the activator protein (AP)-1 (Dlugosz *et al*, 1994; Denning *et al*, 1995; Ruthberg *et al*, 1996). Phorbol esters, such as phorbol 12-myristate 13-acetate (PMA) also stimulate PKC activity, activating AP-1 proteins a sequence that ultimately leads to increased cornification (Welter *et al*, 1995). AP-1 complexes that activate AP-1 response elements comprise members of the Jun and Fos families (Wisdom, 1999; Angel *et al*, 2001). The Fos family includes c-Fos, FosB, Fra-1, and Fra-2, whereas the Jun family includes c-Jun, Jun-B, and JunD. The AP-1 complex is typically comprised of heterodimers between a Fos and a Jun family member, which recognize a 5′-TGAGTCA-3′ consensus sequence in the enhancer region of a variety of genes, including genes that are important for keratinocyte differentiation. Many of the differentiation-related corneocyte proteins, the majority of which are encoded at the epidermal differentiation complex (EDC) on chromosome 1q21 (Volz *et al*, 1993), are regulated by AP-1 response elements in their promoters (Crish *et al*, 1998, 2002).

The ability of AP-1 complexes to interact with an AP-1 response element and to stimulate transcription is controlled by a number of factors, but the primary regulation is at the level of transcription of the genes encoding the AP-1 proteins (Karin, 1995; Wisdom, 1999; Angel *et al*, 2001). Thus, an increase in the levels of AP-1 protein heterodimers generally increases the transcription of AP-1-regulated

Abbreviations: AP, activator protein; LXR, liver X receptor; SC, stratum corneum

genes. Likewise, increases in AP-1 activity may coordinately increase the expression of many of the proteins required for keratinocyte differentiation. Not only calcium, but also several other known stimulators of keratinocyte differentiation activate AP-1. We have recently shown that topical treatment of mice with oxysterols, activators of liver X receptor (LXR), such as 22(R)-OH-cholesterol, stimulates epidermal differentiation. This stimulation of differentiation is specifically mediated by the nuclear hormone receptor, LXR, since it does not occur in mice deficient in LXR-β (Komuves *et al*, 2002). LXR like other members of the class II nuclear hormone receptor family heterodimerizes with RXR, and binds to DR-4 response elements. Two different genes (α and β) are known to encode LXR proteins, and both are activated by oxysterols (Janowski *et al*, 1996; Lehrmann *et al*, 1997). LXR-α and LXR-β are expressed in cultured normal human keratinocytes and in fetal rat skin, but only LXR-β is present in northern blots of normal adult mouse epidermis (Komuves *et al*, 2002). In cultured human keratinocytes, oxysterols increase cornified envelope formation and the expression of both transglutaminase-1 and involucrin (Hanley *et al*, 2000). The increased expression of involucrin can be abolished by either deletion or site-specific mutation of the distal AP-1 response element (−2117 to −2111 bp) within the involucrin promoter region. Moreover, oxysterols increase AP-1 binding in an electrophoretic gel mobility shift assay and increase the expression of an AP-1 reporter construct coupled to luciferase, indicating that LXR activators induce a general increase in expression of AP-1-regulated genes in keratinocytes (Hanley *et al*, 2000a, b). Taken together, these findings suggest that oxysterols activate LXR, thereby increasing the activity of AP-1, which stimulates the expression of involucrin, and perhaps other AP-1-regulated proteins that are crucial for keratinocyte differentiation (Hanley *et al*, 2000). These studies leave unanswered, however, which AP-1 proteins are specifically regulated by LXR activation. In this study, we identified the individual AP-1 proteins that are increased by oxysterol treatment using supershift analysis of proteins in the AP-1 complex. Furthermore, we not only identified which individual members of the AP-1 transcription complex are regulated on the protein level, but also whether regulation occurs at the RNA level using a number of different LXR agonists. Together, our results indicate that the increase in AP-1 activity induced by oxysterols in keratinocytes results from increased expression of Fra-1, Jun-D, and c-Fos.

