Effect of Essential Fatty Acid Deficiency on Cutaneous Sterol Synthesis

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The fact that the skin is a major site of total body sterologenesis, coupled both with the apparent absence of low density lipoprotein receptors on keratinocytes and with the lack of influence of serum cholesterol on epidermal sterologenesis, has created the impression that epidermal lipid synthesis might be autonomous, i.e., nonregulatable. Recent studies have shown, however, that disruption of cutaneous barrier function with acetone or detergents stimulates epidermal sterologenesis (J Lipid Res 26:418–427, 1985). To correlate further sterologenesis with barrier function, we measured de novo synthesis of cholesterol and total nonsaponifiable lipids in essential fatty acid-deficient (EFAD) hairless mice. Animals with defective barrier function, manifested by abnormal transepidermal water loss, demonstrated a 2-fold increase in epidermal cholesterol and total nonsaponifiable lipid synthesis over controls while synthesis in the dermis was unchanged. Epidermal sterologenesis in EFAD animals, repleted with linoleic acid either systematically or topically, returned toward normal as barrier function improved. Moreover, plastic occlusion of EFAD mouse skin normalized epidermal sterologenesis at 1 and 3 days. These results provide further evidence that epidermal sterologenesis is not entirely autonomous, and can be regulated by water barrier requirements. *J Invest Dermatol 87:588–591, 1986*

Sterols are among the most copious lipid products of epidermal differentiation, where they have at least 2 functions. First, they comprise a significant portion of the stratum corneum intercellular domains, where they are thought to participate in the cutaneous permeability barrier [1]. Second, they serve as precursors of vitamin D_3 [2–4]. Finally, the association of several recessively inherited, acquired ichthyotic conditions with aberrant sterol metabolism suggests that sterols in stratum corneum intercellular domains may also regulate desquamation [5]. Studies by this and other laboratories have demonstrated that: (1) circulating sterols are not a major source of epidermal sterols [6]; and (2) the skin is a major site of sterol synthesis in the whole animal [7–10]. Of the sterol synthetic activity in rodent skin, the majority (~ 75%) occurs within the dermis, while the remaining 25% of the cutaneous synthetic activity occurs in the viable epidermis, where it is localized almost exclusively to the basal and spinous layers [11]. Since these layers comprise only a small fraction of the skin, on a weight basis the viable epidermis can be considered among the most active sites of sterol synthesis in the body [11].

The factors that regulate epidermal sterologenesis are poorly understood. Manipulations that result in altered serum sterol levels do not affect the rate of cutaneous sterol synthesis [12]. Similarly, variations in dietary cholesterol content also do not appear to affect cutaneous sterologenesis [8]. Additionally, in vitro studies have demonstrated that sterol synthesis in confluent keratinocytes is not inhibited by the addition of low density lipoprotein (LDL)-cholesterol to the media [13,14], presumably due to the lack of LDL receptors on the plasma membranes of these cells [13–15]. One implication of these studies is that epidermal sterol synthesis might be an autonomous process, not regulated by exogenous factors.

Yet, more recent in vitro studies suggest that sterologenesis can be regulated by polar sterol metabolites [13,14], and that the rate of in vivo epidermal sterol synthesis is modulated by certain local factors [6]. When the permeability barrier of the epidermis was disrupted by treatment either with detergents or with lipid solvents, epidermal sterol synthesis increased 2- to 3-fold. Moreover, epidermal synthetic activity paralleled both the return of barrier function toward normal and the extent of damage to the barrier. Finally, and most importantly, when the increase in permeability was prevented by occlusion with an impermeable film, the expected increase in epidermal sterologenesis that follows barrier disruption was not observed. Together, these studies suggest that one factor that regulates epidermal sterol synthesis is the permeability barrier status of the skin.

