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## Regulation of Skin Lipid Biosynthesis: Role in Transcutaneous Permeation Enhancement

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Stratum corneum lipids form the major portion of skin constituents. Their synthesis and metabolism regulate the epidermal structural organization and barrier function. An alteration of their content is a good indicator of various skin diseases. The barrier function can be modified by employing epidermal lipid metabolic inhibitors. This approach can be advantageously utilized to increase the transcutaneous delivery of drugs. Hence, it is imperative to study the factors that influence the synthesis and metabolism of epidermal ceramide, cholesterol and fatty acids and their role in barrier homeostasis.

Skin is the single largest organ of the body and functions as an effective barrier against environment and environmental chemicals. Stratum corneum lipids especially, ceramide, cholesterol and fatty acids seem to be responsible for maintenance of such a high order of permeability barrier. Perturbation of barrier leads to alteration in generation of these compounds in the epidermis paralleled with synthesis of abnormal lamellar bodies. In addition, certain skin disorders are associated with altered levels of biosynthesis of these constituents (Table 1).

Moreover, inhibition of synthesis of these skin constituents provides a novel means of enhancing the permeation of drugs across skin. Hence, an understanding of the various biochemical processes that occur after abrogation of the barrier provide a great potential for treatment of skin diseases and for designing future strategies for development of transdermal drug delivery systems.

### CERAMIDES:

The presence of ceramides in human epidermis was first reported by Gray and Yardley in 1975<sup>1,2</sup>. Ceramides

contain sphingosine or a related long chain base along with a fatty acid in the amide linkage. They are synthesized *de novo* in the epidermis, via phospholipid intermediates<sup>3</sup> and constitute up to 40% of the total lipids present in the stratum corneum. They act as the main polar, membrane-forming lipid and play an important role in skin barrier function<sup>4,5</sup>. Recently, sphingosine and sphinganine have been demonstrated to possess potent antimicrobial activity against fungi and bacteria, respectively<sup>6</sup>.

### Role in barrier function regulation:

Cornification of the epidermis of terrestrial animals is accomplished by a complex process that involves sequestration of lipids to intercellular domains<sup>7,8</sup> and alterations in lipid biochemical composition<sup>9</sup>. This process decreases the content of phospholipids towards outer cell layers of the epidermis and produces a nonpolar mixture of lipids enriched in cholesterol, free fatty acids and sphingolipids<sup>10</sup>.

It has been established that disruption of barrier functions of skin by either tape stripping or essential fatty acid deficient diet is accompanied by an increased sphingolipid synthesis<sup>4</sup>. Solvents that remove sphingolipids (and other neutral lipids) from the stratum corneum are

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also capable of abrogating the permeability barrier<sup>11</sup>. The activity of serine palmitoyl transferase, the rate limiting enzyme of sphingolipid synthesis is also increased<sup>12,13</sup>, thus suggesting a link between sphingolipid synthesis and barrier recovery.

The incorporation of tritium into sphingolipids increases after acetone treatment of skin with maximum increase (170%) occurring 5-7 h after treatment. It normalizes when the barrier function returns to normal in 24 h. Artificial restoration of the barrier with a water-impermeable membrane prevents the increase in both, tritium incorporation into sphingolipids and serine palmitoyl transferase activity<sup>4</sup>. In addition, the immediate inhibition of sphingolipid synthesis by topical application of  $\beta$ -chloroalanine is reversed by co-application of ceramides<sup>5</sup>. This indicates that sphingolipid synthesis increases after the barrier recovery has taken place. Hence, the *de novo* synthesis of ceramide does not seem to play an important role in the early rapid phase of barrier recovery.

It is noteworthy, that in normal skin, ceramide is present in relatively small quantities in the lower epidermis and accumulates in large amounts in the stratum corneum and granulosum<sup>1,9,14,15</sup>. In murine epidermis, both the activity of serine palmitoyl transferase and rate of tritium incorporation into sphingolipids has been found to be nearly equivalent in the lower and outer epidermis under basal conditions and after acute barrier perturbation (acetone treatment). However, after chronic perturbation (essential fatty acid deficient diet) the serine palmitoyl activity increases only in the lower epidermis<sup>16</sup>. This increase in serine palmitoyl activity in the lower epidermis is probably required for membrane expansion to support cellular proliferation after a chronic perturbation effect.

