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Citation for final published version:

Muñoz-García, Agustí, Thomas, Christopher P., Keeney, Diane S., Zheng, Yuxiang and Brash, Alan R. 2014. The importance of the lipoxygenase-hepoxilin pathway in the mammalian epidermal barrier. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 1841 (3) , pp. 401-408. 10.1016/j.bbalip.2013.08.020 file

Publishers page: <http://dx.doi.org/10.1016/j.bbalip.2013.08.020>  
<<http://dx.doi.org/10.1016/j.bbalip.2013.08.020>>

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The importance of the lipoxygenase-hepoxilin pathway in the mammalian epidermal barrier

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**Keywords:** Lipoxygenase, linoleic acid, hepoxilin, trioxilin, ceramide, ichthyosis, essential fatty  
acid

**Abbreviations:**

ARCI, autosomal recessive congenital ichthyoses; CLE, corneocyte lipid envelope; EFA, essential fatty acids; LOX, lipoxygenase; eLOX3, epidermal lipoxygenase-3; EOS, esterified omega-hydroxyacyl-sphingosine; FATP4, fatty acid transport protein 4; H(P)ETE, hydro(pero)xyeicosatetraenoic acid; H(P)ODE, hydro(pero)xy-octadecadienoic acid; HxA, HxB, hepoxilin A or B; OS, omega-hydroxyacyl-sphingosine; VLFA, very long-chain fatty acid

## Highlights

- The lipoxygenases 12*R*-LOX and eLOX3 act in tandem in epidermal barrier function
- They oxidize linoleic acid esterified in  $\omega$ -hydroxyacyl ceramide to hepoxilin
- This acts as a signal for hydrolysis of the oxidized linoleate from the ceramide
- The free ceramide  $\omega$ -hydroxyl is bonded to protein forming a key barrier component
- Understanding the role of hepoxilins can lead to therapy of skin diseases.

## Summary

This review covers the background to discovery of the two key lipoxygenases (LOX) involved in epidermal barrier function, 12*R*-LOX and eLOX3, and our current views on their functioning. In the outer epidermis, their consecutive actions oxidize linoleic acid esterified in  $\omega$ -hydroxyceramide to a hepoxilin-related derivative. The relevant background to hepoxilin and trioxilin biochemistry is briefly reviewed. We outline the evidence that linoleate in the ceramide is the natural substrate of the two LOX enzymes and our proposal for its importance in construction of the epidermal water barrier. Our hypothesis is that the oxidation promotes hydrolysis of the oxidized linoleate moiety from the ceramide. The resulting free  $\omega$ -hydroxyl of the  $\omega$ -hydroxyceramide is covalently bound to proteins on the surface of the corneocytes to form the corneocyte lipid envelope, a key barrier component. Understanding the role of the LOX enzymes and their hepoxilin products should provide rational approaches to ameliorative therapy for a number of the congenital ichthyoses involving compromised barrier function.

## **The mammalian epidermal water barrier: a short primer**

One of the most important functions of the skin is the maintenance of a barrier to water vapor diffusion to the environment [1-3]. Substantial evidence demonstrates that the main determinant of the permeability properties of the skin is the lipid composition and organization in the outer layers of the epidermis, the stratum corneum [4, 5]. The mammalian stratum corneum (SC) consists of flat, dead cells, called corneocytes, surrounded by a protein layer, the cornified envelope (CE), in turn surrounded by a monolayer of lipids, the corneocyte lipid envelope (CLE), covalently attached to the CE. The spaces between corneocytes are filled with a matrix of intercellular lipids, mainly cholesterol, free fatty acids, and ceramides, which altogether organize in layers called lamellae in mammals. The backbones of the lamellae are the ceramides, molecules formed by a sphingoid base amide-linked to a fatty acid of varying chain length [6-12]. The intercellular lamellae are thought to impede excessive evaporation through the skin, as their disruption leads to increases in transepidermal water loss (TEWL) [13, 14]. Fundamental to the formation of a fully competent barrier is the need of linoleic acid, incorporated in a particular class of ceramides unique to the epidermis (EOS, esterified  $\omega$ -hydroxyacyl-sphingosine) [5, 15, 16]. The activity of epidermal lipoxygenases (LOX) is crucial to understand the physiological process that links the need of essential fatty acids to ensure the normal formation and maintenance of the permeability barrier of the skin. [Figure 1](#) illustrates the main structural components of the barrier relevant to the role of LOX enzymes.