To explore the possibility further that the status of the epidermal permeability barrier regulates cutaneous sterologenesis, we studied another model, the essential fatty acid-deficient (EFAD) hairless mouse. Prior studies have shown that EFAD results in excess epidermal water loss (reviewed in [16,17]). Thus, the purpose of the current investigation was to determine whether the defect in barrier function in EFAD also is associated with a stimulation in epidermal sterol synthesis [18].

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Abbreviations:

EFAD: essential fatty acid deficient (-cy)
LDL: low density lipoprotein
TEWL: transepidermal water loss
TNS: total monoaponifiable
SDS: sodium dodecyl sulfate

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METHODS

Animals Hairless male weanling mice (HR/HR) aged 19–21 days, and weighing 6–12 g, were purchased from Jackson Laboratories (Bar Harbor, Maine). The animals were divided into 2 groups and kept in adjacent cages in a normal light cycle room in the animal facility at the Veterans Administration Medical Center, San Francisco. Control animals were fed a diet consisting of casein, sucrose, choline, a mixture of salt, fat-soluble vitamins A, D, E, and water-soluble B vitamins to which 5% corn oil and inositol (1 mg/g) was added, while the EFAD group received an identical diet, except that 5% hydrogenated coconut oil was substituted for the corn oil. Transepidermal water loss (TEWL) was measured weekly, and sterol synthesis was quantitated when water loss levels exceeded controls by greater than 5-fold.

Topical Applications of Lipids In these experiments, one side of the deficient animals (~5 cm^2) received thrice-daily applications of 2 mg of linoleic acid in 20 μl of vehicle, while the contralateral side was treated similarly with thrice-daily applications of vehicle alone. The purity of the linoleic acid was checked by thin-layer chromatography against known standards, and each was dissolved in propylene glycol:ethanol prior to application (3:7, v/v), as previously described [19,20]. Care was taken to ensure that the solutions were spread evenly and did not run. Transepidermal water loss determinations were made immediately after the oil applications.

Measurement of Transepidermal Water Loss Transepidermal water loss (TEWL) was measured in ether-anesthetized animals under ambient atmospheric conditions, in the morning with a Meeco electrolytic water analyzer [19,20]. "Ultrapure" dry nitrogen gas, 99.99% pure, was passed through the sample cup at 100 cc/min. The sampling cup was separated from its Parafilm cover, and slid onto the site to be measured, thus minimizing contamination with atmospheric air. Contralateral sites were measured on each animal to ensure the reproducibility of the TEWL measurements. As in prior studies on EFAD rodents [19], TEWL rates were virtually identical over all sites on each animal, although there was considerable variation from animal to animal.

Measurement of Sterol Synthesis The animals were injected i.p. with tritiated water (^3H_2O, 20 mCi per mouse). Three hours after the injection the animals were killed, weighed, a blood specimen obtained, and the skin removed and weighed. The epidermal and dermal layers were separated, as in our prior studies [11], by exposure to dry heat for 60 s at 60°C. This procedure yields homogenous sheets of epidermis with no basal cells left attached to the dermis [11]. The separated skin fractions from each animal were saponified by refluxing overnight in a solution of 45% potassium hydroxide, water, and 70% ethanol (2:1:5, volumes). The saponified flasks were cooled and an internal standard of [^{14}C] cholesterol was added to the flasks before extracting the nonsaponifiable material 3 times with petroleum ether. The petroleum ether extract [total nonsaponifiable (TNS) lipids] was dried, dissolved in chloroform, and applied to thin-layer chromatography plates. The plates were developed in ethylacetate:benzene (1:5, volumes) for 50 min at room temperature, and the radioactive bands corresponding to cholesterol, lanosterol, and squalene were excised and counted in scintillation vials containing 10 ml of counting solution. The TNS lipids are the sum of the cholesterol, lanosterol, and squalene bands. It, of course, should be recognized that these bands undoubtedly contain other comigrating labeled nonsaponifiable lipids. Prior published studies from our laboratory have shown that cholesterol comprises over 90% of the free sterol in the epidermis of the hairless mouse with small amounts of 7-dehydrocholesterol and desmosterol present as well [21]. It is therefore likely that the cholesterol band is predominantly comprised of cholesterol. Our counting techniques, including calculations for background, spillover, and specific activity per animal, were performed as described previously [11], and the results were expressed as μmol ^3H_2O incorporated per g total skin wet weight.