Topical application of bromoconduritrol-B epoxide to murine skin selectively inhibits  $\beta$ -glucocerebrosidase and increases the glucoceramide content. But the ceramide content remains unchanged. In addition, immature membrane structures appear in the intercellular spaces throughout the stratum corneum. These biochemical changes are accomplished with decrease in barrier function that is not restored by co-application of ceramide<sup>17</sup>. These findings suggest that extracellular processing of glucosylceramide to ceramide is essential for maintenance of epidermal barrier function. It is interesting to note that the ratio of glucosylceramide to ceramides remain high in oral mucosal epithelium<sup>18</sup> and the  $\beta$ -glucosi-

dase levels are lower in the oral mucosa than in epidermis of the same species<sup>19</sup>. This is perhaps the reason that the oral mucosa is more permeable than the epidermis.

The changes in activities of the enzymes responsible for synthesis of glucosylceramide (GC synthetase) and its conversion to ceramide ( $\beta$ -glucocerebrosidase) provide a clear insight into the development of barrier function. GC synthetase activity rises to a maximum on gestation day 19 and correlates with appearance of epidermal lamellar bodies in rat skin. However, the activity of cerebrosidase rises throughout fetal barrier development and is accompanied by an increase in m-RNA levels indicating increased gene transcription for its activity<sup>20</sup>. Hence, both synthesis and hydrolysis of lamellar body derived glucosylceramide are regulated during fetal development and the enzymatic hydrolysis seems to be essential for barrier ontogenesis.

$\beta$ -glucocerebrosidase requires an acidic pH for optimum activity<sup>17</sup>. Exposure of acetone-treated stratum corneum to pH 7.4 results in persistence of immature, extracellular lamellar membrane structures and a marked decrease in the *in situ* activity of the enzyme paralleled with delay in barrier recovery. In addition, inclusion of calcium and potassium enhances recovery delay while no effect is observed with acidic buffer<sup>21</sup>. Hence, maintaining an acidic stratum corneum pH by either preserving the barrier or exogenously applying acidic pH-based formulations may help in enhancing the recovery of perturbed skin.

Another factor that influences the ceramide synthesis *in vivo* is the sphingolipid activator protein (SAP-C). Decreased levels of prosaposin (p-SAP) have been detected in atopic skin. Deficiency of p-SAP in the epidermis is found to result in accumulation of epidermal glucosylceramides and low levels of ceramide resulting in a thickening of stratum lucidum and abnormality in lamellar membrane maturation within the interstices of stratum corneum<sup>22</sup>.

While intercellular ceramide is essential for maintenance of barrier function, intracellular ceramide takes part in cell signaling (sphingomyelin cycle) and induces cell differentiation or apoptosis<sup>23,24</sup>. The sphingomyelin cycle can be activated by various agents like,  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub><sup>25</sup>, interleukin-1<sup>26</sup>, tumor necrosis factor- $\alpha$ <sup>27,28</sup> and calcipotriol<sup>29</sup>. The activation of this cycle and generation of intracellular ceramide may also provide a

link between an extracellular signal and induction of apoptosis program as a process for elimination of unwanted or damaged cells from multicellular compartments. During this process the cells release matrix, reduce cell volume, condense their chromatin and disintegrate the nuclear lamina. It has been recently reported that exogenous addition of cell-permeable ceramides induce apoptosis as measured by nuclear fragmentation<sup>30</sup>. Hence, regulation of intracellular ceramide seems to be important for preventing apoptosis.

Okadaic acid, a specific inhibitor of 2A-type serine/threonine protein phosphatases inhibits ceramide-induced apoptosis. The other targets of ceramide action include, ceramide-activated protein kinase<sup>31-33</sup> and protein kinase C isoform<sup>34</sup>. Identification and understanding the function of these targets will help in designing strategies for preventing cell death and managing skin diseases.