## **Barrier genes and ichthyosis**

Genetic anomalies in the complex physiological process of epidermal barrier formation result in scaly skin diseases (ichthyoses) with medical complications like dehydration, infections, chronic blistering, and rapid-calorie loss [17-20]. Gene mutations implicated in the autosomal recessive congenital ichthyoses (ARCI) include *ALOX12B*, *ALOX3*, *CYP4F22*, *ichthyin* and *TGM-1* [18, 21-24]. Cytochrome P450 *CYP4F22* may be responsible for synthesis of the  $\omega$ -hydroxyl of  $\omega$ -hydroxy-acyl ceramides. *ichthyin* is a predicted transmembrane protein with undetermined function. *TGM-1* cross-links the proteins forming the corneocyte envelope (CE), and is also implicated through in vitro studies and a knock-in mouse mutation in catalyzing the covalent bond between  $\omega$ -hydroxy-acyl ceramides and the CE, forming the CLE [25, 26], (albeit with human studies showing that lamellar ichthyoses due to mutations in this isozyme have a normal

CLE) [27]. The fact that inactivating mutations in all of these genes produce related skin phenotypes supports the idea that they are elements of the same physiological process.

The genetic evidence establishes that the integrity of the mammalian epidermal water barrier requires the normal functioning of 12*R*-lipoxygenase (12*R*-LOX, human gene *ALOX12B*) and epidermal lipoxygenase-3 (eLOX3, gene *ALOXE3*) [18, 21, 28-30]. LOX enzymes are dioxygenases that oxygenate lipids (polyunsaturated fatty acids and their esters), yet their specific role in skin barrier function is not well understood. In general terms, and taking parallels from better known LOX enzymes, their functions may be to produce oxidized lipids as signaling molecules, to induce structural changes through an enzyme-catalyzed lipid peroxidation, or rarely, to help initiate the mobilization of lipids (reviewed in ref. [31]). Our current model suggests a function mainly related to the second of these possibilities, the induction of structural changes.

The present paper stands as an update and major revision of an earlier review [32]. Before going into specifics on our current model of the workings of 12*R*-LOX and eLOX3 in epidermal barrier function, we present overviews of the background history of the two LOX enzymes and of their essential fatty acid substrate in the skin.

### **Historical perspective on 12*R*-Lipoxygenase**

Mammalian 12*R*-LOX and eLOX3 remained undetected until the late 1990's. The existence of LOX enzymes in the plant kingdom was recognized over 70 years ago [33], and the more modest levels of LOX enzyme activities in animal tissues were first identified in the mid-1970s. That work established the existence of a 12*S*-LOX in blood platelets [34, 35], a 5*S*-LOX in leukocytes (catalyzing the first steps in leukotriene biosynthesis) [36], and a 15*S*-LOX in reticulocytes [37]. Closer examination over the years revealed the expression of these enzymes in many other mammalian tissues [38].

The “*S*” in 12*S*-, 5*S*- and 15*S*-LOX enzymes refers to the mirror image form of the fatty acid hydroperoxide product. “*R*” chirality LOX enzymes were unknown. Then in the mid-1980s enzyme activities in invertebrate animals (corals, starfish, sea urchins) were characterized that

synthesized “*R*” chirality fatty acid hydroperoxides [39]. In the mid-1990s, purification of one of these invertebrate *R*-LOX proteins and its molecular cloning revealed a close sequence homology to the plant and animal *S*-LOX enzymes [40]. Differences in substrate binding were proposed that could explain how structurally related enzymes can form products of one mirror image form or the other [40, 41].

The first indication of a role for oxidized polyunsaturated fatty acids in skin biology came in the mid 1970’s when an accumulation of 12-HETE (12-hydroxyeicosatetraenoic acid, i.e. 12-hydroxy-arachidonic acid) and free arachidonic acid was discovered in the involved epidermis of psoriasis [42]. At the time, it was presumed that the 12-HETE arose from the activity of 12*S*-LOX (subsequently identified in the germinal layer cells of the mammalian epidermis [43]). A decade later the psoriatic product was shown to be mainly 12*R*-HETE [44]. It took another decade of research before the “culprit” enzyme in this case was identified as the uncommon type of *R*-lipoxygenase, 12*R*-LOX, the only *R*-LOX in mammalian tissues [45, 46].

Historically the enzymatic oxidation of arachidonic acid in mammalian biology shows a strong association with the biosynthesis of pro-inflammatory lipid mediators [47], and this remains a focus of eicosanoids in skin inflammation and immunity [48]. Synthesis of the prostaglandin products of the cyclooxygenase pathway is inhibited by NSAIDs, and the leukotriene products of 5-LOX have their pro-inflammatory actions blocked by receptor antagonists such as Singulair<sup>®</sup> and Accolate<sup>®</sup> [47]. Initially therefore, it was natural to equate the synthesis of 12*R*-HETE in psoriasis with the inflammation in the epidermis. Yet 12*R*-HETE itself showed only modest pro-inflammatory activity [49]. From a current perspective, the abundance of 12*R*-HETE in psoriasis may reflect merely the co-occurrence of free arachidonic acid (not present normally, but a facet of the inflammation in psoriasis) with an over-abundance of the granular cells of the upper epidermis, the natural site of 12*R*-LOX expression in healthy skin. Having the arachidonic acid available as substrate together with high expression of 12*R*-LOX may account for the excess of 12*R*-HETE in psoriatic epidermis. In fact in normal epidermis, there is only one definitive account of the synthesis of 12*R*-HETE, barely detectable as a product of [<sup>14</sup>C]arachidonic acid metabolism in human hair roots [50].

