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Parallel regulation of sterol regulatory element binding protein-2 and the enzymes of cholesterol and fatty acid synthesis but not ceramide synthesis in cultured human keratinocytes and murine epidermis

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Abstract After permeability barrier perturbation there is an increase in the mRNA levels for key enzymes necessary for lipid synthesis in the epidermis. The mechanism(s) responsible for this regulation is unknown. Sterol regulatory element binding proteins-1a, 1c, and -2 (SREBPs) control the transcription of enzymes required for cholesterol and fatty acid synthesis in response to modulations of sterol levels. We now demonstrate that SREBP-2 is the predominant SREBP in human keratinocytes and murine epidermis, while SREBP-1 is not detected. Sterols regulate SREBP-2 mRNA levels in keratinocytes and the epidermis and the proteolytic cleavage of SREBP-2 to the mature active form in keratinocytes. In parallel to the increase in mature active SREBP, there is a coordinate increase in mRNA levels for cholesterol (HMG-CoA reductase, HMG-CoA synthase, farnesyl diphosphate synthase, and squalene synthase) and fatty acid (acetyl-CoA carboxylase, fatty acid synthase) synthetic enzymes. However, mRNA levels for serine palmitoyl transferase (SPT), the first committed step for ceramide synthesis, do not increase in parallel. The increase of mRNA for enzymes required for epidermal cholesterol and fatty acid synthesis is consistent with both the previously described early increase of cholesterol and fatty acid synthesis after barrier disruption and a role for SREBP-2 in the regulation of cholesterol and fatty acid synthesis for epidermal barrier homeostasis. In contrast, SPT appears to be regulated by different mechanisms, consistent with the different time course of its stimulation after barrier disruption.—**Harris, I. R., A. M. Farrell, W. M. Holleran, S. Jackson, C. Grunfeld, P. M. Elias, and K. R. Feingold.** Parallel regulation of sterol regulatory element binding protein-2 and the enzymes of cholesterol and fatty acid synthesis but not ceramide synthesis in cultured human keratinocytes and murine epidermis. *J. Lipid Res.* 1998. **39:** 412–422.

Supplementary key words HMG-CoA reductase • HMG-CoA synthase • fatty acid synthase • serine palmitoyl transferase

Keratinocytes synthesize large quantities of lipids both for cell growth and for maintenance of the epidermal permeability barrier (1). The lipids of the stratum corneum, which are critical ingredients of the barrier, comprise mainly cholesterol, fatty acids, and ceramides (2, 3). Lipid synthesis increases rapidly, within 1–2 h after barrier abrogation, in parallel with increased activities of HMG-CoA reductase, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), key enzymes of cholesterol and fatty acid synthesis, respectively (4–7). In contrast, the increase in sphingolipid synthesis and the activity of serine palmitoyl transferase (SPT), the first committed enzyme in sphingolipid synthesis, are delayed (approximately 6 h) after barrier disruption (8). Recent studies have demonstrated that mRNA levels for HMG-CoA reductase, HMG-CoA synthase, farnesyl diphosphate synthase (FPPS), squalene synthase, ACC, FAS, and SPT all increase after barrier disruption (9, 10). As the mRNA levels for these proteins increase after either tape stripping or acetone treatment, these changes are independent of the method of barrier perturbation (9, 10). Moreover, with the exception of FPPS, the increase in mRNA levels for these enzymes is prevented by immediate occlusion with a Latex wrap which provides an artificial permeability barrier (10). These data indicate that the increase in mRNA levels for these enzymes is regulated by barrier function rather than simply representing an injury response.

Abbreviations: TEWL, transepidermal water loss; SREBP, sterol regulatory element binding protein; FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase; SPT, serine palmitoyl transferase; FPPS, farnesyl diphosphate synthase; SRD, sterol regulation defective; LPDS, lipoprotein-deficient serum.

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However, the mechanism(s) by which the mRNA levels for these enzymes are regulated in keratinocytes is unknown.

The recent discovery of sterol regulatory element binding proteins-1a, -1c, and -2 (SREBPs), transcription factors that regulate cholesterol synthesis in response to sterols, has provided important insights into the regulation of lipid synthesis (11). SREBP-1 and -2 are separate gene products of approximately 125 kDa located in the membranes of the endoplasmic reticulum and nuclear envelope. The gene for SREBP-1 has two different transcription start sites that generate two mRNAs and proteins, SREBP-1a and -1c. Whereas SREBP-1 and -2 are both detected in placenta, muscle, kidney, and liver, SREBP-2 alone is detected in heart, brain, and lung (12, 13). The proportions of SREBP-1a and -1c also are tissue-dependent (14). It is hypothesized that the relative proportions of SREBP-1a, -1c, and -2 may determine the response of specific tissues to changing sterol levels.

Activation of SREBPs is regulated primarily by cellular sterol levels (11). When the sterol content of the cell decreases, two proteases clip the membrane spanning loop of the precursor SREBP, releasing the mature NH_2-terminal ~68 kDa fragment, which then is free to enter the nucleus and bind to the sterol response element (SRE) in the promoter of genes important for cholesterol homeostasis. Inhibitors of cholesterol synthesis, such as lovastatin and fluindostatin, which reduce the level of endogenous sterols, stimulate proteolytic cleavage of SREBPs to their mature active forms (11). Conversely, proteolytic cleavage of SREBP to the mature active form is inhibited by incubating cells with 25-OH-cholesterol or cholesterol. SREBPs stimulate the transcription of key genes of cholesterol metabolism such as the LDL receptor, HMG-CoA synthase. HMG-CoA reductase, FPPS, and squalene synthase (11–13, 15–18). Recent studies have demonstrated that SREBPs also regulate the transcription of ACC and FAS, key enzymes for fatty acid synthesis (19–23). The simultaneous regulation of enzymes in both cholesterol and fatty acid synthesis pathways may allow for coordinate regulation of the synthesis of both lipid species.