Skin xerosis is a condition in which the skin condition effectively improves by topical application of lactic acid<sup>35</sup>. The incorporation of L-lactic acid into keratinocytes has been found to be more than D-lactic acid. The increased levels of ceramide are accompanied with improvement in barrier function as judged by transepidermal water loss (TEWL)<sup>36</sup>. This indicates the influence of enhanced ceramide synthesis in decreasing the desquamatory process and improving the corneocyte cohesiveness, which may help in improving the skin condition in winter xerosis. A recent development in this area is the observation of significant increase in ceramide level in keratinocytes (*in vitro*) after culturing with *Streptococcus thermophilus*. The increase is due to increased levels of GSH-sensitive shingomyelinase and not due to *de novo* synthesis of ceramide<sup>37</sup>. Development of a topical formulation containing sonicated *S. thermophilus* is in progress that will provide a means for restoring ceramide content in skin of patients suffering from desquamatory disease.

#### CHOLESTEROL:

Skin is the major site for sterologenesi s in both rodents<sup>38</sup> and primates<sup>39</sup>. About 80% of the total cholesterol synthesis takes place in the dermal layers and the remaining 20% is derived from the basal and spinous layers of the viable epidermis<sup>40</sup>. This synthesis occurs *de novo* in the viable epidermis and is not affected by circulating blood cholesterol levels<sup>41</sup>, possibly because epithelial cells lack LDL receptors<sup>42,43</sup>. Skin in various parts

of the body contain appreciably different cholesterol content. Additionally, the content of free cholesterol and cholesteryl ester are altered in diseases like, psoriasis and ichthyosis<sup>44</sup>. Hence, synthesis of cholesterol in skin seems to be important for maintaining proper functioning of skin.

#### Role in barrier function regulation:

Cholesterol synthesis responds to certain specific regulatory influences. Skin damage by treatment with either detergents or solvents leads to a burst of non-saponifiable lipid synthesis activity that is limited to the epidermis. The synthesis normalizes when the TEWL returns to normal after occlusion of damaged skin with latex film<sup>45</sup>. This suggests that barrier perturbation regulates sterol synthesis. However, there appears to be a difference between the turnover rates of cholesterol and ceramide during barrier recovery phase. Studies with tritium incorporation have shown that cholesterol synthesis is accelerated during the early phase of barrier recovery (0-4 h) and returns to normal levels within 6 h<sup>45,46</sup>. This is accompanied by an increase in the amount of HMG-CoA reductase (within 2 h) after barrier disruption with acetone that returns to normal by 7 h<sup>47</sup>. But, neither incorporation of tritium into sphingolipids nor serine palmitoyl transferase activity has been found to increase significantly during the first 5 h after barrier disruption with acetone<sup>4</sup>. These findings suggest that new cholesterol synthesis is required for early barrier repair. Additionally, it is possible that a pre-stored pool of sphingolipids in the stratum granulosum may be sufficient for early repair while the preformed cholesterol pool may not be sufficient for this purpose. Hence, the newly synthesized cholesterol seems to be mainly responsible for barrier repair immediately after perturbation whereas, the delayed synthesis of ceramide seems to be necessary for later stages of barrier repair. Further, barrier perturbation has been also found to modulate the activation state (dephosphorylated form) of HMG-CoA reductase. The incorporation of [<sup>14</sup>C] acetate (starting point of cholesterol biosynthesis) into cholesterol increases considerably whereas, that of [<sup>3</sup>H] mevalonolactone (converted form of HMG-CoA) remains unchanged following skin treatment with acetone. This demonstrates that the initial increase in cholesterol synthesis induced by barrier disruption occurs before the formation of mevalonate. A higher degree of barrier disruption (TEWL>550 ppm/0.5 cm<sup>2</sup>/h) following acetone treatment or essential fatty acid

deficiency diet results in an increase in total HMG-CoA reductase activity, whereas lower degree of barrier disruption (TEWL > 300 ppm/0.5 cm<sup>2</sup>/h) leads to changes in activation state<sup>47</sup>. This indicates that cholesterol synthesis after profound barrier disruption occurs via changes both in the amount and activation state, whereas moderate barrier disruption influences synthesis by only modulating the activation state of HMG-CoA reductase.