In primary cultures of human keratinocytes there is no detectable 12*R*-LOX activity (they produce only traces of 15-HETE [51, 52]). The absence of 12*R*-LOX is likely attributable to its association with the late stages of keratinocyte differentiation, a condition that requires special long-term treatments to mimic in culture [53], and not studied so far with regard to LOX enzyme activities. (A prominent 12-LOX activity was detected in normal human epidermis, but the *R/S* chirality of the 12-HETE was not determined [54]). Despite the hard to detect activity, immunohistochemical evidence clearly defines the occurrence of 12*R*-LOX (and eLOX3) in the outermost cells of the stratum granulosum in normal mammalian epidermis [29, 30]. We also have evidence from *in situ* analysis in human skin that 12*R*-LOX mRNA expression occurs in the outermost epidermal cells that retain a nucleus (Figure 2). Although 12*R*-LOX expression is not entirely skin-specific, no symptomology or phenotype is reported in relation to the limited expression in other tissues. While mRNA transcripts for 12*R*-LOX are detectable by RT-PCR in several other tissues (and similarly for eLOX3) [55, 56], the only instance confirmed so far with catalytically active enzyme is a weak activity of 12*R*-LOX in human tonsils [57].

The role of 12*R*-LOX in healthy skin is quite different from the earlier implications of its appearance in psoriasis, and we now know that the natural substrate of 12*R*-LOX is a linoleate ester rather than arachidonic acid. As firmly established from analysis of the gene inactivation in humans and mice, the biological role of 12*R*-LOX is helping to seal the permeability barrier of the outer epidermis, an activity that entails intimate cooperation with eLOX3 in synthesis of linoleate-derived “hepoxilins”.

### **“Hepoxilins”**

Fatty acid hydroperoxides are easily broken down through non-enzymatic transformations, including into fatty acids in which the original two oxygen atoms of the hydroperoxide moiety are rearranged in the molecule to form one epoxide and one hydroxyl group [58]. Non-enzymatically, these epoxy-hydroxy (also called epoxyalcohol) derivatives are produced as a mixture of isomers.

The name “hepoxilin” was coined in the mid-1980s to describe epoxyalcohol products of the 12*S*-LOX-derived arachidonic acid hydroperoxide, 12*S*-HPETE [59]. They were considered to

be derived enzymatically, although at the time there was scant evidence to support the contention. “Hepox” stood for hydroxy-epoxy and “ilin” came from an activity as an insulin secretagogue (albeit an activity not confirmed to be of physiological significance). All fatty acid hydroperoxides readily give rise to epoxyalcohol derivatives, although strictly only derivatives of the arachidonic acid hydroperoxide 12*S*-HPETE merit the connotation “hepoxilin”. Nonetheless, all the others can be considered as hepoxilin-related or hepoxilin-type fatty acid derivatives [32]. For example, the term is commonly applied to the epoxyalcohols formed by eLOX3 from linoleate hydroperoxides.

Hepoxilins are classified as A-type or B-type [60]. When a fatty acid hydroperoxide rearranges into epoxyalcohols, the new OH group can be separated from the new epoxy group by a double bond (hepoxilin A, HxA, chemically an “allylic” epoxide, readily hydrolyzed in mild acid e.g. pH 3), or the OH can be next to the epoxide (hepoxilin B, HxB, more stable to mild hydrolysis conditions) (Figure 3). Non-enzymatic synthesis produces a mixture of HxA- and HxB-type epoxyalcohols, and each of these is a mixture of *R*- and *S*-hydroxy isomers. So, non-enzymatically, four major hepoxilin products are formed from 12*S*-HPETE. Because of the differences in chemical stability, the HxB-type is the more commonly detected in tissue extracts, whereas the HxA-type may appear as the corresponding hydrolysis products, the trioxilins (Figure 3). The detection of hepoxilin and trioxilin derivatives in human epidermis occurred along these lines.

Analysis of the hepoxilin/trioxilins in human and murine epidermis suggests that the majority of the free products are “true” hepoxilins originating from 12*S*-HPETE. Freshly isolated human epidermal cells catalyze the stereoselective biosynthesis of HxB<sub>3</sub> from arachidonic acid and 12*S*-HPETE [54, 61], and the levels are greatly increased in the lesion epidermis in psoriasis, along with the corresponding trioxilins [62]. The recent studies in *Alox3* knockout mice indicate that free hepoxilins are measurable in the normal murine epidermis (along with purely 12*S*-HETE) and that the synthesis is eLOX3-dependent [30].