The purposes of the present study were to determine *1*) which SREBP transcripts are predominant in the epidermis and cultured keratinocytes; *2*) whether manipulation of sterol levels in cultured keratinocytes regulates the transcription of SREBPs and their activity; *3*) whether altering sterol levels in the epidermis and keratinocytes regulates mRNA levels for enzymes required for cholesterol and fatty acid synthesis; and lastly *4*) whether SPT and sphingolipid synthesis are also regulated by a SREBP-dependent mechanism.

MATERIALS AND METHODS

Hairless male mice (Crl:SKHI-hrBR) were purchased from Charles River Laboratories (Wilmington, MA). Molecular Biology grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and Fischer Scientific (Fairlawn, NJ). [α-^{32}P] dCTP (3000 Ci/mmol, 10 mCi/ml) was purchased from NEN Research Products (Boston, MA). The Multiprime Labeling System was purchased from Amersham International (Amersham, UK). Mini-spin columns (G-50) were purchased from Worthington Biochemical Corporation (Freehold, NJ). Oligo(dT)-cellulose, type 77F, was purchased from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). Nytran Plus and Protran nitrocellulose membrane was purchased from Schleicher and Schuell (Keene, NH). Western light chemiluminescent detection kit was purchased from Tropix (Bedford, MA). BCA protein assay kit was purchased from Pierce (Rockford, IL). Spin-X centrifuge filters were purchased from Corning Costar Corporation (Cambridge, MA). cDNA for HMG-CoA reductase (pH Red-102 ATCC No. 57042), SREBP-1, and SREBP-2 (ATCC No 87012, 79816) were purchased from the American Type Tissue Culture Collection (Rockville, MD). cDNA for rat HMG-CoA synthase (LA IIA), and FPPS (CR39), were kindly provided by Dr. P. Edwards, University of California, Los Angeles, CA. cDNA for rat squalene synthase was kindly provided by Dr. I. Shechter, Uniformed Services, University of Health Services, Bethesda, MD. cDNA for rat ACC was kindly provided by Dr. K-H. Kim, Purdue University, Lafayette, IN. cDNA for rat FAS was kindly provided by Dr. C. M. Amy and Dr. S. Smith, Oakland Children's Hospital, CA. cDNA for mouse SPT (LCB2) was kindly provided by Dr. R. C. Dickson, University of Kentucky, Lexington, KY. cDNA for human actin (pHFβA1) was kindly provided by Dr. A. Pollock, University of California, San Francisco, CA. cDNA for rat cyclophilin cDNA (pCD15:8-1) was kindly provided by Dr. G. Strewler, Harvard Medical School, Boston, MA. IgG-IC6 SREBP-2 monoclonal antibody was kindly provided by Drs. M. S. Brown and J. Goldstein, Southwestern University, Dallas, TX. Fuji RX film was used for autoradiography.

Topical application of fluindostatin

The skin of 6–8-week-old male hairless mice was treated daily for 6 days with vehicle (propylene glycol–ethanol, 7:3) with and without 1.5 mg fluindostatin, with and without 1.5 mg cholesterol and 0.1 mg 25-OH-cholesterol. Transepidermal water loss (TEWL) levels were measured immediately before treatment, using a Meeco electrolytic water analyzer, as described previously (4–6). Animals with average TEWL rates of less

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than 0.3 mg/cm^2 per h (normal < 0.3 mg/cm^2 per h) at the end of treatment were included in this study (i.e., only animals with normal barrier function were studied). Previous studies by our laboratory have shown that prolonged treatment with topical HMG-CoA reductase inhibitors increases TEWL (24, 25). Because increases in TEWL stimulate lipid synthesis, in the present studies we have specifically avoided utilizing animals with altered TEWL rates.

Isolation of the epidermis

Four hours after the last treatment, the animals were killed by Isoflurane anesthesia (Abbott Laboratories, Chicago IL) and the skin was excised. The subcutaneous fat was removed by scraping with a scalpel blade, and the skin was then placed in 10 mm EDTA in calcium- and magnesium-free PBS, pH 7.4, for 35 min at 37°C. The epidermis was removed by scraping and snap-frozen in liquid nitrogen and stored below −70°C.

Isolation of epidermal mRNA and Northern blotting

Total RNA was prepared by a variation of the guanidinium thiocyanate method as described previously (9). Total RNA was purified and added to oligo(dT)-cellulose to obtain poly (A)$^+$ RNA. Quantitation was determined by measuring the absorbance at 260 nm. Northern blots were prepared and hybridization was carried out as described previously (9). Film exposed in the linear range of sensitivity was analyzed using a Bio-Rad laboratories (Hercules, CA) densitometer. The densitometry quantitation was adjusted for actin or cyclophilin levels on the same blot and expressed as fold of control, where the control equals one.

Cell culture

Keratinocytes. Normal human foreskin keratinocytes, second passage, were maintained in 0.07 mm Ca^{2+} KGM. At confluence keratinocytes were incubated for 12 or 24 h with 10 μm lovastatin, 12.5 μm 25-OH cholesterol, or vehicle alone (<0.1% ethanol). Trypan blue exclusion assays demonstrated that these treatments did not affect cell viability.

SRD Cells. SRD-2 and SRD-6 cells were cultured in DMEM/Hams F12 (1:1) medium supplemented with 5% LPDS and either 25-OH-cholesterol (0.5 μg/ml) or cholesterol (5 μg/ml) and mevalonic acid (100 μm) as described previously (16).

Isolation of poly (A)$^+$ RNA from keratinocytes

Poly (A)$^+$ RNA was isolated using a variation of the proteinase-K extraction method. Briefly, cells from three 100-mm dishes were washed with PBS and scraped into 5 ml solution A (0.5 m NaCl, 10 mm Tris, pH 7.5, mm EDTA, 1% SDS, and 200 μg/ml proteinase-K). The viscosity of the solution was reduced by passing through a 25-gauge needle, and then incubating for 1 h at 37°C. Oligo(dT)-cellulose, 7.5 mg, was added to each sample and incubated for 1 h. The oligo(dT)-cellulose was washed and the poly (A)$^+$ RNA was eluted. Northern blots were prepared as described previously (9).

Isolation of total RNA from SRD cells

Total RNA was isolated from confluent 100-mm dishes using Trizol Reagent (Gibco BRL) according to the manufacturer's instructions. Ten μg of total RNA was resolved on 1% agarose/formaldehyde gels and probed as described previously (16).