Essential fatty acid deficiency diet causes abnormality in permeability barrier function<sup>48</sup> and increases the epidermal cell turnover<sup>49</sup>. In mice with such skin, the epidermal sterol synthesis increases, while dermal sterol synthesis remains unaffected. Occlusion with water impermeable latex normalizes the transdermal water loss and leads to normalization of sterol synthesis even in the presence of fatty acid deficient diet<sup>24,50</sup>. The fact that the transport of extracutaneously derived fatty acid to perturbed skin is not increased<sup>24</sup>, suggests that cutaneous water barrier status regulates epidermal sterologogenesis.

HMG-CoA reductase is inhibited by cholesterol sulphate. The low ratio (1:1) of free cholesterol-to-cholesterol sulphate in stratum corneum of X-linked ichthyosis<sup>51</sup> has been suggested to be due to rapid uptake of cholesterol sulphate than free cholesterol by keratinocytes. This cholesterol sulphate is then not washed out of keratinocytes<sup>52</sup>. Hence, cholesterol sulphate-cholesterol homeostasis in the epidermis seems to be important for desquamation.

It is noteworthy that TEWL recovery after skin treatment with acetone is inhibited by calcium (0.01 mM). Moreover, both verapamil and nifedipine prevent the calcium-induced inhibition of TEWL recovery. In addition, potassium alone (10 mM) and phosphate alone (5 mM) also produce slight inhibition of barrier repair. A synergistic inhibitory effect is exhibited by a combination of calcium and potassium and this is paralleled by inhibition of HMG-CoA reductase activity<sup>53</sup>. Hence, the barrier recovery appears to signal a decrease in the concentration of these ions from the epidermis due to increased water flux that leads to excessive loss of these ions.

Topical application of lovastatin, a competitive inhibitor of HMG-CoA reductase is found to impair normalization of cholesterol in stratum corneum treated with acetone. This treatment results in both delayed secretion of extracellular bodies and appearance of abnormal lamellar bodies, indicating inhibition of secretory proc-

ess and altered/decreased secretion of cholesterol, respectively. Co-application of either mevalonate or cholesterol with lovastatin normalizes the TEWL, indicating that cholesterol synthesis is required for maintenance of barrier structure and function<sup>54</sup>.

It is interesting to note that after topical application of lovastatin (0.75 mg, once daily) to mice, the epidermal cholesterol level decreases initially and then returns to normal at the 10<sup>th</sup> day. However, epidermal fatty acid synthesis increases by 277% and HMG-CoA reductase activity increases by 231%. This is accompanied by structural abnormalities in the lamellar body secretory system of epidermis. This indicates a compensatory response in cholesterol synthesis due to chronic barrier abnormality produced by repeated application of lovastatin<sup>55</sup>. Hence, an altered sterol:fatty acid ratio rather than decreased cholesterol may be responsible for appearance of abnormal barrier function upon repeated application of lovastatin.

Cholesterol has a greater role in skin of aged as compared to young mice. HMG-CoA reductase activity and sterologogenesis is decreased in aged mice and it fails to attain the levels reached in young epidermis following acute perturbations. In contrast, fatty acid and sphingolipid synthesis increase sufficiently in aged mice so as to approach the levels attained in young epidermis. In addition, application of either a mixture containing stratum corneum lipids in equimolar concentration<sup>56</sup> or cholesterol alone<sup>57</sup> to perturbed skin results in production of normal lamellar structures and enhances barrier recovery. This finding implies decreased ability of aged epidermis to repair following an injury and hence, may also lead to altered rate of drug delivery as compared to young epidermis. This strongly suggests the need for careful adjustment of transdermal dose for elderly patients.