Aside from hydrolysis promoted by the acidic conditions used for fatty acid extraction, formation of trioxilins in cells is likely to be catalyzed by epoxide hydrolases (EH). In 1989 a specific

hepoxilin EH was isolated from rat liver with an observed preference for hepoxilin A<sub>3</sub> over leukotriene A<sub>4</sub> [63]. However, it was shown recently that this activity is indistinguishable from mammalian soluble epoxide hydrolase (sEH) [64]. Because moderate expression of sEH has been shown in human epidermis, the presence of trioxilins both from AA and LA is possible, warranting further investigation [65]. The substrate specificity with regard to possible EOS trioxilin moieties should also be examined. Novel candidate EH genes were described recently and the first of these enzymes to be characterized (termed EH3) is highly active in the hydrolysis of fatty acid epoxides [66]. “Skin” is among the tissues showing strong expression of EH3 [66], and clearly it is of interest to define the location of its expression more precisely.

### **A historical perspective on eLOX3**

eLOX3 was cloned from mouse epidermis cDNA in the late 1990s by Fürstenberger, Krieg and colleagues as one of three newly discovered LOX genes [67, 68]. Two of the expressed proteins were characterized as the mouse 8*S*-LOX and 12*R*-LOX. No catalytic activity could be found with the third, so it could only be named as epidermal lipoxygenase-3 (eLOX3, human gene *ALOXE3*) [69].

The paradigm-shifting report that mutations in the human 12*R*-LOX or eLOX3 genes are associated with congenital ichthyosis included the insightful suggestion that because a mutation in either of the two LOX genes produces a similar phenotype, the two LOX enzymes may function in the same metabolic pathway [21]. This prompted further investigation of the biochemical activities of eLOX3, with the finding that the enzyme exhibits hydroperoxide isomerase activity, converting fatty acid hydroperoxides to specific hepoxilin-type derivatives (and also to fatty acid ketones) [70]. In contrast to the non-enzymatic hepoxilin synthesis discussed in the previous section, the hepoxilin products of eLOX3 are specific epoxyalcohol isomers, with just one chiral form of the new hydroxy group. For example, eLOX3 converts 12*R*-HPETE specifically to an 8*R*-hydroxy-11*R*,12*R*-epoxide, a single HxA-type epoxyalcohol [70]. Mechanistic studies on eLOX3 suggest that its unusual hydroperoxide isomerase activity is related to a lack of access of molecular oxygen within the active site, which allows redox cycling of the catalytic iron and efficient metabolism of fatty acid hydroperoxides [71, 72].

We hypothesize that it is linoleate esterified in epidermal-specific ceramides that are the natural substrates for 12*R*-LOX and eLOX3 and that these activities underlie the role of the LOX genes in skin barrier function. Actually, the linoleate hydroperoxides 9-HPODE and 13-HPODE are not outstandingly good substrates of eLOX3, at least not as the free fatty acids [73]. But this is not directly relevant according to our current hypothesis on the natural physiological substrates of 12*R*-LOX and eLOX3.

### **The crucial requirement for one essential fatty acid, linoleate**

When the hydroperoxide isomerase activity of eLOX3 was first discovered it was shown that 12*R*-HPETE is a particularly good substrate [70]. If 12*R*-LOX and eLOX cooperate in their activities to produce a hepoxilin-type product, it appeared to make sense that the primary 12*R*-LOX product from arachidonic acid (12*R*-HPETE) should be the substrate for eLOX3. But in retrospect this was discounting a long line of evidence from studies of essential fatty acids and their role in the epidermis. The fact is that arachidonic acid is almost absent from the outermost cells of the mammalian epidermis. There is excellent biochemical evidence from fatty acid analyses and from feeding studies in animals that the physiologically relevant essential fatty acid in relation to mammalian epidermal barrier function is solely linoleic acid [74].

The need for an essential fatty acid in barrier function was first recognized through the classic work of George and Mildred Burr, who discovered the dietary requirement for an “essential fatty acid” (EFA). They showed that one of the obvious symptoms of a dietary deficiency is increased TEWL and the development of a scaly skin phenotype [75, 76]. Topical application of EFA alleviates the skin symptoms [77]. Later it was shown that although feeding arachidonic acid cures the symptoms, the mechanism involves its retroconversion to linoleate [78]. The scaly skin also develops in EFA deficiency in humans and is reversible by adding linoleate back to the diet [79].

A key observation, highly relevant to the involvement of LOX enzymes, is that EFA deficiency is associated with a loss of linoleate (C18:2 $\omega$ 6) in the epidermis and its replacement with oleate (C18:1 $\omega$ 9). Oleate has only one double bond and is not a LOX substrate. Thus, either

inactivating mutations in one of the key LOX enzymes or a deficiency in the epidermal LOX substrate (linoleate) results in loss of integrity of the epidermal water barrier.

### **EFA and ceramides, intimately linked in the epidermis**

Linoleate is by far the most abundant EFA in the epidermis, wherein it is mainly esterified to the omega hydroxyl of the amide-linked very long-chain fatty acid (VLFA, C<sub>28</sub>-C<sub>36</sub>) in a class of ceramide specific to the epidermis, esterified omega-hydroxyacyl-sphingosine (EOS) [16]. EOS is an important structural ceramide in the intercellular lamellae. In addition, a portion is further converted to omega-hydroxyacyl-sphingosine (OS) and omega-hydroxy-VLFA that are covalently attached to the external face of the cornified cell envelope (CE) composed of cross-linked proteins (Figure 4) [25, 80]. These covalently bound lipids form the corneocyte lipid envelope (CLE) [81].