Preparation of Microsomes

Cultured keratinocytes were washed with PBS and scraped from three 100-mm dishes in 0.5 ml/dish homogenizing buffer (0.25 m sucrose, 15 mm EGTA, 15 mm EDTA, 10 mm Tris-HCl, pH 7.0, 5 mm DTT, 5 μg/ml pepstatin, 2.6 μg/ml aprotinin, 0.5 mm phenylmethylsulfonyl fluoride (PMSF), 25 μg/ml leupetin, 25 μg/ml N-acetyl-leucyl-leucyl-norleucinal (ALLN), and sonicated for two 10-sec bursts at 35% intensity. The homogenate was removed and centrifuged at 10,000 *g* for 15 min at 4°C; the supernatant was removed and centrifuged at 100,000 *g* for 1 h at 4°C, and the microsomes were stored at −70°C. The pellets were resuspended in 50 μl lysis buffer (4% SDS, 5 μg/ml pepstatin, 2.6 μg/ml aprotinin, 0.5 mm PMSF, 25 μg/ml leupetin, 25 μg/ml ALLN). Protein content was estimated using the BCA method (Pierce) with BSA standards.

Immunoblot analysis

Microsomal protein, 10 μg, was mixed with an equal volume of loading buffer (10% glycerol, 0.1% bromophenol blue, 0.05 m Tris-HCl, pH 6.8, 25 mm DTT) prior to SDS-PAGE using 7.5% mini-acrylamide gels. After electrophoretic separation, protein was transferred to a nitrocellulose membrane. Monoclonal antibody, 5 μg/ml IgG-IC6, against human SREBP-2 COOH-terminal (amino acids 833–1141) was used followed by an alkaline phosphatase-conjugated secondary antibody and chemiluminescent detection, as described in the manufacturer's instructions (Tropix, Bedford, MA).

Statistics

Statistical significance was determined using a two-tailed unpaired Student's *t*-test; a Mann-Whitney U test also was used when the SD of the two groups was significantly different. Results are expressed as mean ± standard error of the mean (SEM).

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RESULTS

SREBP-2 is the predominant sterol regulatory element binding protein in the epidermis

To determine whether either of the SREBP-1 isoforms and/or SREBP-2 are present in murine epidermis, we first probed poly $(A)^+$ Northern blots with their respective cDNA probes. Two transcripts of 4.0 and 5.4 kb for SREBP-2 were detected (**Fig. 1A**), as previously described in liver (13). In contrast, SREBP-1 mRNA was not detected, despite its abundance in murine liver (Fig. 1B). Thus, SREBP-2 is the most abundant SREBP in epidermis, and therefore it is likely to be the major regulator of epidermal cholesterol and fatty acid synthesis in response to changes in sterols.

Epidermal SREBP-2 expression is regulated by sterol levels

To determine the effect of sterol levels on SREBP-2 mRNA levels, mice were treated topically with fluindostatin which inhibits the activity of HMG-CoA reductase, thereby reducing epidermal sterol levels. As permeability barrier requirements regulate the mRNA levels for many enzymes required for lipid synthesis (9, 10), and transepidermal water loss increases with repeated fluindostatin treatment (24, 25), mice were studied prior to development of a barrier abnormality (see Methods). As shown in **Fig. 2A**, mRNA levels of SREBP-2 increase (up to 4.9-fold) with fluindostatin treatment, while SREBP-1 mRNA remained undetectable (data not shown). Actin mRNA levels also were unchanged (data not shown).

SREBP-2 also predominates in human cultured keratinocytes

To further investigate the mechanism by which SREBPs are regulated, we next determined whether cultured human keratinocytes express the same SREBPs as murine epidermis. As observed in murine epidermis, SREBP-2 mRNA was detected (Fig. 2B), whereas SREBP-1 mRNA was not detected in cultured human keratinocytes by Northern blotting. Next, lovastatin, another HMG-CoA reductase inhibitor, was added to keratinocytes, for 12 h to inhibit cholesterol synthesis. SREBP-2 mRNA levels increased slightly (1.3-fold) (Fig. 2B), and again, SREBP-1 mRNA was not detected by Northern blotting (data not shown). To demonstrate that the effect of lovastatin occurred as a result of reduced sterol levels, keratinocytes were treated simultaneously with both 25-OH-cholesterol and lovastatin. The co-application of 25-OH-cholesterol and lovastatin not only blocked the increase in SREBP-2 mRNA levels induced by lovastatin alone but decreased levels below vehicle control (Fig. 2B). Moreover, 25-OH-cholesterol alone decreased SREBP-2 mRNA levels by 60% compared to vehicle control (Fig. 2B). As cyclophilin mRNA levels were unchanged by all treatments, the changes in sterol levels did not affect mRNA levels of all proteins. Using an identical protocol, similar results were seen after 24 h of treatment with lovastatin ± sterols (data not shown). Therefore, SREBP-2 is also the predominant SREBP in cultured human keratinocytes, and the transcription of SREBP-2 is regulated by sterol levels in keratinocytes. These studies indicate that human keratinocytes are a relevant model for mechanistic studies of the regulation of epidermal lipid synthesis.