Results

Fra-1 is the predominant protein in the AP-1 complex of oxysterol-treated keratinocytes A variety of differentiation-promoting agents, such as calcium, phorbol esters, cholesterol sulfate, and oxysterols induce AP-1 DNA binding activity (Eckert and Green, 1986; Fuchs, 1990; Welter *et al*, 1995; Hanley *et al*, 2000; Hanley *et al*, 2001). We previously showed increased binding of nuclear proteins to AP-1 DNA by electrophoretic gel mobility shift analysis (Hanley *et al*, 2000); i.e., addition of nuclear extracts from oxysterol-treated keratinocytes to AP-1 oligonucleotides resulted in a band shift, and the intensity of the band increased in extracts from human cultured keratinocytes treated with oxysterols (Hanley *et al*, 2000). In this study, we employed supershift analysis to determine which AP-1 protein members are present in the oxysterol-induced AP-1 complex. In nuclear extracts from control keratinocytes (Fig 1, *upper panel*), Fra-1 was the predominant supershifted band, but Fra-2 and Jun-D were also observed. In cultured keratinocytes, treated with 10 M 22(R)OH-cholesterol for 24 h, we observed a supershifted band in nuclear extracts from cells incubated with an antibody to Fra-1, which demonstrates that Fra-1 is a predominant protein in the AP-1 complex of these cells (Fig 1). In contrast, no distinct bands were observed when antibodies against Fra-2, Fos-B, c-Fos, Jun-D, c-Jun (cJ), and Jun-B (JB) were pre-incubated with the nuclear extract. These data indicate that the AP-1 complex that is increased in oxysterol-treated keratinocytes contains predominantly Fra-1.

Oxysterols increase Fra-1, Jun-D, and c-Fos protein levels in keratinocytes Although the supershift experiment

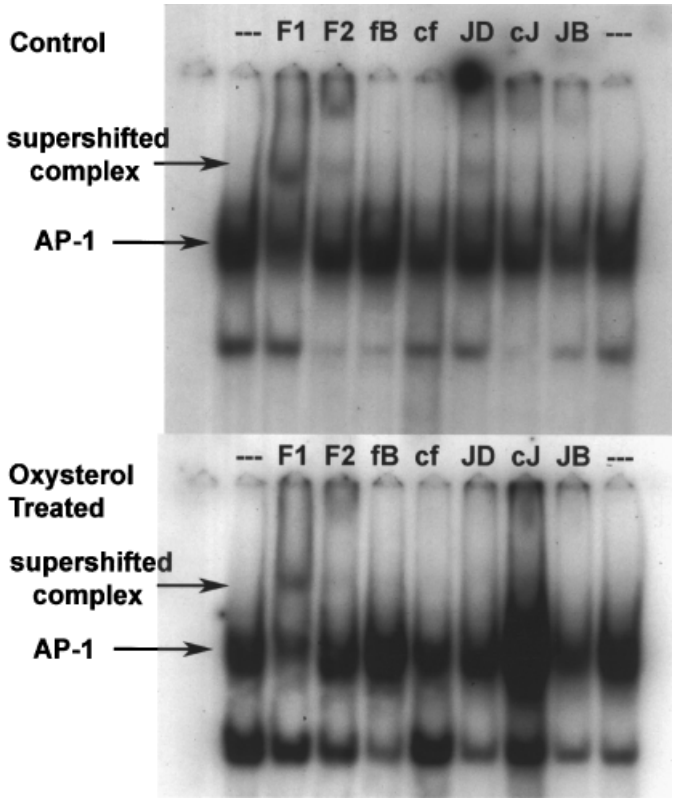

Figure 1
Activator protein (AP)-1–DNA complex composition in oxysterol-treated keratinocytes. Cultured keratinocytes were treated with vehicle (*upper panel*) or 10 μM 22(R)OH-cholesterol (*lower panel*) for 24 h and nuclear extracts were isolated as described in Methods. The extracts were pre-incubated for 2 h at 4°C in the presence of 20 μg of antibody and then incubated in the presence of ^{32}P-labeled AP-1 oligonucleotides as described in Methods. The incubations were electrophoresed on non-denaturing gels, dried, and autoradiographed. The autoradiographs shown are representative of results obtained in three separate experiments. F1, Fra-1; F2, Fra-2; fB, Fos-B; cf, c-Fos; JD, Jun-D; cJ, c-Jun; and JB, Jun-B; ---, negative control (no antibody).

clearly shows Fra-1 content, other AP-1 factors may not be detected by this technique. Therefore, we compared the individual components of the AP-1 complex from oxysterol-*versus* vehicle-treated keratinocytes using western blot analysis. Total cellular protein was isolated from keratinocytes 24 h after treatment with either 22(R)OH-cholesterol (10 μM) or vehicle. Western blot analyses showed that oxysterols increased Fra-1 protein levels (Fig 2). In addition, we observed an increase in JunD protein levels, as well as a moderate increase in c-Fos (Fig 2), both of which were not detectable in the supershift assays. In contrast, the protein levels of Fra-2, c-Jun and Jun-B did not change with oxysterol treatment. Together, these results show that treatment of cultured keratinocytes with the LXR activator, 22(R)OH-cholesterol, increases the protein levels of Fra-1, Jun-D, and c-Fos.