#### FATTY ACIDS:

Linoleic acid and arachidonic acid are the most abundant essential fatty acids in the epidermis. Linoleic acid is solely derived from the diet. Arachidonic acid originates from the liver because skin cannot convert linoleic acid to arachidonic acid<sup>58</sup>. Ultrastructure of essential fatty acid deficient mouse epidermis shows normal number of lamellar bodies that are empty or only partially filled and after secretion, their contents do not fill the intercellular spaces<sup>59</sup>. The deficiency also causes epidermal hyperproliferation in rat<sup>60</sup> and presumably occurs due to a decrease in epidermal PGE<sub>2</sub> levels<sup>61</sup>. The evidence that

TABLE 1 : SOME IMPORTANT SKIN DISORDERS ASSOCIATED WITH ALTERED SKIN CONSTITUENTS

Skin disease	Metabolic Defect	Altered Skin Constituent	Ref.
1. Acne	Unknown	Decreased ceramide	77
2. Atopic dermatitis	Deficiency of sphingomyelinase and sphingomyelinacylase induction	Decreased ceramide	78
3. Atopic skin	Elevated ceramidase activity	Decreased ceramide	79
4. Fabry's disease	Deficiency of $\alpha$ -galactosidase	Increased globotriosyl ceramide	80
5. Farber disease	Deficiency of ceramidase	Enhanced ceramide synthesis	80
6. Gaucher disease	Deficiency of $\beta$ -glucosidase	Increased glucosylceramide	81
7. Lamellar ichthyosis	Unknown	Decreased ceramide Increased free cholesterol and decreased cholesterol ester	82 44, 83
8. Limited lamellar ichthyosis	Unknown	Increased ceramide	82
9. Bullous ichthyosiform erythroderma	Unkown	Decreased ceramide	82
10. Psoriasis	Unknown	Decreased cholesterol ester Decreased C <sub>1</sub> , C <sub>3</sub> , C <sub>4</sub> , C <sub>5</sub> , C <sub>6</sub> Increased C <sub>2</sub> , C <sub>5</sub>	44 84
11. Atopic eczema		Increased free sterols	85
12. Palmoplantar keratoderma		Imbalance of cholesteryl sulphate/cholesterol	86
13. Recessive X-linked ichthyosis	Deficiency of sterol sulphatase	Increased cholesterol sulphate	51
14. Congenital ichthyosiform erythroderma		Increased n-alkanes	87

C<sub>1</sub>-C<sub>6</sub>: Fatty acids with respective carbon chain length.

Table summarizing few clinically important skin diseases that are associated with altered skin lipid composition.

topical or intraperitoneal administration of linoleic acid restores the barrier function without improving the skin scaliness and arachidonic acid heals scaliness without repairing barrier function<sup>62, 63</sup>, indicates that linoleic acid plays a specific role in barrier integrity whereas, arachidonic acid is involved in epidermal homeostasis.

#### Role in barrier function regulation:

The rate of TEWL is known to reduce with application of sunflower-seed oil, which is rich in linoleic acid<sup>64</sup>. Skin deficient in fatty acid develops a 'leaky' barrier, which is associated with loss of intercellular lipid. As a result of

this, water soluble tracers gain access to normally inaccessible cellular domains<sup>59</sup> and the lipid soluble tracers preferentially traverse intercellular domains<sup>65</sup>. The stratum corneum membrane complex contains about 50% lipid that accounts for approximately 80% total stratum corneum lipid<sup>66</sup>. Moreover, the intercellular spaces occupy 10-30% of the stratum corneum volume as compared to 0.5-1.5% in other tissues<sup>67</sup>. This may account for high permeation of lipophilic molecules across skin.

Like the other two major constituents, the biosynthesis of fatty acids is also regulated by cutaneous

barrier requirements. Perturbation of cutaneous permeability by acetone increases only epidermal but not dermal fatty acid biosynthesis as measured by tritium incorporation into fatty acid. This synthesis normalizes after 12 h. However, essential fatty acid deficiency also displays elevated levels of dermal fatty acid synthesis. Transport of extracutaneously derived fatty acid to perturbed skin as evidenced by no uptake of albumin-bound [<sup>14</sup>C] stearic acid by epidermis and dermis indicates *de novo* synthesis of fatty acid after perturbation. In addition, normalization of TEWL by occlusion with latex film despite the presence of fatty acid deficiency, indicates that water flux possibly provides a signal for *de novo* synthesis of fatty acid<sup>23</sup>.