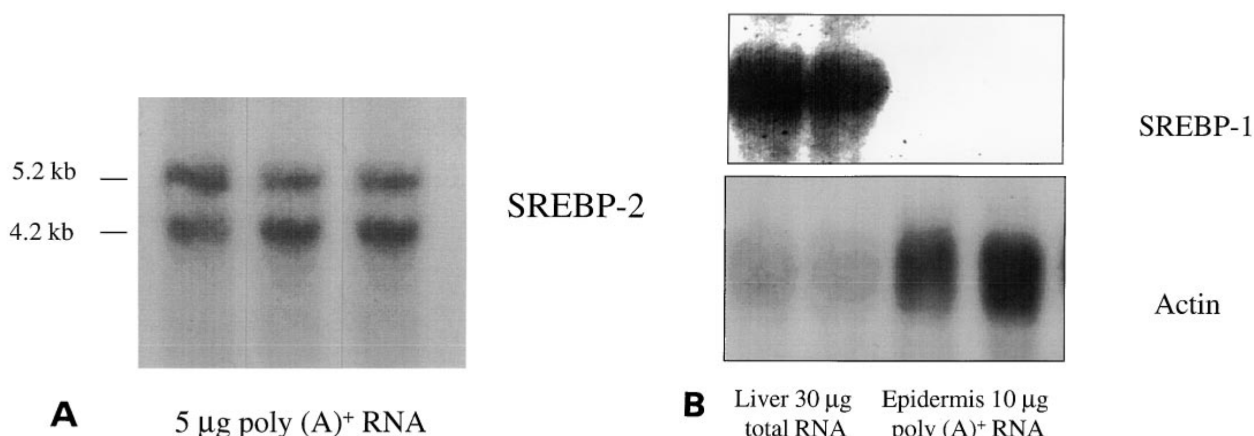

Fig. 1. SREBP-2 but not SREBP-1 is detected in epidermis by Northern blotting. A) Northern blot of murine epidermis poly $(A)^+$ RNA probed with a cDNA probe for SREBP-2. Each lane represents the epidermis of two animals. B) Northern blot of 30 µg murine liver total RNA and 10 µg murine epidermal poly $(A)^+$ RNA probed with cDNA probes for SREBP-1 and actin.

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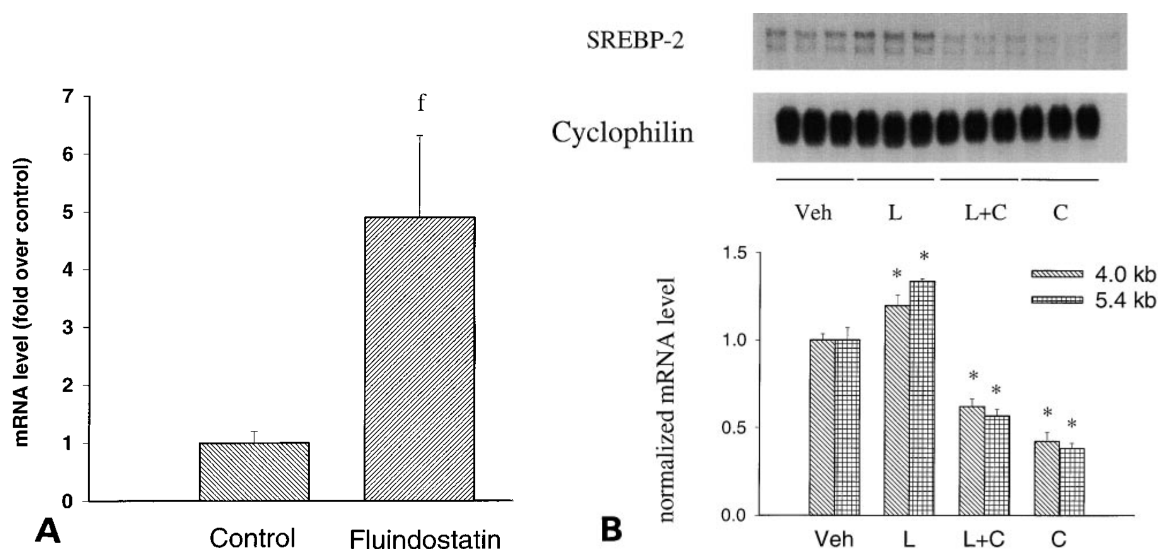

Fig. 2. SREBP-2 mRNA is present in the epidermis and cultured keratinocytes and levels change in response to sterol levels. Northern blot of poly $(A)^+$ RNA showing: A) SREBP-2 levels in mouse epidermis topically treated for 6 d with 1.5 mg fluindostatin or vehicle (control) (n = 5). B) SREBP-2 and cyclophilin mRNA levels in human cultured keratinocytes treated for 12 h with vehicle (Veh), 10 μm lovastatin (L), 10 μm lovastatin + 12.5 μm 25-OH-cholesterol (L+C), or 12.5 μm 25-OH-cholesterol alone (C) (n = 3). Data are presented as mean ± SEM and expressed as fold of control corrected for loading using cyclophilin levels. $^*P < 0.05$ Student's two-tailed *t*-test, $^fP < 0.05$ Mann-Whitney U test.

Sterols regulate the proteolytic activation of SREBP-2 in human keratinocytes

To determine the effect of sterols on the cleavage of SREBP-2 to its mature active form, we used Western blots with a monoclonal antibody specific to the human SREBP-2 COOH-terminal. Whereas the precursor SREBP-2 in the endoplasmic reticulum membrane is ~125 kDa, the COOH-terminal SREBP-2 fragment resulting from the release of the mature NH_2-fragment is ~65 kDa peptide. In these studies, the quality of COOH-terminal SREBP-2 remaining in the microsomes reflects the rate of cleavage of SREBP-2 and directly indicates the rate of formation of the NH_2 termi-

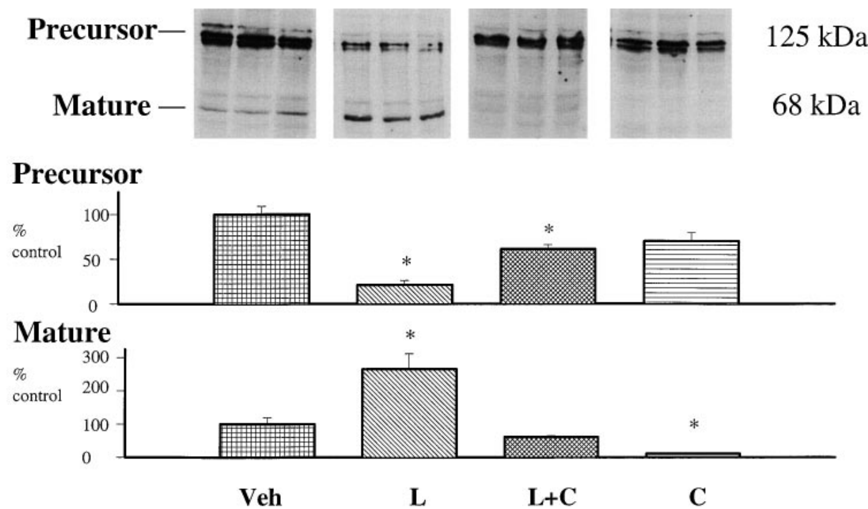

Fig. 3. Cleavage of SREBP-2 to the mature form in keratinocytes is regulated by sterol levels. Western blot using a monoclonal antibody to the COOH-terminus of human SREBP-2. Human cultured keratinocytes were treated for 12 h with vehicle (Veh), 10 μm lovastatin (L), 10 μm lovastatin + 12.5 μm 25-OH-cholesterol (L + C), or 12.5 μm 25-OH−cholesterol alone (C). Data are presented as mean ± SEM and expressed as a percentage of the control. $^*P < 0.05$ Student's two-tailed *t*-test.