Oxysterols increase mRNA levels of Fra-1, Jun-D, and c-Fos in keratinocytes We next determined whether oxysterol-induced alterations in the AP-1 complex are due to increased RNA levels by northern blot analysis. Treatment of human-cultured keratinocytes with 22(R)OH-cholesterol (10μM) for 24 h increased mRNA levels of Fra-1, c-Fos, and Jun-D ($p < 0.05$, Fig 3), whereas, in contrast, oxysterol treatment did not affect mRNA levels of Fra-2, Jun-B, and c-Jun. These results show that the oxysterol-induced increase in Fra-1, c-Fos, and Jun-D proteins can be attributed to increased mRNA levels for these factors. To assess further, whether these changes in AP-1 proteins can be attributed to activation of LXR, we next assessed changes in AP-1 factor mRNA levels after treatment with a number of alternative LXR agonists. We observed a comparable induction of Fra-1, when keratinocytes were treated not only with another naturally occurring oxysterol, 25-OH-cholesterol ($p < 0.01$), but also with two additional, non-sterol activators of LXR (i.e., GW3965 and TO-901317) ($p < 0.05$, Fig 4). Moreover, as with the oxysterols, 22(R)OH-cholesterol, the non-steroidal LXR activator, TO-901317, also induced Jun-D and c-Fos mRNA expression ($p < 0.01$, Fig 5). Together, these results show that LXR activation increases Fra-1, Jun-D, and c-Fos in cultured keratinocytes, but only Fra-1 increased in the complex binding the AP-1 site contained by the oligonucleotide.

