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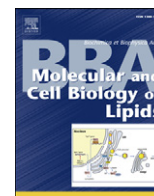
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Review

sPLA2 and the epidermal barrier[☆]Dusko Ilic^a, James M. Bollinger^b, Michael Gelb^b, Theodora M. Mauro^{c,*}^a Human Embryonic Stem Cell Laboratories, Guy's Assisted Conception Unit, Division of Women's Health, King's College London School of Medicine, London, UK^b Departments of Chemistry and Biochemistry, University of Washington, Seattle, WA, USA^c Department of Dermatology, University of California, San Francisco, San Francisco Veterans Medical Center, San Francisco, CA, USA

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ABSTRACT

The mammalian epidermis provides both an interface and a protective barrier between the organism and its environment. Lipid, processed into water-impermeable bilayers between the outermost layers of the epidermal cells, forms the major barrier that prevents water from exiting the organism, and also prevents toxins and infectious agents from entering. The secretory phospholipase 2 (sPLA2) enzymes control important processes in skin and other organs, including inflammation and differentiation. sPLA2 activity contributes to epidermal barrier formation and homeostasis by generating free fatty acids, which are required both for formation of lamellar membranes and also for acidification of the stratum corneum (SC). sPLA2 is especially important in controlling SC acidification and establishment of an optimum epidermal barrier during the first postnatal week. Several sPLA2 isoforms are present in the epidermis. We find that two of these isoforms, sPLA2 IIA and sPLA2 IIF, localize to the upper stratum granulosum and increase in response to experimental barrier perturbation. sPLA2F^{-/-} mice also demonstrate a more neutral SC pH than do their normal littermates, and their initial recovery from barrier perturbation is delayed. These findings confirm that sPLA2 enzymes perform important roles in epidermal development, and suggest that the sPLA2IIF isoform may be central to SC acidification and barrier function. This article is part of a Special Issue entitled The Important Role of Lipids in the Epidermis and their Role in the Formation and Maintenance of the Cutaneous Barrier. Guest Editors: Kenneth R. Feingold and Peter Elias.

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1. sPLA2 – general aspects

Secretory phospholipases A2 (sPLA2) comprise a large and widely distributed family of enzymes. sPLA2s are distributed throughout the epidermis [1–3]. These enzymes hydrolyze the glycerophospholipid ester bond at the *sn*-2 position to generate a free fatty acid and a lysophospholipid. A subset of sPLA2s has been shown to initiate and augment arachidonic acid release, which then can be converted to eicosanoids. sPLA2 activity requires micromolar to millimolar Ca²⁺ concentrations, and is relatively non-selective in the fatty acids that can be released. Two pathways, termed the external plasma membrane pathway and the heparin sulfate (HSPG)-shuttling pathway, have been described (reviewed in Murakami and Kudo 2004) [4]. In the HSPG-shuttling pathway, sPLA2-IIA, -IID, and -V bind to the heparan sulfate chains of HSPG glypican, are endocytosed through caveolae/rafts, and supply arachidonic acid to cyclooxygenases in activated cells. This pathway results in preferential release of cellular arachidonate and is

stimulus-dependent. An alternative pathway is the external plasma membrane pathway. In this pathway, sPLA2-V and -X release arachidonic acid from phosphatidylcholine located in the extracellular face of the plasma membrane. The resulting arachidonic acid diffuses or is transported through the cell's plasma membrane, supplying perinuclear cyclooxygenases. sPLA2-IIF also can utilize the external pathway. This pathway leads to nonselective fatty acid release (see Section 4.0, below), and is stimulus independent, often described as acting on quiescent cells.

sPLA2's act through these, and likely additional pathways that are not yet fully defined to produce a multitude of effects in mammalian tissues. In addition to pancreatic sPLA2-IB, whose role in digestion is well defined, sPLA2 activity controls aspects of lipoprotein metabolism/atherosclerosis; lung surfactant hydrolysis/acute respiratory distress syndrome; bacterial membrane hydrolysis/innate immunity; spermatozoa acrosome reaction; and formation and regulation of the epidermal water permeability barrier (reviewed in Lambeau and Gelb, 2008 and Murakami and Kudo 2004)[4,5].