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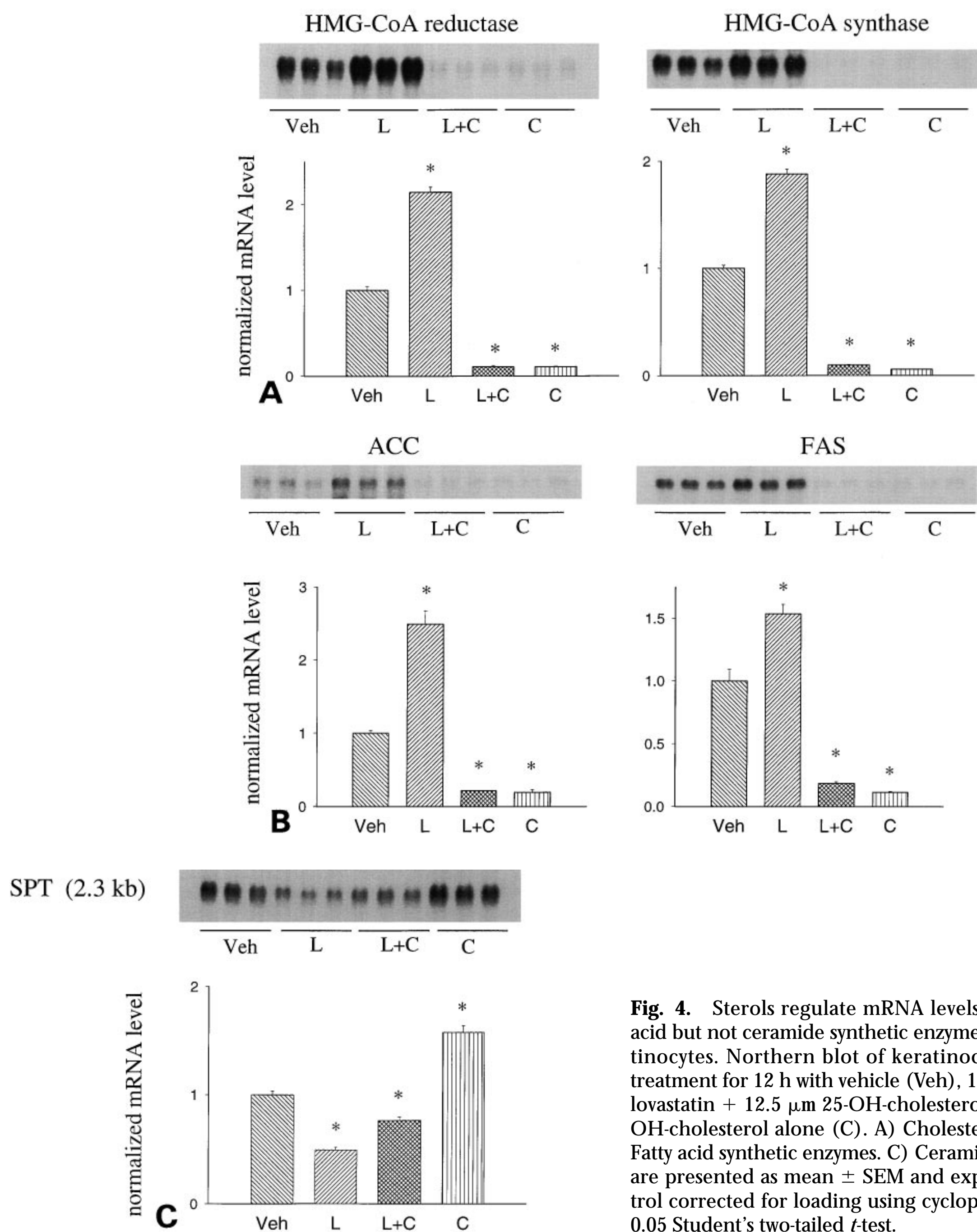

Fig. 4. Sterols regulate mRNA levels for cholesterol and fatty acid but not ceramide synthetic enzymes in cultured human keratinocytes. Northern blot of keratinocyte poly $(A)^+$ RNA after treatment for 12 h with vehicle (Veh), 10 μm lovastatin (L), 10 μm lovastatin + 12.5 μm 25-OH-cholesterol (L + C), or 12.5 μm 25-OH-cholesterol alone (C). A) Cholesterol synthetic enzymes. B) Fatty acid synthetic enzymes. C) Ceramide synthetic enzyme. Data are presented as mean ± SEM and expressed as fold of the control corrected for loading using cyclophilin levels (n = 3). $^*P <$ 0.05 Student's two-tailed *t*-test.

nal fragment that stimulates transcription. As shown in **Fig. 3**, lovastatin treatment increased the COOH terminal fragment indicating increased proteolytic release of mature SREBP-2 protein and this increase was blocked by simultaneous treatment with 25-OH-cholesterol. Treatment of keratinocytes with 25-OH-cholesterol alone reduced the levels of both precursor and COOH terminal SREBP-2 to below the vehicle control. These results show that changes in the activation of SREBP-2 parallel the regulation of SREBP-2 mRNA levels. Thus, manipulation of sterol levels in cultured human keratinocytes regulates both SREBP-2 proteolytic activation and mRNA levels.