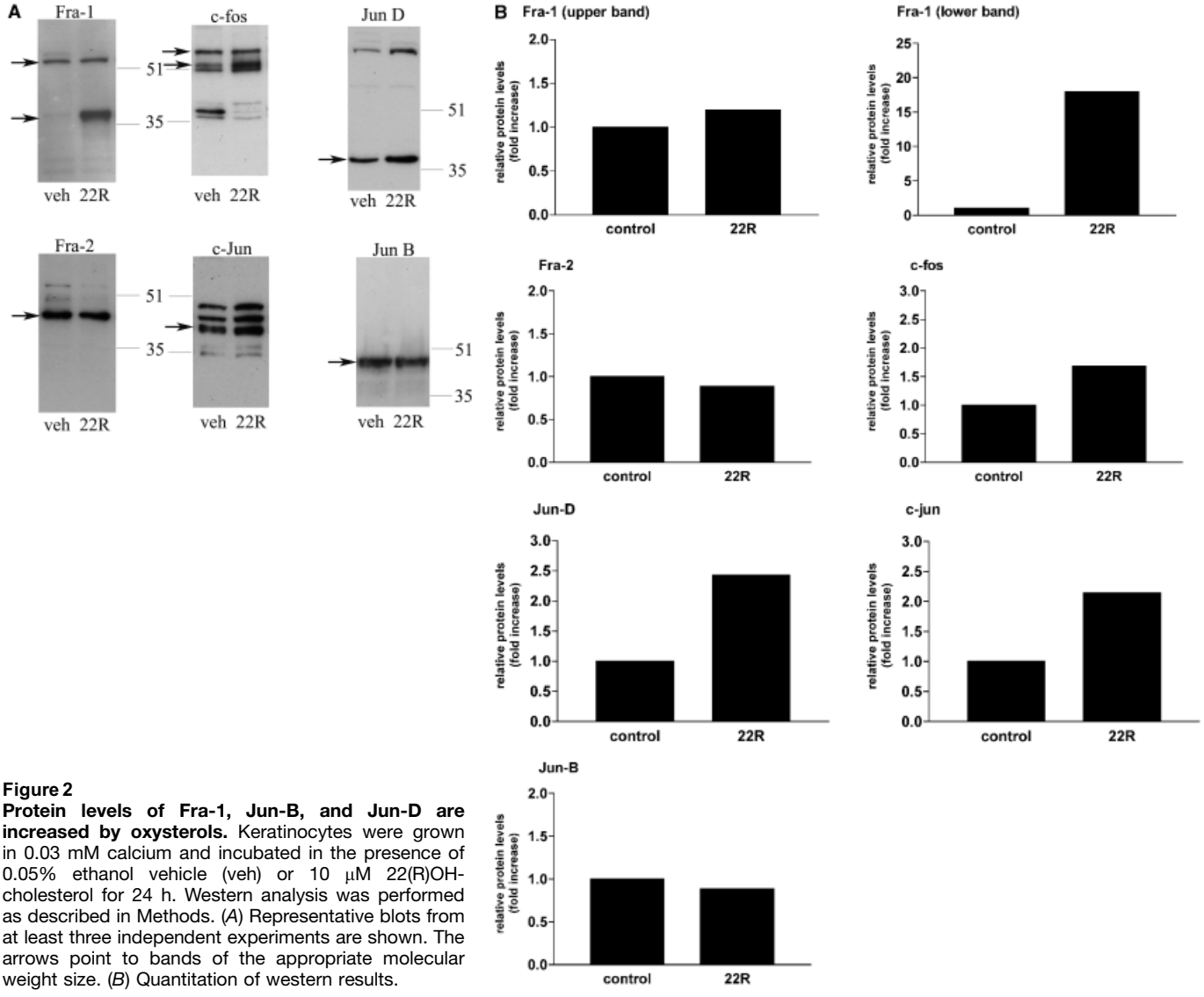

Figure 2
Protein levels of Fra-1, Jun-B, and Jun-D are increased by oxysterols. Keratinocytes were grown in 0.03 mM calcium and incubated in the presence of 0.05% ethanol vehicle (veh) or 10 μM 22(R)OH-cholesterol for 24 h. Western analysis was performed as described in Methods. (*A*) Representative blots from at least three independent experiments are shown. The arrows point to bands of the appropriate molecular weight size. (*B*) Quantitation of western results.

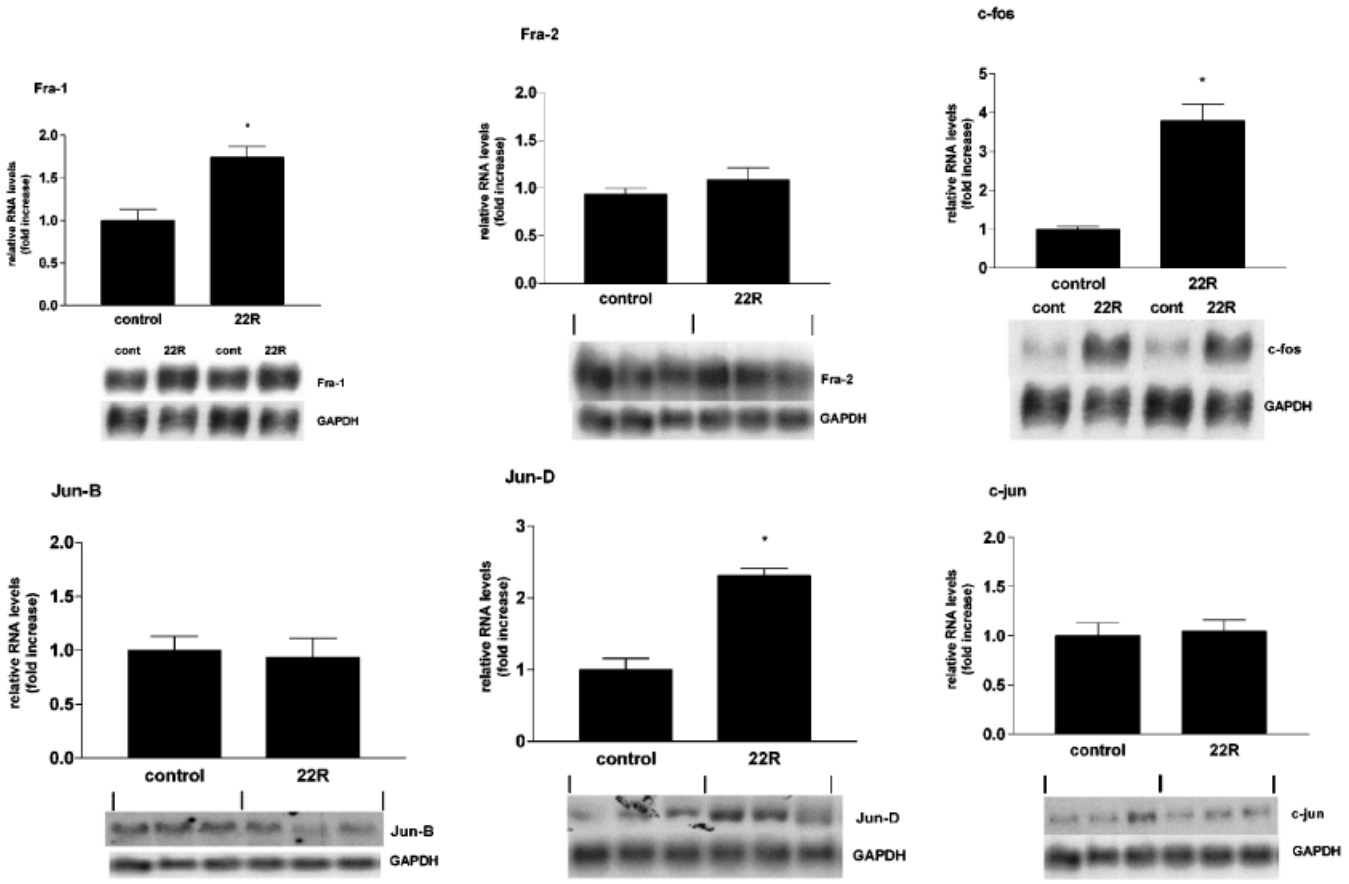

Figure 3
mRNA levels of Fra-1 and Jun-D are increased by oxysterols. Keratinocytes were grown in 0.03 mM calcium and incubated in the presence of 0.05% ethanol vehicle (veh) or 10 μM 22(R)OH-cholesterol for 24 h. Total RNA (Fra-1, Fra-2, c-Fos, Jun-D) or Poly(A) + mRNA (Jun-B, c-Jun) was isolated and northern analysis was performed as described in Methods. Representative blots are shown. Graphs are representative of two to three independent experiments and show the results of densitometric analysis of specific bands for the respective genes expressed as relative expression compared to housekeeping genes. Data are presented as mean ± SEM ($n = 3-4$). $^*p < 0.05$.