2. sPLA2 in the skin

Studies examining the presence and localization of sPLA2 subtypes in the skin have not been consistent for some subtypes (notably sPLA2X). The discrepancies noted in Table 1, below, likely result from two factors: 1) expression differs in mouse vs. human skin; and

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Table 1
sPLA2 expression in skin (M = mouse; H = human).

Subtype	Epidermal Localization	Citation
IB	Suprabasal keratinocytes (M) SG – SC junction (H)	Gurrieri 2003 Ma:ereeuw-Hautier DOW Haas 2005
IIA	Throughout epidermis (M) Upper SC (H)	Gurrieri 2003 Haas 2005
IIC	Throughout epidermis	Gurrieri 2003
IID	Throughout epidermis (M)	Gurrieri 2003
IIE	Perinuclear localization basal keratinocytes (H)	Haas 2005
IIF	Suprabasal keratinocytes (M)	Gurrieri 2003 Sato 2009 Haas 2005
IIF	Suprabasal keratinocytes (M, H)	Sato 2009 Gurrieri 2003
III	Keratinocytes (M)	Sato 2009
	Basal keratinocytes (H)	Haas 2004
	Hyperproliferative epidermis (H)	Rys-Sihora 2003
V	Suprabasal keratinocytes (M)	Gurrieri 2003
	Basal and spinous keratinocytes (H)	Haas 2004
X	Basal and suprabasal keratinocytes (M)	Gurrieri 2003
	Suprabasal layers (H)	Schadolo 2001 Haas 2005
	Present only in hair follicles (M)	Yamamoto 2010
XII	Suprabasal keratinocytes (M)	Gurrieri 2003

2) expression differs in cultured keratinocytes vs. native skin. However, studies consistently show that a variety of sPLA2 isoform are expressed in epidermis, and that subtypes vary in location and with differentiation state.

3. Key roles of sPLA2 in epidermis

sPLA2 actions are involved in several essential epidermal processes. The most extensively studied is the role of sPLA2s in inflammation. Transgenic mice overexpressing sPLA2-IIA develop epidermal hyperplasia and alopecia [6]. Similarly, transgenic mice overexpressing endogenous group III sPLA2 (sPLA2G3) develop skin inflammation, acanthosis and sebaceous gland hyperplasia [2]. sPLA2IIA also appears to be upregulated in psoriatic skin, along with sPLA2-IID and IB [3]. sPLA-IIA and IID also have been shown to release arachidonic acid [7]. Pharmacologic inactivation of cytosolic phospholipase A2 inhibits TNF-induced NF kappa B activation [8] in HaCat keratinocytes, while TPA-induced inflammation and arachidonic acid release from mouse keratinocytes are modulated by cytosolic and secretory PLA2 [9], further identified as sPLA2-IA, IID and IB [3]. In contrast, transgenic mice overexpressing sPLA2-X develop enhanced differentiation, alopecia and epidermal hyperplasia but no signs of inflammation [10]. Finally, sPLA2-IID is expressed in antigen-presenting skin dendritic cells, where it acts to down-regulate inflammation, in contrast to its role in keratinocytes [11]. In humans, SC acidity influences skin inflammation via various mechanisms. First, impaired SC acidity, via impaired barrier function [12], could lead to increased antigen presentation or microbial invasion. Enhanced antigen presentation, via both the innate and adaptive immune systems, provides inflammatory responses [13]. Impaired SC acidity could act in concert with dysregulation of the immune system to produce a vicious cycle of inflammation and impaired barrier where a leaky barrier leads to antigen ingress and exaggerated inflammation which in turn leads to further barrier deterioration. This has been proposed for skin inflammation in HIV + patients [14]. Finally, a more neutral pH, acting through increased kallikrein-mediated interleukin 1 activation, can produce increased skin irritation (Nygaard & Egeland).

sPLA2 expression also varies with keratinocyte differentiation state and there is some evidence that sPLA2 can direct keratinocyte differentiation [3,6,10]. Finally, sPLA2 has been shown to control SC acidification (see below).