Changes in keratinocyte mRNA levels for cholesterol and fatty acid synthetic enzymes occur in parallel with modulations in mature SREBP-2 levels

To determine whether generation of mature SREBP-2 correlates with mRNA levels for enzymes required for

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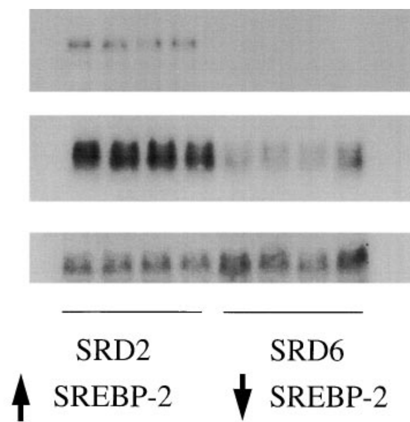

Fig. 5. SREBP-2 does not regulate SPT mRNA levels. Northern blots of total RNA prepared from Chinese hamster ovary cell lines that have either high levels of mature active SREBP-2 (SRD2) or low levels of mature SREBP-2 (SRD6). Northern blots were probed with cDNA probes to enzymes required for cholesterol synthesis, FPPS and HMG-CoA synthase, and SPT (n = 4).

lipid synthesis, we next measured the mRNA levels for the key enzymes of cholesterol synthesis (HMG-CoA synthase, HMG-CoA reductase), fatty acid synthesis (FAS and ACC), and the rate-limiting enzyme for de novo ceramide synthesis (SPT, 2.3 kb transcript) in keratinocytes. Lovastatin increased mRNA levels for the cholesterol (**Fig. 4A**) and fatty acid synthetic enzymes at 12 h post treatment (Fig. 4B). However, SPT mRNA levels did not increase and, in fact, decreased in response to lovastatin treatment (Fig. 4C). The simultaneous addition of 25-OH-cholesterol inhibited the lovastatin-induced increase of cholesterol and fatty acid synthetic enzyme mRNA levels (Fig. 4A, B). Moreover, 25-OH-cholesterol treatment alone also dramatically reduced the mRNA levels of the enzymes required for cholesterol and fatty acid synthesis (Figs. 4A, B). In contrast, SPT mRNA levels were increased by 25-OH-cholesterol treatment (Fig. 4C). Using identical protocols, similar results were observed after 24 h of treatment with lovastatin ± sterols (data not shown). Therefore, in cul-

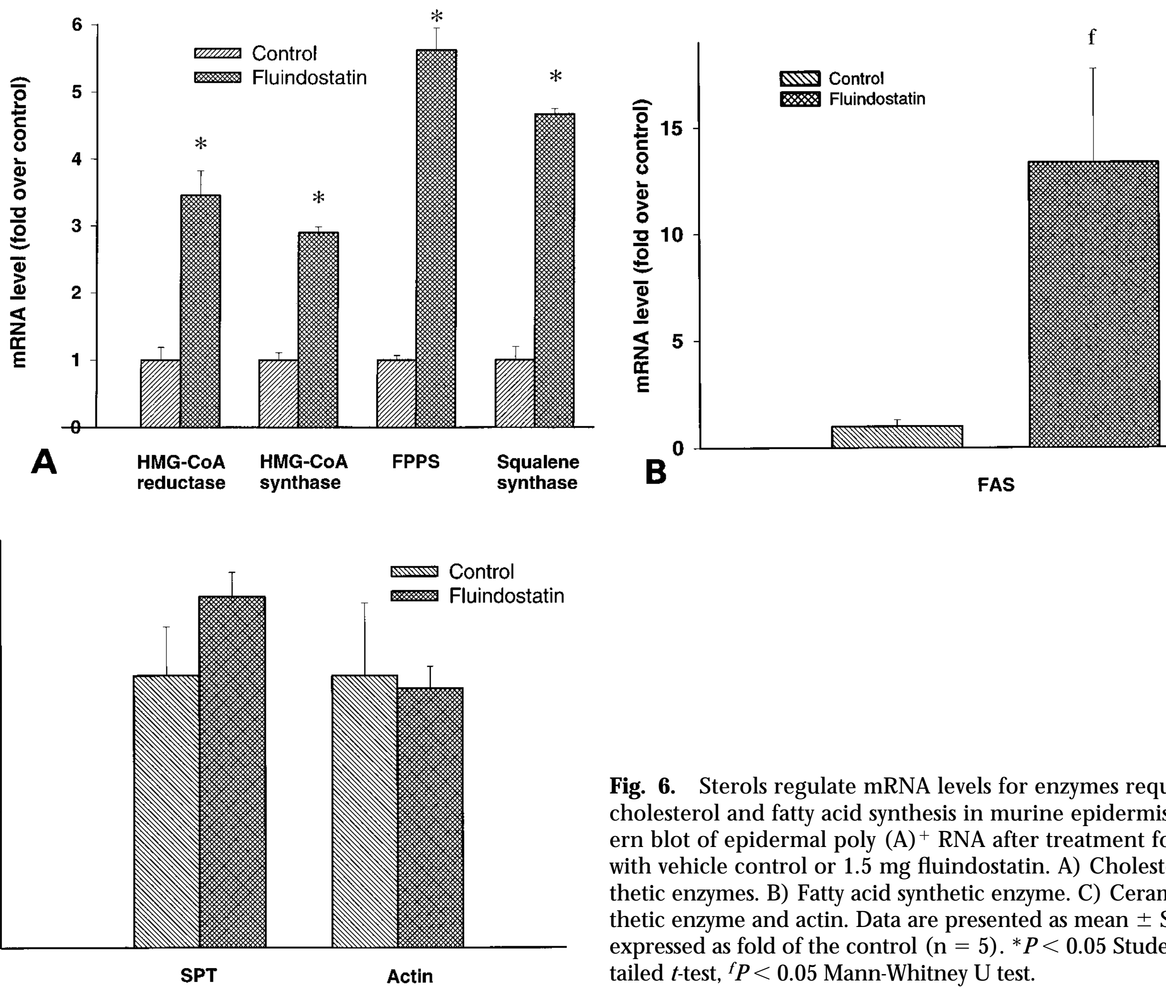

Fig. 6. Sterols regulate mRNA levels for enzymes required for cholesterol and fatty acid synthesis in murine epidermis. Northern blot of epidermal poly (A)$^+$ RNA after treatment for 6 days with vehicle control or 1.5 mg fluindostatin. A) Cholesterol synthetic enzymes. B) Fatty acid synthetic enzyme. C) Ceramide synthetic enzyme and actin. Data are presented as mean ± SEM and expressed as fold of the control (n = 5). $^*P < 0.05$ Student's two-tailed *t*-test, $^fP < 0.05$ Mann-Whitney U test.