Discussion

A large number of different genes essential for keratinocyte differentiation are regulated by AP-1 response elements. The expression of the major proteins that comprise the cornified envelope, including loricrin, involucrin, and small proline-rich proteins (SPRR), as well as the enzyme primarily responsible for protein cross-linking of the cornified envelope, transglutaminase-1, are all stimulated by activation of AP-1 (Yamada *et al*, 1994; Disepio *et al*, 1995; Eckert *et al*, 1997; Lohman *et al*, 1997; Banks *et al*, 1998; Sark *et al*, 1998; Disepio *et al*, 1999; Fischer *et al*, 1999; Jessen *et al*, 2000; Johansen *et al*, 2000; Ng *et al*, 2000; Phillips *et al*, 2000). In addition, the expression of differentiation-linked, cytosolic proteins, such as profilaggrin and certain keratins, e.g., K1, K5, and K6, are also stimulated by AP-1 activation (Rothnagel *et al*, 1993; Casatorres *et al*, 1994; Lu *et al*, 1994; Navarro *et al*, 1995; Jang *et al*, 1996; Ma *et al*, 1997). Since many treatments, including increased calcium, phorbol esters, and cholesterol sulfate, that are known to induce keratinocyte differentiation, also stimulate AP-1 activity, the AP-1 mechanism appears central to the whole scenario of epidermal differentiation. Yet, while all Fos and Jun members of the AP-family are expressed in keratinocytes, the various stimuli of differentiation activate divergent spectra of AP-1 proteins (Rossi *et al*, 1998; Angel *et al*, 2001; Yates and Rayner, 2002). For example, high extracellular calcium, which is well known to promote keratinocyte differentiation, increases c-Fos and actually decreases Fra-1 (Ng *et al*, 2000). In contrast, cholesterol sulfate stimulates DNA binding of AP-1–complexes that express increased Fra-1, Fra-2, and Jun-D (Hanley *et al*, 2001).

Previous studies have shown that oxysterols, by activating LXR, also stimulate keratinocyte differentiation. Specifically, in cultured human keratinocytes, oxysterols increase the expression of involucrin, and this increase requires an intact distal AP-1 response element (−2117 to −2111 bp) (Hanley *et al*, 2000). Additionally, these experiments also showed that treatment of keratinocytes with oxysterols increased the binding of nuclear extracts to an AP-1 response element (increased gel shifting) and increased the transactivation of an AP-1 response elements linked to luciferase (increased AP-1 reporter expression). In this study, we confirm that oxysterols increase the binding of nuclear extracts to that same AP-1 response element, and demonstrate further, using a supershift assay, that the