3.1. Specific sPLA2 isoforms and epidermal barrier development

To assess the role of the various sPLA2 isoforms in skin, we first examined their relative abundance. Because functional studies demonstrate that sPLA2 activity is especially important in barrier homeostasis during the first week of life [15,16], we compared sPLA2 isoform abundance in perinatal mice (1–6 days) with young adult mice (3–6 month). We found that the relative abundance of the different sPLA2 isoforms varied widely, and some isoforms (notably IID, IIE and V) changed as mice matured (Table 2). These data are consistent with earlier studies that show that some sPLA2 isoforms change as keratinocytes differentiate [3].

Real-time RT-PCR was used to determine the expression patterns of genes implicated in skin barrier formation in newborn mice. RT-PCR was performed, using the methods outlined in Ilic et al (2006) [17]. Normalized relative gene copy numbers (GCNs) were then compared for the different sPLA2 isoforms. Most gene expression patterns remained relatively constant during development, with the exceptions of IIE, which increased; which IID and V decreased. sPLA2 IIF was the most abundant isoform expressed, especially during the first week of life.

Confirming previous studies [3], we found that the sPLA2 isoform IIA localized to the upper SG/SC (Fig. 1), while isoform X is found throughout much of the epidermis. We also found that IIF localized to the upper SG (Fig 1).

To further delineate the roles of sPLA2 isoforms IIA and IIF, we examined their behavior after barrier disruption in normal hairless mouse skin. As assessed by immunostaining, both isoforms increased after barrier disruption, peaking at 6 h and returning to baseline by 24 h. Occlusion blocked the increases for both isoforms seen after barrier disruption demonstrating that this increase is a specific response to barrier requirements. Nuclear hormone receptor agonists such as LXR and PPAR α are known to activate sPLA₂ [18–20]. We therefore assessed whether application of LXR or PPAR agonists changed sPLA2 IIA or IIF expression *in vivo*. We found that sPLA2 IIF expression was increased by LXR and PPAR alpha, beta and gamma agonists, while sPLA2 IIA expression was unchanged after agonist applications. These findings suggest that sPLA2 isoform expression is controlled by epidermal barrier status, and that sPLA2 IIF, in particular, might be important in epidermal barrier homeostasis.

3.2. sPLA2 enhances epidermal barrier formation via lipid metabolism and SC acidification

The epidermal barrier is critical for terrestrial life, preventing water and ion loss, and also preventing toxins, infectious agents and bacteria from entering [21]. Pathological states in which this barrier is lost, such as prematurity or burns, are associated with high mortality, stressing the importance of this barrier [22].

The epidermal barrier is formed by the outermost layer of the skin, the stratum corneum (SC). A two-compartment complex formed by the anucleate SC corneocytes and the interspersed lipid bilayers, originally likened to ‘bricks and mortar’ [23,24], constitute the major permeability barrier. Several coordinated processes are required to develop this barrier. First, the epidermis must develop completely, forming normal anucleate SC corneocytes. Second, lipid must be secreted from the stratum granulosum (SG) cells before they transition into SC corneocytes [25,26]. In contrast to the phospholipid-containing plasma membrane of the viable epidermal keratinocytes, lamellar membranes are composed of ceramides, cholesterol and fatty acids. These lipids are delivered to the SC by the secretion of lamellar body contents. sPLA2 activity polar epidermal lipids into less polar species. Together with ceramides, these apolar lipids are major components of the lipid found between corneocytes in the SC. sPLA2 enzymes have been localized within the lamellar bodies, and pharmacologic sPLA2 inhibition reduces free fatty acid content, increases phospholipid content, inhibits barrier recovery and compromises epidermal barrier function [27].

Table 2
Relative sPLA2 isoform expression in neonatal and young adult mice.