Measurement of Transport of Sterols to the Skin To assess whether barrier disruption, associated with an EFAD diet, resulted in an increased transport of cholesterol from the circulation to the epidermis, animals were injected i.p. with approximately 2 million dpm of [^{14}C]cholesterol, solubilized in monopalmitate (Tween 40, Sigma). After 6 h the entire epidermis from each animal was isolated, and the recovery of label determined after saponification, extraction, and thin-layer chromatography, as described above.

Statistical significance was determined by a 2-tailed Student's *t*-test.

RESULTS

The incorporation of ^3H_2O into cholesterol, lanosterol, squalene, and TNS lipids in the epidermis and dermis of control and EFAD animals is shown in Table I. In these studies all of the deficient animals demonstrated TEWL rates that were greater than 100 ppm/cm^2/h (mean, 155 ± 12, n = 16), while the rates in control animals never exceeded 20 ppm/cm^2/h. Cholesterol, lanosterol, and TNS lipid synthesis were increased approximately 2-fold in the epidermis of EFAD animals. In contrast, dermal sterologenesis was similar in the control and EFAD groups. As noted in our previous studies [11], the majority of the total cutaneous sterol synthesis was localized to the dermal layer, even in EFAD animals. These observations demonstrate that EFAD results in the enhancement in sterol synthesis which is localized to the epidermal layer.

In order to determine whether the increased labeling was due to altered delivery of serum-derived sterols to EFAD skin, we administered an i.p. bolus of [^{14}C]cholesterol to control and EFAD animals. No significant difference in recovery of label in the epidermal layers was observed (control, 9188 ± 1917 vs EFAD, 9126 ± 1980 dpm, n = 4 each group). Additionally, it should

Table I. Effect of Essential Fatty Acid Deficiency on Cutaneous Sterol Synthesis

	μmol ^3H_2O Incorporated/h/g Wet Skin Weight							
	Epidermis				Dermis			
	Cholesterol	Lanosterol	Squalene	TNS Lipids	Cholesterol	Lanosterol	Squalene	TNS Lipids
Control (n = 11)	0.163 ± 0.013 0.001	0.067 ± 0.010 0.001	0.057 ± 0.013 NS	0.286 ± 0.027 0.001	0.943 ± 0.073 NS	0.377 ± 0.037 NS	0.243 ± 0.057 NS	1.560 ± 0.137 NS
Essential fatty acid-deficient (n = 16)	0.363 ± 0.037 0.01	0.130 ± 0.010 0.001	0.090 ± 0.017 0.02	0.587 ± 0.053 0.001	0.907 ± 0.053 0.01	0.390 ± 0.30 NS	0.300 ± 0.047 0.01	1.597 ± 0.097 NS
Revertant (n = 4)	0.227 ± 0.017	0.077 ± 0.007	0.020 ± 0.003	0.323 ± 0.017	1.217 ± 0.093	0.303 ± 0.060	0.130 ± 0.010	1.650 ± 0.123

Epidermal and dermal de novo sterol synthesis are compared in control mice, essential fatty acid-deficient mice, and essential fatty acid-deficient mice fed a normal diet for 3 days. Results are expressed as mean ± SEM. n = number of animals; NS = not significant. Significance data are presented between data for each group.

be noted that the percentage of the administered label that was transported to the epidermis was very small in either group (0.5% of total label administered). These results indicate that the increase in the incorporation of ^3H_2O into sterols in the epidermis of EFAD animals is not accounted for by transport of newly synthesized sterols from extracutaneous sites to the epidermis.