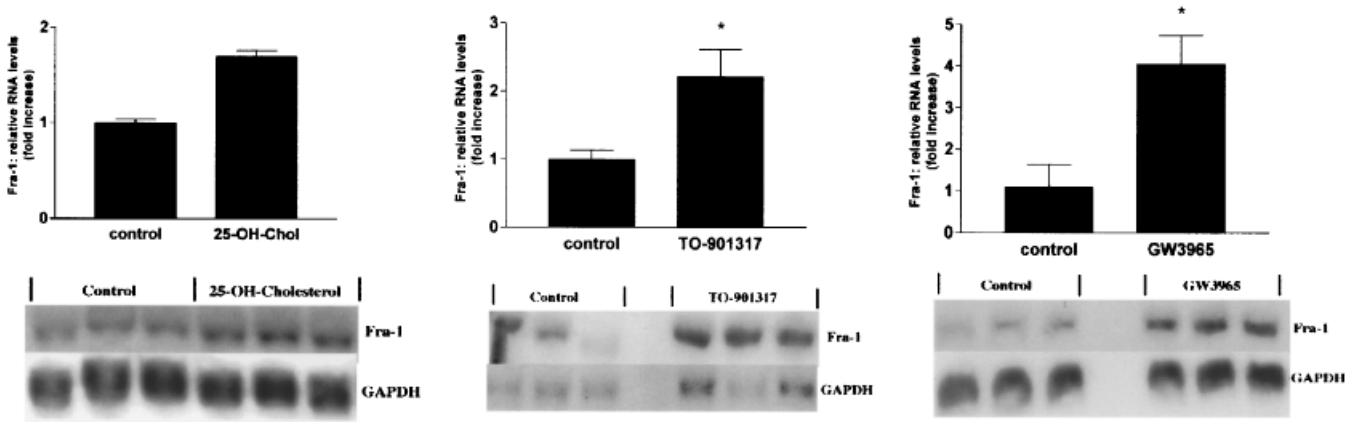

Figure 4
Non-sterol liver X receptor agonists induce Fra-1 mRNA expression. Keratinocytes were grown in 0.03 mM calcium and incubated in 5 μM 25-OH-cholesterol, 10 μM TO-901317 for 24 h. Total RNA was isolated and northern analysis was performed as described in Methods. Representative blots are shown. Graphs show the results of densitometric analysis of specific bands for Fra-1 expressed as relative expression compared to housekeeping genes. Data are presented as mean ± SEM ($n = 3–4$). $^*p < 0.05$.

AP-1 complex contains predominately Fra-1. Moreover, using Western and Northern blot analysis, we show that the protein and mRNA levels of Fra-1 increase in keratinocytes treated with either oxysterol or non-sterol activators of LXR. In addition, we further demonstrate that the protein and mRNA levels of Jun-D and c-Fos increase in keratinocytes treated with LXR activators. These studies indicate that LXR activators increase the levels of specific AP-1 proteins that can then activate gene transcription by binding to AP-1 response elements in the promoters of various genes. Whereas supershifting detects Fra-1 predominantly, using Western and Northern blotting we demonstrate that an increase in Jun-D and c-Fos also appears to play a role. These differences between the supershift experiments and Western blotting are most likely due to differences in assay sensitivity (gel shift *vs* Western blot). It is well recognized that supershifting analysis is not as sensitive as Western blotting for determining the levels of a specific protein in cells. Together, the results show that different spectra of AP-1 proteins are stimulated in response to various inducers of differentiation, suggesting that a variety of mechanistic pathways can activate AP-1 in keratinocytes.

In the epidermis, a multi-step process of keratinocyte differentiation ultimately results in the formation of the outermost sheath of the skin, the SC, which consists of protein-enriched corneocytes embedded in a continuous, lipid-enriched, extracellular matrix (Jackson *et al*, 1993). The combination of corneocytes (the bricks) and extracellular lipid membranes (the mortar) provides a barrier between the organism and the environment that is required for survival (Jackson *et al*, 1993). Corneocytes are formed when keratinocytes lose their nucleus above the stratum granulosum, and deposit a 10-nm thick, insoluble, external protein envelope that replaces the plasma membrane, termed the cornified envelope (CE) (Kalinin *et al*, 2002). This structure provides both rigidity and strength to the skin, while providing a scaffold for the supramolecular organization of the extracellular lipid matrix. The lipids in the extracellular spaces of the SC derive from the secretion of the lamellar body (LB) contents from stratum granulosum cells. Both the hydrophobicity of these lipids and their organization into membrane bilayers account for the barrier to water movement (Jackson *et al*, 1993). The formation of LB requires lipids, including cholesterol, which derive mainly from local epidermal lipid synthesis, but some lipids, e.g., essential fatty acids, derive from extracutaneous sites (Feingold, 1991). Whereas the protein *versus* lipid arms of keratinocyte differentiation that lead to SC formation are traditionally viewed as concurrent, but independent processes, the ability of oxysterol metabolites of cholesterol to activate LXR, and to stimulate the expression of proteins that comprise the cornified envelope, suggests a mechanism for cross-talk between the two arms of keratinocyte differentiation (Fig 6). Similarly, the ability of cholesterol sulfate, another compound synthesized from cholesterol, to stimulate keratinocyte differentiation provides further support for the coordination of the protein and lipid arms of keratinocyte differentiation. One can speculate that as cholesterol levels increase in stratum spinosum/granulosum cells for lamellar body formation, would also result in increased generation of its metabolic products, including oxysterols and cholesterol sulfate. In fact, cholesterol sulfate levels are known to peak in the granular layer (Elias *et al*, 1984). Thus, these molecules are in a position to regulate keratinocyte differentiation by the coordinated formation of the dual-compartment SC.

In summary, these results suggest that activation of LXR by oxysterols stimulates keratinocyte differentiation by inducing specific AP-1 transcription factors, which in turn activate the genes required for the differentiation process (Fig 6). Oxysterols may be important signal molecules in the epidermis that can coordinately regulate keratinocyte differentiation by increasing AP-1 activity.