Topical application of 5-(tetradecyloxy)-2-furancarboxylic acid (TOFA), an inhibitor of acetyl CoA carboxylase after disruption of the barrier by acetone or tape stripping inhibits epidermal fatty acid and delays barrier recovery. The accompanying abnormalities in organization of lamellar bodies are corrected by co-administration of palmitate which in turn normalizes the barrier function<sup>68</sup>. In fact, barrier disruption has been found to increase fatty acid synthesis by increasing the activities of both epidermal acetyl CoA carboxylase and fatty acid synthetase<sup>69</sup>. Hence, in addition to essential fatty acids, bulk fatty acids also seem to be essential for barrier homeostasis.

In addition, an optimum ratio of free fatty acids-to-cholesterol and free fatty acids-to-ceramide appears to be necessary for proper skin barrier function. Stratum corneum of patients with lamellar ichthyosis exhibits an extra peak in the X-ray diffractogram, which can be ascribed to crystalline cholesterol. The reduced free fatty acid-to-cholesterol ratio and free fatty acid-to-ceramide ratio are accompanied with increased TEWL<sup>70</sup>. This suggests that quality of the stratum corneum depends on both composition and organization.

Although, skin synthesizes fatty acids *de novo*, the transport of exogenous essential fatty acids to the epidermis is necessary. Fatty acid uptake by keratinocytes exhibits preference for essential fatty acids and is mediated by a transport system. In cultured human keratinocytes, increased differentiation does not affect fatty acid transport protein mRNA levels, but results in an increase (70%) in fatty acid acyl CoA synthetase mRNA. The fatty acid binding protein mRNA is decreased, while fatty acid translocase mRNA is not detectable<sup>71</sup>.

However, in murine epidermis fatty acid translocase mRNA in addition to the other proteins has also been detected. Barrier disruption increases its levels, while occlusion with impermeable membrane completely blocks this increase<sup>72</sup>. Fatty acyl CoA esters are then utilized in anabolic or catabolic pathways and also facilitate fatty acid uptake into cells<sup>73</sup>. Hence, an increase in its activity in differentiated keratinocytes could enhance fatty acid uptake which is consistent with the high requirements of differentiated keratinocytes for fatty acid that serve as a precursor of extracellular lamellar membranes. The increased levels of fatty acid translocase mRNA in murine epidermis indicates that barrier disruption is directly responsible for its increase, which may possibly enhance fatty acid transport.

## CONCLUSIONS:

The existence of a definite proportion of ceramide, cholesterol and fatty acids is essential for maintaining normal structure and permeability functions of skin. The content of these critical skin constituents varies in different parts of the body. Therefore, site of application of a transdermal formulation seems to be clinically important. Both acute (solvent extraction) and chronic (fatty acid deficient diet) perturbation lead to altered permeability of the skin. Because lipophilic drug molecules exhibit greater permeation due to high lipidoidal content of skin, extraction with solvents reduces the permeation of highly lipophilic drug molecules. But the permeation of ionized<sup>74</sup> or hydrophilic molecules like mannitol<sup>75</sup>, is profoundly increased. This has been attributed to the formation of hydrophilic compartments in solvent-extracted skin<sup>75</sup>. Therefore, a critical evaluation of these skin constituents that may act as rate limiting step for transcutaneous permeation of a drug molecule will help in optimizing drug delivery through skin.

Topical application of synthesis inhibitors of these skin constituents delays barrier recovery of skin. This aspect is being increasingly investigated to increase the transdermal delivery of drugs that exhibit poor permeation across normal skin. However, extrapolation of *in vitro* results to *in vivo* situation need to be carefully examined. For example, although, fluvastatin increases the octanol/water partition coefficient of lidocaine hydrochloride by 50 times, the *in vivo* uptake increases only 2-fold<sup>76</sup>. In addition, dose optimization of these metabolic inhibitors seems to be important and their pharmacological effects upon systemic absorption should be critically

evaluated, especially after chronic use. Moreover, repeated topical application of formulations containing these chemicals as absorption enhancers to the same site may result in altered permeability of the skin and lead to altered rates of drug delivery. Hence, better understanding of the role of these metabolic inhibitors will provide new strategies for skin disease treatment and enhancement of percutaneous permeation of drugs.

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