### **Proposed role of linoleate and LOX enzymes in construction of the epidermal barrier**

Figure 5 presents our proposal on the involvement of the two LOX enzymes in construction of the epidermal water barrier. The eponymous organelles of the stratum granulosum, the lamellar granules (or lamellar bodies), contain lipid precursors and a battery of hydrolytic enzymes [82, 83]. As illustrated at step 1 in Figure 5, glucosyl-EOS is a constituent of the limiting membrane of the lamellar bodies, with its glucose moiety on the inside [84, 85]. At stage 2, as the granules fuse with the plasma membrane, the molecules of glucosyl-EOS expose the glucose moieties on the outside, the very long chain fatty acid of EOS spans the bilayer, and in this rendering the molecule is folded in a U-shape with the linoleate chain in the membrane. As differentiation proceeds, the glucosyl-EOS is deglycosylated and the two LOX enzymes oxidize the linoleate tail (Figure 5, step 3), producing an “oxygen signal” as the more polar carbon chain no longer strongly favors the lipid environment. We hypothesize that the oxidized linoleate will serve as substrate for an esterase/hydrolase, which cleaves the oxidized linoleate to form OS with a free  $\omega$ -hydroxyl (Figure 5, step 4). This lipase (not identified to date) could be released from the lamellar bodies and activated in the intercellular spaces of the SC (see ref [86]), or be intracellular, as currently assumed for the two LOX enzymes. Finally the ceramide OS will be covalently coupled to the polymerized protein of the CE via the action of transglutaminase, thus

forming the corneocyte lipid envelope (CLE) of the completed epidermal barrier (Figure 5, step 5).

### **Evidence supporting the role of the LOX enzymes in barrier function**

The skin phenotype in gene inactivation and knockout studies highlights the essential role of 12R-LOX and eLOX3 in barrier formation and prevention of transepidermal water loss, and the specific location of the LOX enzyme expression in the outer epidermis is consistent with this role. Furthermore, biochemical analyses show that linoleate (C18:2 $\omega$ 6, a LOX substrate) is the sole EFA required for skin barrier integrity and that its replacement by oleate (C18:1 $\omega$ 9, not a LOX substrate) in EFA deficiency results in barrier defects. Our working hypothesis of the involvement of the two LOX enzymes in the oxidation of linoleate-containing ceramides and formation of the CLE is supported by the following observations [87]:

- i) Normal pig and murine epidermis contain low levels of oxidized species of linoleate in the EOS ceramides.
- ii) These oxidized species of linoleate in the ceramides are chiral, with the hallmark of 12R-LOX and eLOX3 activity. The oxidized species are predominantly 9R-HODE, 9-keto-linoleate, and hepxilin-related 9R,10R-epoxy-13R-hydroxy and/or 9R,10R-epoxy-13-oxo-linoleates.
- iii) These oxidized ceramides are almost absent in the epidermis of 12R-LOX knockout mice.
- iv) The epidermis of 12R-LOX knockout mice contains ~2-fold higher amounts of unoxidized EOS, indicating accumulation of the LOX substrate.
- v) Covalently bound ceramides in the epidermis of 12R-LOX knockout mice are severely reduced to only a few percent of the wild-type level, and in line with this, the CLE is largely absent in the epidermis of 12R-LOX knockout mice.
- vii) eLOX3 knockout animals show a slightly less severe phenotype than the 12R-LOX knockouts (albeit neonatal lethal due to TEWL by ~10 hours), and this is associated with a reduction in covalently bound ceramides to about half of the normal levels [30].

### **Speculations on additional roles of oxidized linoleate**

*Structural versus signaling roles* - Although ceramides EOS with oxygenated linoleate are present in the epidermis of pig and mouse [87], and probably in humans as well, it appears that

the quantities present are insufficient to support a direct structural role. Indeed, if the hepoxilin (or trioxilin) tails serve as a signal for esterases and are hydrolyzed to form ceramides OS as we propose, then the majority of the 12R-LOX/eLOX3-derived hepoxilins should be present in the SC in their free form. The linoleate-related hepoxilins released in this manner might exert a signaling function in several processes related to components of the permeability barrier of the skin. This is also the case for the arachidonate-derived hepoxilins [30, 61, 62]. For example, it has been hypothesized that hepoxilins might be ligands for the plasma membrane receptor “ichthyin” [88]. Although the functions of ichthyin are not clear to date, ichthyin seems to interact with a fatty acid transporter of the endoplasmic reticulum, FATP4, which incorporates exogenous fatty acids into the cells [22, 89]. Patients with mutations in *ALOX12B*, the gene that codes for 12R-LOX, show a decrease in the expression of FATP4 but in an up-regulation of ichthyin [22]. Mutations in FATP4 itself are associated with ichthyosis prematurity syndrome [90, 91].