Materials and Methods

Reagents The natural LXR activators, 22(R)OH-cholesterol and 25-OH-cholesterol, were purchased from Sigma Chem. Co. (St Louis, Missouri). *N*-(2,2,2-trifluoro-ethyl)-*N*-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]-benzenesulfonamide (TO-901317)

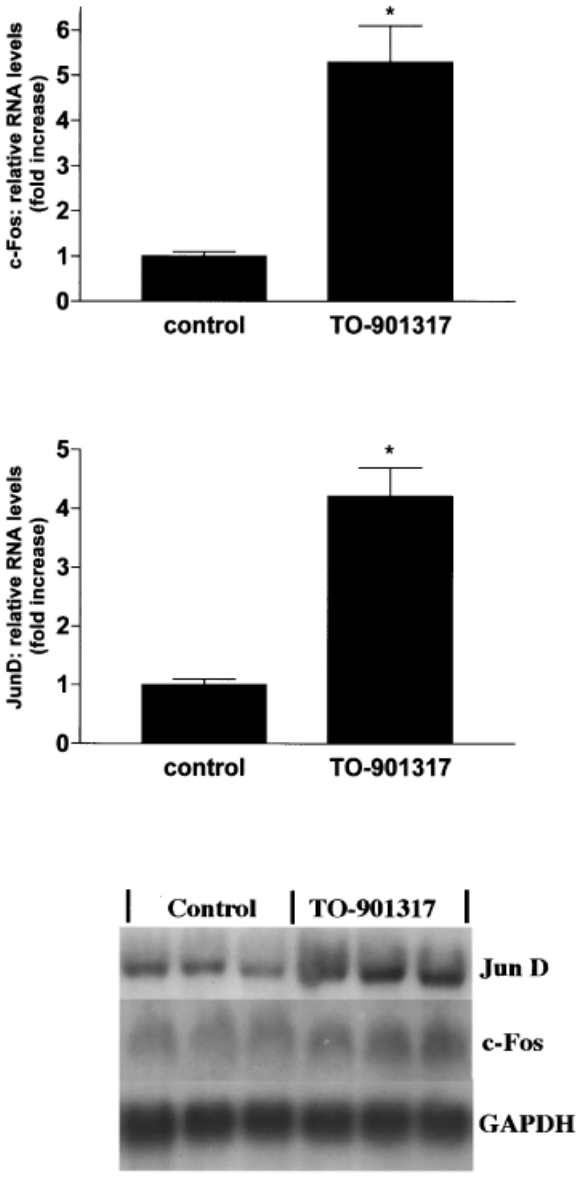

Figure 5
Non-sterol liver X receptor agonist induces c-Fos and JunD mRNA expression. Keratinocytes were grown in 0.03 mM calcium and incubated in 10 μM TO-901317 for 24 h. Total RNA was isolated and northern analysis was performed as described in Methods. Representative blots are shown. Graphs show the results of densitometric analysis of specific bands for the respective genes expressed as relative expression compared to GAPDH. Data are presented as mean ± SEM ($n = 3$–4). $^{*}p < 0.05$.

is a synthetic, non-steroidal LXR activator and was purchased from Cayman Chemical (Ann Arbor, Michigan). GW3965 was synthesized at GlaxoSmithKline (Research Triangle Park, North Carolina) and diluted in an ethanol vehicle for *in vitro* (Collins *et al*, 2002) experiments. [32P] dCTP (3000 Ci per mmol, 10 mCi per mL) was purchased from NEN Research Products (Boston, Massachusetts). Minispin columns (G50) were purchased from Worthington Biochemical (Freehold, New Jersey). cDNA for rat cyclophilin was kindly provided by Dr G. Strewler (Harvard Medical School, Massachusetts). Fuji RX (Fuji, Vahalla, NY) film was used for autoradiography.

Cell culture Human epidermis was isolated from newborn foreskins, and keratinocytes were plated in serum-free keratinocyte growth medium (KGM; Clonetics, San Diego, California), as

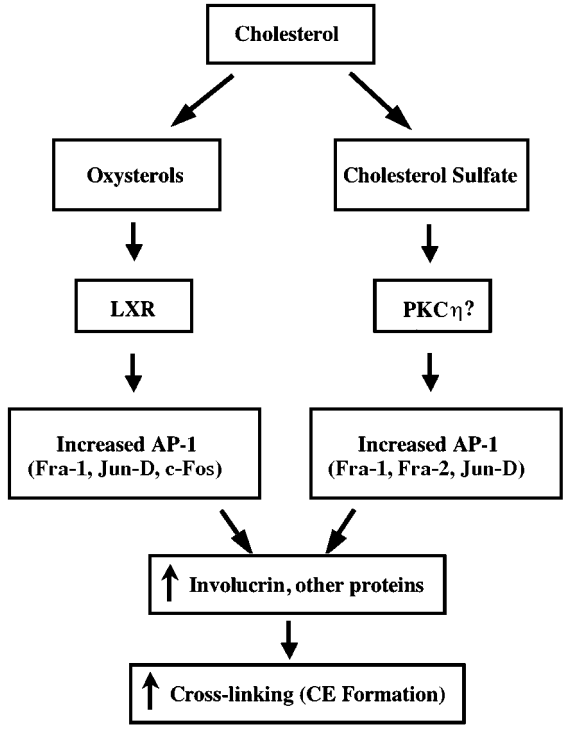

Figure 6
Proposed flow chart for the effects of sterol metabolites on keratinocyte differentiation. Activation of liver X receptor (LXR) by increased oxysterols and cholesterol sulfate stimulate keratinocyte differentiation by inducing specific activator protein (AP)-1 transcription factors, which in turn activate the genes required for the differentiation process.