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tured keratinocytes, conditions that generate the mature form of SREBP are associated with an increase in mRNA levels for enzymes required for cholesterol and fatty acid synthesis, while conditions that prevent the cleavage of SREBP-2 are associated with a decrease in mRNA levels of the enzymes required for cholesterol and fatty acid synthesis. In contrast, SPT mRNA levels do not change in parallel with the cleavage of SREBP-2 and the changes in cholesterol and fatty acid synthetic enzymes.

SREBP-2 does not regulate SPT mRNA levels

Because of the differences in regulation of SPT mRNA, we next used two Chinese hamster ovary cell lines (SRD2 and SRD6) that have defects in the regulation of mature active SREBP levels to determine directly whether the changes in SPT mRNA levels are regulated by SREBP-2. SRD2 cells express a dominant-positive form of SREBP-2 that is constitutively active (26), while SRD6 cells fail to cleave the precursor SREBP to the mature active form (27). The mRNA levels for enzymes required for cholesterol synthesis (HMG-CoA synthase and FPPS) are regulated in parallel with the levels of mature SREBP in these cell lines, i.e., increased in SRD2 cells and decreased in SRD6 cells (**Fig. 5**). However, SPT mRNA levels are similar in both cell lines, further indicating that SPT is not regulated by SREBP-2 (Fig. 5).

Sterol modulations also regulate mRNA levels for key enzymes of cholesterol and fatty acid synthesis in epidermis

To determine whether sterol-induced changes in SREBP-2 also correlate with changes in mRNA levels for lipid synthetic enzymes in the epidermis of intact mice, we next measured mRNA levels for cholesterol (HMG-CoA synthase, HMG-CoA reductase, FPPS, and squalene synthase), fatty acid synthetic enzymes (ACC and FAS), and ceramide (SPT) synthetic enzymes. As in cultured human keratinocytes, fluindostatin treatment resulted in an increase in the mRNA levels for all cholesterol synthetic enzymes (**Fig. 6A**) and FAS (Fig. 6B). However, ACC mRNA levels increased only slightly. Furthermore, simultaneous treatment with exogenous sterols blunted the increase in mRNA levels of these enzymes (**Fig. 7**). In contrast, mRNA levels for SPT and actin did not change with fluindostatin treatment (Fig. 6C). Topical applications of sterols alone to the epidermis failed to decrease mRNA levels for SREBP-2 or enzymes required for cholesterol and fatty acid synthesis (data not shown). These studies show that in the epidermis of intact mice, mRNA levels for the key enzymes of cholesterol and fatty acid synthesis change in parallel with alterations in SREBP-2 levels whereas the mRNA for SPT does not.

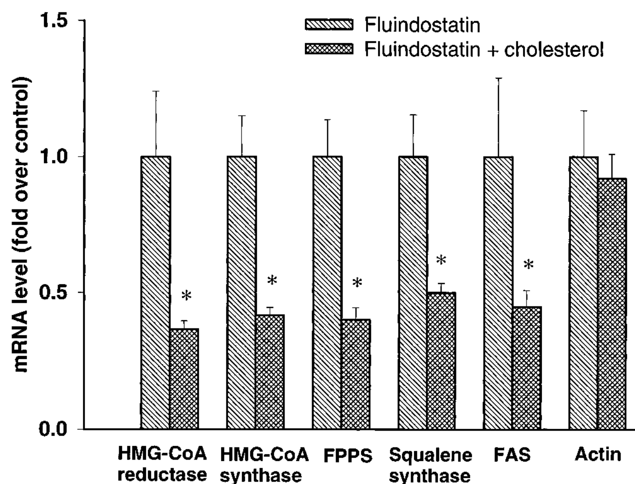

Fig. 7. Co-application of sterols prevents the fluindostatin-induced increase in mRNA levels for enzymes required for cholesterol and fatty acid synthesis in murine epidermis. Northern blot of epidermal poly $(A)^+$ RNA after treatment with 1.5 mg fluindostatin or 1.5 mg fluindostatin + 0.1 mg 25-OH-cholesterol and 1.5 mg cholesterol. Data are presented as mean ± SEM and expressed as fold of the fluindostatin treated group (n = 5). $^*P < 0.05$ Student's two-tailed *t*-test.

DISCUSSION

Previous studies have demonstrated an increase in cholesterol, fatty acid, and sphingolipid synthesis after barrier disruption, which has been attributed to increases in the activity of key enzymes in these biosynthetic pathways (4–8). Moreover, the stimulation of enzyme activity can be accounted for by increases in the mRNA levels of these key enzymes (9, 10). However, the mechanism(s) by which these mRNAs are regulated is unknown.

In the present study, we demonstrate first that SREBP-2 mRNA is the predominant SREBP present both in cultured human keratinocytes and murine epidermis, while SREBP-1 mRNA is not detected. These results indicate that SREBP-2 is the predominant SREBP in the epidermis, where it may be the major regulator of epidermal lipid synthesis in response to changes in sterol levels. Furthermore, we have demonstrated that modulations in cellular sterols results in changes in both SREBP-2 mRNA levels and the proteolytic activation of SREBP-2.