*Potential interactions with epidermal PPARs* - Hepoxilin-type products released from the ceramides have also been hypothesized to be activators of peroxisome proliferator activated receptors (PPARs). Although 12R-HPETE-derived (arachidonate) hepoxilins and trioxilins are activators of PPAR $\alpha$  [92], this has not been tested using the linoleate-derived hepoxilins. PPARs in the skin are associated with various functions, including differentiation, proliferation, immune response, wound healing and lipid metabolism [93]. In relation to barrier function, Hanley and Feingold established an interesting role for PPAR $\alpha$  as a transcriptional regulator of transglutaminase [94]. Quite possibly the LOX metabolism of linoleate may serve a dual purpose within skin: firstly, allowing cleavage of the linoleate moiety from the long chain ceramides, and secondly, by up-regulation of transglutaminase (responsible for ceramide coupling to corneocyte proteins), thus forming an efficient self-regulating system and reinforcing the crucial role of LOX within the skin via a novel-signaling pathway.

*Barrier restoration* - After disruption or following a change in environmental conditions that requires adjustment of TEWL, the permeability barrier needs to be built at a high rate. In this scenario, lamellar bodies will extrude their contents at a higher rate, and free hepoxilin concentration might be higher, which might promote the formation of lamellar bodies through

the activation of fatty acid transporters, such as FATP4, via interaction with ichthyin. Activation of PPARs will also increase cell proliferation and differentiation (Figure 6). Altogether, these mechanisms will result in a prompt response of the permeability barrier of the skin to diverse environmental conditions.

### **Future directions**

*Identification and characterization of the putative lipase* - According to our working hypothesis on LOX enzymes and barrier function, 12R-LOX and eLOX3 oxidize the linoleate in EOS ceramide, allowing an esterase or hydrolase to recognize and cleave the oxidized linoleate ester, thus providing OS for synthesis of the CLE. The key lipase/esterase/ hydrolase enzyme that uses oxidized-EOS as substrate remains to be identified. Although it is well precedented that lipases will preferentially cleave a variety of oxidized lipids from cell membranes [95-97], the specificity implied in our working model is remarkable. The putative esterase/hydrolase has to recognize and cleave oxidized linoleate of a particular chirality and chemical structure, resulting from the coordinated action of *both* 12R-LOX and eLOX3 on ceramides EOS. There is no evidence so far for an esterase that will recognize and cleave a particular oxidized species, as opposed to merely peroxidized lipid, yet that is what might be inferred in this case.

*Characterization of 12R-LOX/eLOX3 products in human epidermis* - The enzymatic activity of 12R-LOX is relatively low in the conditions of the SC, and because the oxygenated hepxilin-EOS ceramides are present in small concentrations in the epidermis, their detection and identification has posed a challenge. Epoxyalcohols, hydroxides and ketones of ceramides EOS have been detected in the epidermis of pig and mouse using HPLC coupled with UV and MS detection [87]. The presence of these molecules in human epidermis has not been firmly established yet. The qualitative and quantitative characterization of hepxilin-EOS ceramides in normal and diseased human skin will have clinical implications in the search for a rational basis for treatment of several pathologies. If hepxilin-EOS ceramides perform a structural function, they should be distributed throughout the entire depth of the SC. If they act exclusively as signals for esterase activity, they would be intermediates of a complex enzymatic chain reaction. In this case, only small amounts would remain in the lower layers of the SC, where 12R-LOX and eLOX3 are located.

*Implications for therapeutics* – Based on our model of the actions of the LOX enzymes, rational approaches can be tested to correct of the skin symptomatology in ichthyosis patients with several different gene mutations. For patients with inactivating mutations in *ALOX12B* or *ALOXE3*, the prediction is that they fail to oxidize EOS, thus it is not cleaved, and consequently there is a lack of OS available for construction of the CLE. Thus, topical application of OS is predicted to be palliative. The same treatment could be effective for mutations in the cytochrome P450 gene *CYP4F22*. CYP4F enzymes are fatty acid omega-hydroxylases and the CYP4F22 mutations likely result in a deficit in omega-hydroxylated ceramides, although this remains to be shown. Again, topical application of OS (and for *CYP4F22* mutations, also EOS) may by-pass the block in the pathway. In addition to the potential therapeutic benefits, the results should be informative on the roles of key players (*ALOX12B*, *ALOXE3*, *CYP4F22*, and *ICHTHYIN*) and the requirement for esterified or free hepxilin-type products in barrier function.