described previously (Hanley *et al*, 2001). Second passage cells were seeded and maintained in 0.07 mM Ca^{++} KGM. When the cells were approximately 60% confluent, the medium was changed in the presence/absence of specific treatment and cells were cultured for 24 h.

Electrophoretic gel mobility shift assays Nuclear proteins were prepared using the method described previously (Hanley *et al*, 2001). The AP-1 oligonucleotides used were 5′-TCGA-TATGCCG-TGAGTCA-GAGGGC-3′ and 5′-TCGA-GCCCTC-TGACTCA-CGG-CATA-3′; (AP-1 sites are underlined). Double-stranded oligonucleotides were end labeled using 50 μCi of ^{32}P-ATP (3000 Ci per mmol) in the presence of T4 polynucleotide kinase (Amersham Life Sci., Illinois) for 60 min at 37°C and were purified using G-50 microcolumns (Amersham). Binding reactions were performed for 30 min at 4°C using 5 μg nuclear extracts, buffer (10 mM Tris, pH 7.5, 1 mM DTT, 1 mM EDTA, 5% glycerol), 2 μg poly-(dI-dC), and 60,000 cpm of ^{32}P-labeled oligonucleotide in a final volume of 15 μL. Radioinert competitor DNA was added at a 100-fold molar excess. For supershift analyses, 5 μg of nuclear extract was pre-incubated for 2 h at 4°C with 20 μg of antibody (Fra-1, Fra-2, Fos-B, c-Fos, Jun-D, c-Jun, or Jun-B; all from Santa Cruz Biotech, California). The samples were electrophoresed for 1.5 h on 5% acrylamide gels in a 0.5 × TBE buffer, dried, and autoradiographed.

Western blot analysis Levels of different proteins forming the AP-1 complex were assessed by protein electrophoresis and western blotting. Briefly, 25 μg of nuclear extract was fractionated in 10% SDS-PAGE and transferred onto a PVDF membrane (Hybond P, Amersham), which was then incubated in PBS with 5% non-fat dry milk and 0.05% Tween 20 for 1 h at 20°C. The membrane was then incubated for 16 h at 4°C with rabbit antibodies (1:5000, Santa Cruz Biotech) specific for each AP-1 family member. Following four washes in 0.05% Tween 20 in PBS for 15 min each, the membranes were incubated with horseradish peroxidase conju-

gated goat anti-rabbit Ig antibodies (NA934, Amersham) for 1 h at 20°C. After washing, membranes were incubated with SuperSignal Ultra chemiluminescent substrate (Pierce, Rockford, Illinois) and then exposed to film. Pre-stained standards (BioRad, Hercules, California) were used to determine molecular weight.

Northern blot analysis Total RNA was isolated by using Tri-Reagent (Sigma) following the manufacturer's protocol. Poly-A RNA was isolated using oligo(dT) cellulose. RNA yields were quantified by measuring absorption at 260 nm. After fractionation through a 1.2% agarose–formaldehyde gel, RNA integrity in the ethidium bromide-stained gels was assessed under a fluorescent light source and the RNA was transferred to a nylon membrane (NytranSuPerCharge, Schleicher & Schuell, Dassel, Germany). Membranes were pre-hybridized for 1 h at 65°C in Ultrahyb (Ambion, Austin, Texas). Hybridization was conducted in the same buffer in the presence of ^{32}P-radiolabeled partial cDNA fragments coding for AP-1 proteins, as indicated. Probes were ^{32}P-labeled by random priming (RediPrime, Amersham). After overnight hybridization, the membranes were washed for 30 min in 0.2 × SSC, 0.1% SDS at room temperature and subsequently for 60 min at 65°C. Autoradiography was performed at −70°C. Bands of the reported size were quantified by densitometry using QuantityOne Software (BioRad, Hercules, California). Blots were probed with GAPDH or cyclophilin to confirm equal loading. cDNA probes comprised human Fra-2 (clone from the American Type Culture Collection), rat Fra-1, human c-Jun, human Jun-B, mouse Jun-D, and rat c-Fos (gift from Dr D. Gardner, University of California, San Francisco).

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

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