We have also demonstrated that alterations in sterol levels coordinately regulated both SREBP-2 and the

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mRNA levels for enzymes required for cholesterol and fatty acid synthesis in both cultured human keratinocytes and intact murine epidermis. Specifically, treatment of keratinocytes with 25-OH-cholesterol, which markedly reduced both SREBP-2 mRNA levels and proteolytic cleavage to the mature active form, resulted in a dramatic decrease in mRNA levels for HMG-CoA reductase and HMG-CoA synthase, enzymes required for cholesterol synthesis, and a significant decrease in mRNA levels for ACC and FAS, enzymes required for fatty acid synthesis. Conversely, lovastatin, which inhibits cholesterol synthesis, increased SREBP-2 mRNA and cleavage to the mature active form and resulted in an increase in mRNA levels for enzymes required for cholesterol and fatty acid synthesis. Similarly, topical application of fluindostatin, another inhibitor of cholesterol synthesis, to the skin of hairless mice increased SREBP-2 mRNA levels and the mRNA levels of enzymes required for cholesterol and fatty acid synthesis. Together these observations clearly demonstrate that, in both cultured human keratinocytes and intact mouse epidermis, changes in SREBP-2 levels and mRNA levels for key enzymes for cholesterol and fatty acid synthesis occur in parallel. This may allow for the balanced synthesis of cholesterol and fatty acids for the formation of extracellular lamellar membranes that mediate the epidermal permeability barrier.

When sterol synthesis was inhibited by either fluindostatin or lovastatin, the magnitude of the increase in mRNA for SREBP and the enzymes required for cholesterol and fatty acid synthesis was less in cultured keratinocytes than in epidermis. This difference is most likely due to the high levels of basal sterol synthesis in cultured keratinocytes grown in serum-free medium. Thus, under these conditions, further lowering of sterol levels by inhibiting cholesterol synthesis would be expected to result in only modest further increases in mRNA levels, as indeed occurred. In contrast, addition of exogenous sterols to the culture medium markedly decreased mRNA levels and the proportion of SREBP-2 in the mature active form, an observation that would be expected in keratinocytes under these culture conditions. Moreover, in the epidermis, lipid synthesis is low under basal conditions; hence, we observed up to a 13-fold increase in mRNA levels for enzymes required for cholesterol and fatty acid synthesis after inhibiting sterol synthesis, while topical sterols did not further decrease mRNA levels for SREBP-2 and enzymes for fatty acid and cholesterol synthesis, presumably due to the low basal levels of sterol synthesis. Alternatively, the topically applied sterols may not have achieved a sufficiently high concentration in the appropriate cellular sites to affect SREBP regulation.

In contrast to the linkage of SREBP-2 with enzymes required for cholesterol and fatty acid synthesis, mRNA levels for SPT, the initial enzyme in ceramide synthesis, were not regulated in the same manner. In murine epidermis, SPT mRNA levels were not affected by treatment with fluindostatin, which changes sterol availability and regulates SREBP-2. Likewise, in cultured keratinocytes, SPT mRNA levels changed in the opposite direction to SREBP-2 levels and mRNA levels for enzymes required for cholesterol and fatty acid synthesis. Other investigators have demonstrated that 25-OH-cholesterol treatment of Chinese hamster ovary cells (CHO) increased sphingomyelin synthesis, consistent with our results (28). In the present study, we also show that SPT mRNA levels were unchanged in Chinese hamster ovary cells that either express a dominant-positive form of SREBP-2 (SRD2) or that fail to cleave SREBPs in response to limited sterols (SRD6), providing direct evidence that SPT is not regulated by SREBP-2. These differences may account for the disparate kinetics of cholesterol and fatty acid versus ceramide synthesis during epidermal barrier recovery after acute disruption (4–8). During barrier recovery there is a rapid increase in the synthesis of cholesterol and fatty acids, with a parallel increase in activity and mRNA levels of key enzymes required for fatty acid and cholesterol synthesis (4–7). In contrast, the increase in SPT activity and ceramide synthesis is delayed, first occurring at 4 and 6 h, respectively, after barrier disruption (8). Thus, it is likely that different transcription factors regulate ceramide synthesis.

The mechanism for the increase in mRNA levels of key lipid synthetic enzymes after barrier disruption is unknown. These results suggest that the removal of cellular lipids due to lamellar body secretion after barrier disruption leads to the activation of SREBP-2, increasing gene expression for enzymes of cholesterol and fatty acid synthesis and for the LDL receptor, as described previously. It is not yet possible to determine whether SREBP-2 is activated in murine epidermis after barrier disruption, because antibodies are not yet available for murine SREBP-2. Although SREBP-2 may not regulate transcription of SPT, the rate-limiting enzyme for ceramide synthesis, it is possible that SREBP-2 may affect ceramide synthesis indirectly by controlling the levels of fatty acids available for ceramide synthesis. Additionally, it is possible that increased formation of oxysterols activates LXR or SF-1 nuclear receptors which may regulate SPT gene expression, thereby linking increases in sterol synthesis with increases in ceramide synthesis. The increase in SPT mRNA levels and sphingolipid synthesis (28) induced by 25-OH-cholesterol would be consistent with this hypothesis.

Previous studies in our laboratory have shown that prolonged, repeated treatment of murine skin with the

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HMG-CoA reductase inhibitors, lovastatin or fluindostatin, results in a progressive increase in transepidermal water loss (24, 25). The perturbation in the permeability barrier was accompanied by morphologic abnormalities of both lamellar bodies and the lamellar bilayers. An increase in fatty acid levels was demonstrated, while cholesterol levels remained unchanged (24, 25). It was therefore proposed that the altered cholesterol and fatty acid levels changed the ratio of lipids in the lamellar membranes, resulting in a barrier defect. The present study provides further insights into the mechanism by which chronic inhibition of HMG-CoA reductase alters barrier function. Inhibition of cholesterol synthesis lowers, or limits, endogenous sterol levels which results in an increase in the cleavage of SREBP-2, which, in turn, causes an increase in mRNA levels for SREBP-2 and enzymes required for both cholesterol and fatty acid synthesis. In the presence of an HMG-CoA reductase inhibitor, this parallel regulation results in a relative increase in fatty acid synthesis and an excess in fatty acids relative to cholesterol that could disrupt normal membrane structure.

In summary, we have demonstrated that SREBP-2 is the predominant SREBP in the epidermis and keratinocytes. Sterols regulate the proteolytic activation and mRNA levels of SREBP-2, and the mRNA levels for the enzymes required for fatty acid and cholesterol synthesis change in parallel with alterations in SREBP-2. SREBP-2 may therefore be an important regulator of cholesterol and fatty acid synthesis after acute barrier disruption.

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