## **Conclusions**

We hypothesize that two epidermal lipoxygenases, 12R-LOX and eLOX3 act in tandem to oxidize the linoleic acid molecules of the ceramides EOS. Hepoxilins, as oxidation intermediates, would act as a signal which allows the recognition and hydrolysis of the linoleic acid and the conversion of ceramides EOS into ceramides OS. Thereafter, the  $\omega$ -hydroxy fatty acid chain of the resulting ceramides OS covalently binds to proteins of the corneocytes to form the lipid envelope. This step is necessary to form a competent skin barrier. Understanding the role of hepxilins in the epidermis can lead to a rational basis for therapy of diseases that disrupt the normal development of the barrier to water vapor diffusion of the skin, such as autosomal recessive congenital ichthyoses.

## **Acknowledgements**

This work was supported by NIH grant AR-051968.

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## Figure Legends

### **Figure 1. Structural components of the epidermal barrier**

This cartoon shows the arrangement in and around corneocytes in the barrier layer, with a close-up illustrated in the segment below. In the stratum corneum, a coat of polymerized protein, the corneocyte envelope (CE) lies just inside the periphery of the flattened corneocytes. Bonded to the CE is a monolayer of covalently-bound ceramides and fatty acids, the corneocyte lipid envelope (CLE). The CLE is considered to act as a scaffold for organization of the lamellae of extracellular lipids comprised of cholesterol, ceramides, and fatty acids. This figure was originally published in The Journal of Biological Chemistry [87] © the American Society for Biochemistry and Molecular Biology.

### **Figure 2. Expression of 12R-LOX mRNA in human epidermis**

Top: the bright-field view shows an H & E stained section of human abdominal skin. Below: the dark-field view shows hybridization of the antisense probe (radiolabeled cRNA specific for human 12R-LOX) over the outermost epidermal cells that retain a nucleus (upper stratum granulosum). Discarded human skin tissue was obtained according to protocols approved by Vanderbilt University Institutional Review Board. *In situ* was performed as described [98-100].

### **Figure 3. Lipoxygenase catalysis to hepxilins and trioxilins**

In this example, linoleic acid (top) is metabolized by 12R-LOX to 9R-hydroperoxy-linoleic acid, a potential substrate for conversion to HxA-type or HxB-type hepxilins by eLOX3. Hydrolysis of the epoxide gives trihydroxy fatty acids (Trioxilin-type triols).

### **Figure 4. Epidermal specific ceramides**

EOS in the outer epidermis is esterified mainly with linoleic acid (C18:2) and is glucosylated at C-1 of the sphingosine (Glc-EOS) early in differentiation. After hydrolysis of the glycosidic and ester bonds, the resulting OS is esterified by transglutaminase (TGase) to glutamines in the cross-linked proteins of the CE [25]. The ester linkage in the resulting glutamates bonds the monomolecular lipid coating, the CLE, to the outer face of the CE.  $\omega$ -Hydroxy-VLFA (OAcid, shown on the *right side*) are also ester-linked components of the CLE. This figure was originally

published in The Journal of Biological Chemistry [87] © the American Society for Biochemistry and Molecular Biology.

**Figure 5. Ceramides, essential fatty acids and lipoxygenases and their proposed role on maturation of the epidermal water barrier**

(1) Lamellar granules contain lamellar lipid discs with acylglucosylceramides (Glc-EOS, with linoleate coupled to the omega-hydroxyl) in the limiting membrane. (2) Fusion combines the lamellar limiting membrane with the cell plasma membrane, an early stage in formation of the corneocyte lipid envelope (CLE, shown greatly expanded) and extrudes the lamellar contents extracellularly. (Glucoyl)-EOS has the very long chain fatty acid component (long red line) spanning the lipid bilayer, with linoleate (black line with two (white) double bonds) folded into the bilayer. (3) Progression towards the mature barrier entails LOX-catalyzed oxygenation of the linoleate in the ceramide by the consecutive actions of 12*R*-LOX and eLOX3. (4) The resultant “oxygen signal” permits esterase-catalyzed hydrolysis of the oxidized linoleate, freeing the ceramide omega-hydroxyl. (5) Transglutaminase-catalyzed covalent coupling of the CLE to the cross-linked proteins of the CE, thus helping to seal the barrier. A part of this figure was originally published in The Journal of Biological Chemistry [87] © the American Society for Biochemistry and Molecular Biology.

**Figure 6. Proposed pathway of the LOX products in the skin and its relationship with the formation of the permeability barrier.** LOX = Lipoxygenases; FATP4 = Fatty acid transport protein 4; PPAR = Peroxisome proliferator activated receptor; RXR = Retinoid X receptor, a molecule with which PPAR forms a heterodimer, which binds to the DNA of the target gene; EFA = Essential fatty acid; Cer-EOS = Ceramide EOS; Cer-OS = Ceramide OS; Hx = Hepoxilin; HX-EOS = Hepoxilin-EOS.