The effect of feeding a diet containing essential fatty acids for 3 days to a group of EFAD animals was studied next. Three days of the control diet resulted in a marked decrease of TEWL (mean, 54.8 ± 11.5 ppm/cm^2/h, n = 4), and as shown in Table I, decreased the incorporation of ^3H_2O into epidermal cholesterol, lanosterol, squalene, and TNS lipids to approximately half that observed in the untreated EFAD animals, i.e., to the same range as observed in the controls. These observations indicate that the abnormalities in permeability barrier function and the increase in epidermal sterol synthesis, associated with EFAD can be reversed by systemic repletion of the essential fatty acids.

The effect of topical applications of linoleic acid for 24 h prior to study to one flank of EFAD animals was investigated next. The mean TEWL on the vehicle-treated control flank was 212 ppm/cm^2/h, while the mean TEWL on the linoleic acid-treated side was 125 ppm/cm^2/h. As shown in Table II, both epidermal cholesterol and TNS lipid synthesis were reduced by approximately 50% in skin regions treated topically with linoleic acid; topical linoleic acid administration did not affect sterol synthesis in the dermis. These results indicate that the abnormalities in permeability barrier function and epidermal sterol synthesis, associated with EFAD, can be rapidly reversed by topical linoleic acid treatment.

In our prior studies we had demonstrated that the increase in epidermal sterol synthesis, that occurs following treatment with lipid solvents, could be prevented if the barrier defect was corrected by occlusion [6]. The effects of occlusion on epidermal cholesterol synthesis in EFAD and control animals are shown in Fig 1. In EFAD animals, after either 1 or 3 days of occlusion, the incorporation of tritiated water into cholesterol was decreased by approximately two-thirds. Comparable decreases in the incorporation of ^3H_2O into TNS lipids were also observed in the EFAD animals when permeability barrier was corrected by occlusion (data not shown). In the control animals, epidermal cholesterol synthesis was not significantly affected by occlusion (Fig 1). These observations indicate that the correction of the permeability barrier in EFAD animals is associated with a decrease in epidermal sterologenesis.

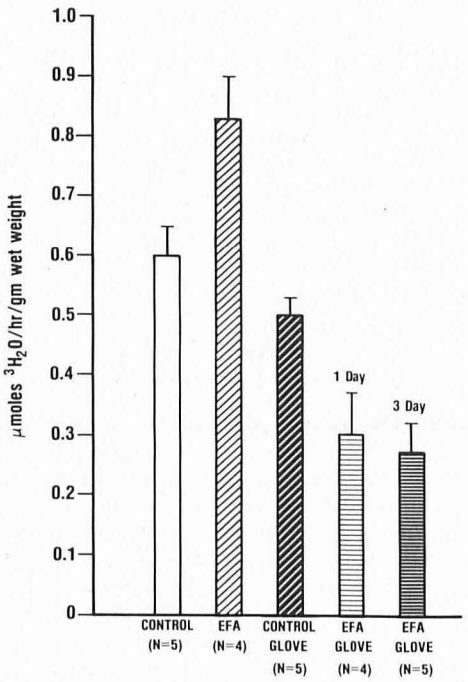

Figure 1. Epidermal cholesterol synthesis is compared in control mice, EFAD mice, control mice covered with a plastic glove for 1 day, and EFAD mice covered with a plastic glove for either 1 or 3 days. Results are expressed as mean, with the *bars* indicating the SEM. N = Number of animals. Control vs EFAD $p < 0.05$, control vs gloved control $p > 0.05$, EFAD vs gloved EFAD 1 day $p < 0.01$, 3 day $p < 0.001$.

DISCUSSION

Previous studies in this laboratory have suggested that there is a relationship between the barrier to transcutaneous water loss and epidermal sterol synthesis [6]. When the barrier was damaged with either detergents (sodium-dodecyl sulfate, SDS) or lipid solvents (acetone, ether), there was an increase in epidermal sterologenesis, proportional to the degree of barrier disruption. The time course of this increase in synthetic action closely paralleled return of TEWL toward normal, with both barrier function and sterol synthesis reaching normal levels by 24 h following a single application of acetone. Last, and most importantly, when the defect in the permeability barrier was corrected with an impermeable film, the levels of sterologenesis remained normal. Taken together these observations suggest that the cutaneous water barrier status is one factor that regulates epidermal sterol synthesis.