Figure 1

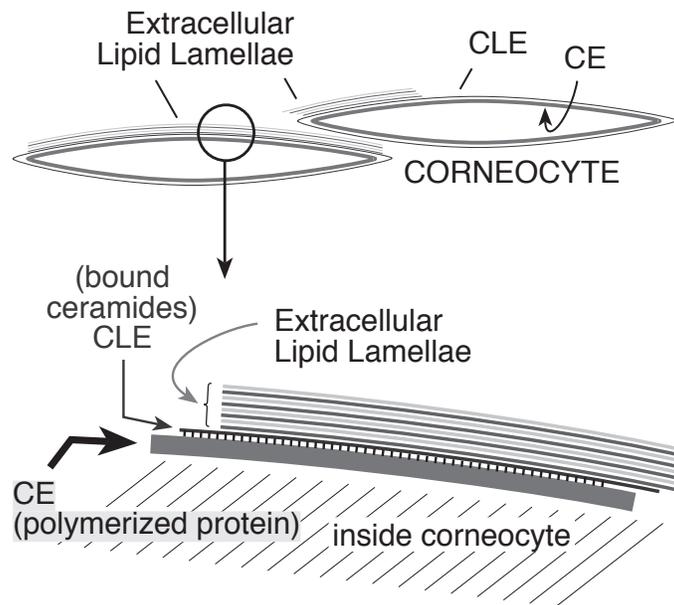


Figure 2

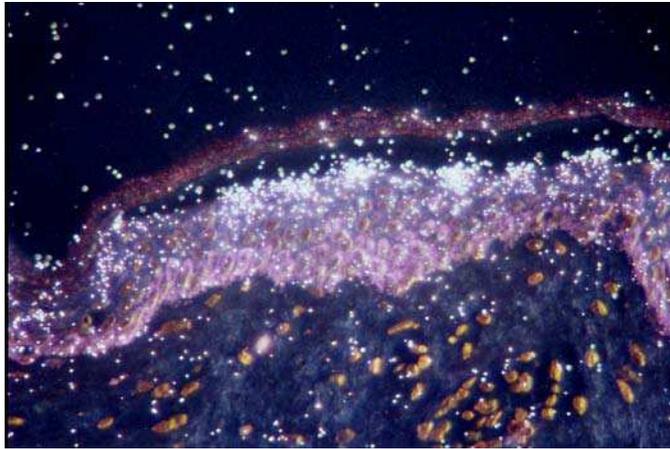
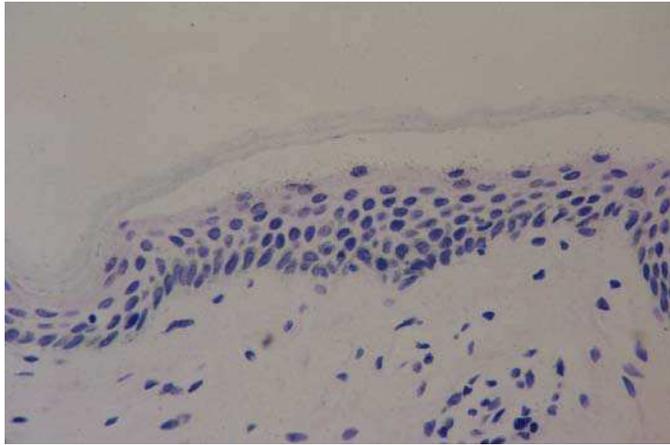


Figure 3

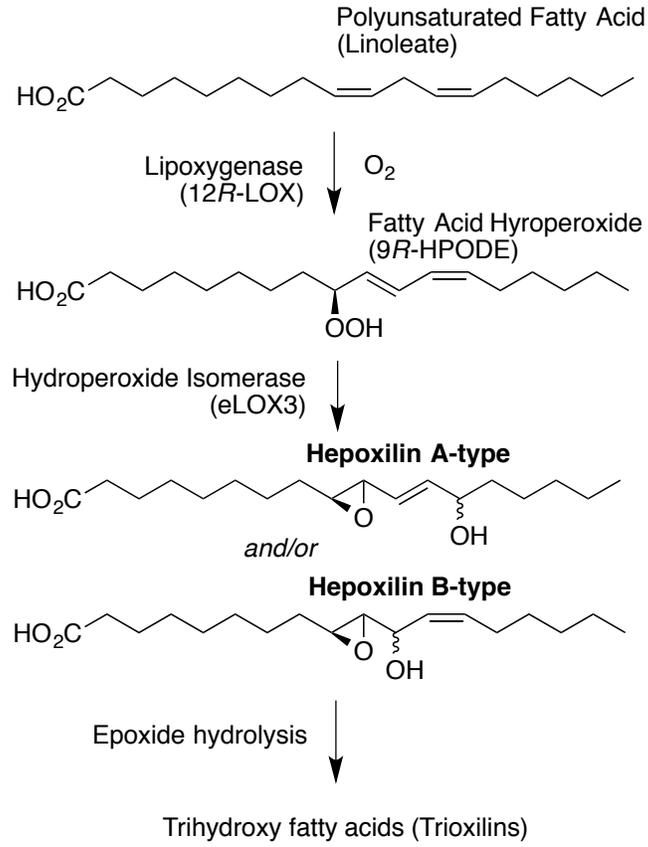




Figure 5