In the present study we have quantitated cutaneous sterol synthesis in EFAD, a condition that is well known to cause an abnormality in permeability barrier function (reviewed in [16,17]), but also is characterized by accelerated epidermal cell turnover, as well [22]. In EFAD skin, epidermal sterol synthesis was increased approximately 2-fold as compared with controls, while in contrast, dermal sterol synthesis was similar in the controls and EFAD animals. Moreover, when the EFAD was corrected by either oral or topical essential fatty acid administration, the permeability barrier improved and the rate of sterologenesis in the epidermis decreased correspondingly. Lastly and most importantly, when the permeability barrier defect in the EFAD animals was corrected by occlusion, the expected increase in epidermal sterol synthesis was not observed. These observations all are consistent with our previous results, and lend further support, in yet another model, that permeability barrier requirements and epidermal sterol synthesis are linked.

Although the permeability barrier defect in EFAD has been appreciated for years [16,19], until recently it was not known whether the defective barrier in EFAD was due to a lack of linoleic acid per se in stratum corneum membranes, or whether it was secondary to the epidermis' concurrent high-turnover state [22]. Linoleic acid is an obligate precursor of arachidonic acid, which in turn, is metabolized via the cyclooxygenase and lipoxygenase pathways to prostaglandins and a variety of hydroxy acids [16,23]. Since these arachidonic acid metabolites regulate epidermal cell turnover, the barrier defect might be construed to be secondary to the high-turnover state. Recent evidence, from several sources,

Table II. Effect of Topical Linoleic Acid Administration on Sterol Synthesis in Essential Fatty Acid-Deficient Animals

	μmol ^3H_2O Incorporated/h/g Wet Skin Weight			
	Epidermis		Dermis	
	Cholesterol	TNS Lipids	Cholesterol	TNS Lipids
Vehicle-treated side (n = 4)	0.77 ± 0.02	1.20 ± 0.04	0.63 ± 0.08	1.08 ± 0.12
Linoleic acid-treated side (n = 4)	0.46 ± 0.06	0.66 ± 0.09	0.67 ± 0.12	0.89 ± 0.16
	($p < 0.01$)	($p < 0.01$)	NS	NS

Epidermal and dermal de novo sterol synthesis was quantitated in essential fatty acid-deficient mice, in which one flank was treated topically with linoleic acid, and the other with vehicle alone. n = number of animals; NS = not significant.

however, has demonstrated that linoleic acid itself is the critical ingredient in the barrier (reviewed in [17]). The present studies suggest that linoleic acid and sterol metabolism are somehow linked, revitalizing former speculations that sterol esterification might be important for barrier function (reviewed in [24]). Alternatively, accelerated cholesterol synthesis simply may represent a compensatory mechanism for the barrier defect. Or finally, the boost in sterol synthesis could reflect the increased cell turnover in EFAD. However, the rapidity with which sterologenesis normalizes following occlusion makes the last possibility unlikely. Moreover, topical prostaglandins, which reportedly correct the turnover state, but not the barrier defect (reviewed in [16,17,23]), did not modulate sterologenesis as effectively as did linoleic acid (Feingold et al, unpublished observations). It still remains possible, however, that a portion of the boost in sterologenesis that occurs in EFAD could be ascribed to accelerated cell turnover.

In conclusion, the present study presents further evidence that abnormalities in the cutaneous barrier to water loss play a role in regulating the rate of epidermal sterol synthesis. Whether the synthesis of other lipids is controlled similarly by barrier status, and the actual nature of the molecular signal to increased sterologenesis, remains unknown.

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