Relative gene copy number	IB	IIA	IIC	IID	III	IIF	V	X	XIIA
Neonatal (1–6 day)	184 ± 517 n = 9	704 ± 946 n = 10	1674 ± 691 n = 4	253,026 ± 97,670 n = 5	745,646 ± 286,046 n = 10	13,004,451 ± 4,949,792 n = 10	674,990 ± 292,527 n = 10	7236 ± 3420 n = 10	103,215 ± 23,447 n = 9
Young Adult (3–6 month)	3436 ± 768 n = 5	8805 ± 24,646 n = 10	1305 ± 2918 n = 5	79,291 ± 56,543 n = 5	9,948,017 ± 2,220,239 n = 5	14,119,680 ± 8,408,130 n = 5	23,200 ± 6298 n = 5	24,288 ± 16,494 n = 5	105,612 ± 17,781 n = 5

Even though these studies use relatively non-selective pharmacologic inhibitors, they suggest that sPLA2 might play an important role in generating the SC lipids required for establishing a competent epidermal permeability barrier. We were able to test this using more specific deletion of the sPLA2 IIF isoform in mice (see 4.0 below).

In addition to generating lipids required for structural barrier formation, sPLA2 activity also generates fatty acids that acidify the SC. Two enzymes, sphingomyelinase (SM) and beta-glucocerebrosidase (GCS) generate ceramides destined for the lamellar bilayers. While differentiation and lipid secretion are controlled by a number of factors, including Ca^{2+} [28] the SM and GCS enzymes are activated by acidity [29–31]. SC lipid metabolism and acidity, produced partly by sPLA2 lipid hydrolysis, regulates lipid processing in the SC, producing a barrier that protects the organism from toxins, water loss and infection [29,32].

3.3. Other functions of SC acidity

SC acidity also functions independently as a deterrent to skin infections. An acidic SC inhibits skin colonization with skin pathogens such as *Staphylococcus aureus* and *Streptococcus pyogenes* [33,34]. Skin alkalinity, as seen in infant skin exposed to urea in the diaper area, predisposes to bacterial and yeast infections [35]. Finally, SC acidity controls SC integrity. Loss of SC acidity increases serine protease activity, leading to abnormal corneodesmosome degradation [12,32], and loss of SC integrity and cohesion. The importance of SC acidity for antimicrobial function has been recognized since 1892, when Heuss coined the term “acid mantle” [36].

4. sPLA2 subtypes and SC acidity

Although acidification is essential for normal epidermal barrier function, it takes place within a narrow, strictly defined area of the epidermis. The viable epidermis maintains a neutral pH. Discrete microdomains of acidity, localized between cells of neutral pH, begin to appear at the base of the SC [37], both at equilibrium and as acidity develops after birth and is restored during barrier recovery [38]. Overall, the SC progressively acidifies, with the outermost layers 1–2 pH units more acid than the viable epidermis [39]. Increased acidity in the upper SC is due to more numerous acidic microdomains rather than increasing acidity in a fixed number of acidic microdomains [37,38]. SC acidity varies among groups, with men generally having a more acidic SC than women, post-pubertal skin more acidic than pre-pubertal skin, and certain areas of the body more acidic than others [40–46]. As noted in Table 1, above, sPLA2 subtypes are distributed throughout the epidermis. sPLA2 activity increases in the first week after birth, and its distribution broadens to include all SC layers by five days after birth [15]. Pharmacologic inhibition of sPLA2 increases SC pH in the first week after birth [16]. The most likely candidates for SC acidification, based on distribution, are sPLA2-1B, IIA [8,47,48], and IIF, as noted above.

Because SPLA2 1B was expressed in very low abundance, and because SPLA2 IIA expression did not change in response to LXR or PPAR agonist application, our initial studies focused on SPLA2 IIF, which is abundant in both neonatal and adult skin; upregulated by barrier perturbation; and upregulated by PPAR or LXR agonists. To assess whether sPLA2 IIF was important for SC acidification, we measured SC acidity in transgenic mice in which sPLA2 IIF had been deleted. We found that the SC pH is significantly less acidic in mice lacking sPLA2 IIF, compared with their normal littermates (Fig 2). Furthermore, initial barrier recovery was delayed in sPLA2 $^{-/-}$ mice (Fig. 3).

Specific clinical conditions with defects in SC acidity are discussed below (Section 4.2).

4.1. Other sources of SC acidity

SC acidity was originally attributed to exogenous sources such as sebaceous gland secretion, lactic acid from keratinocyte metabolism,

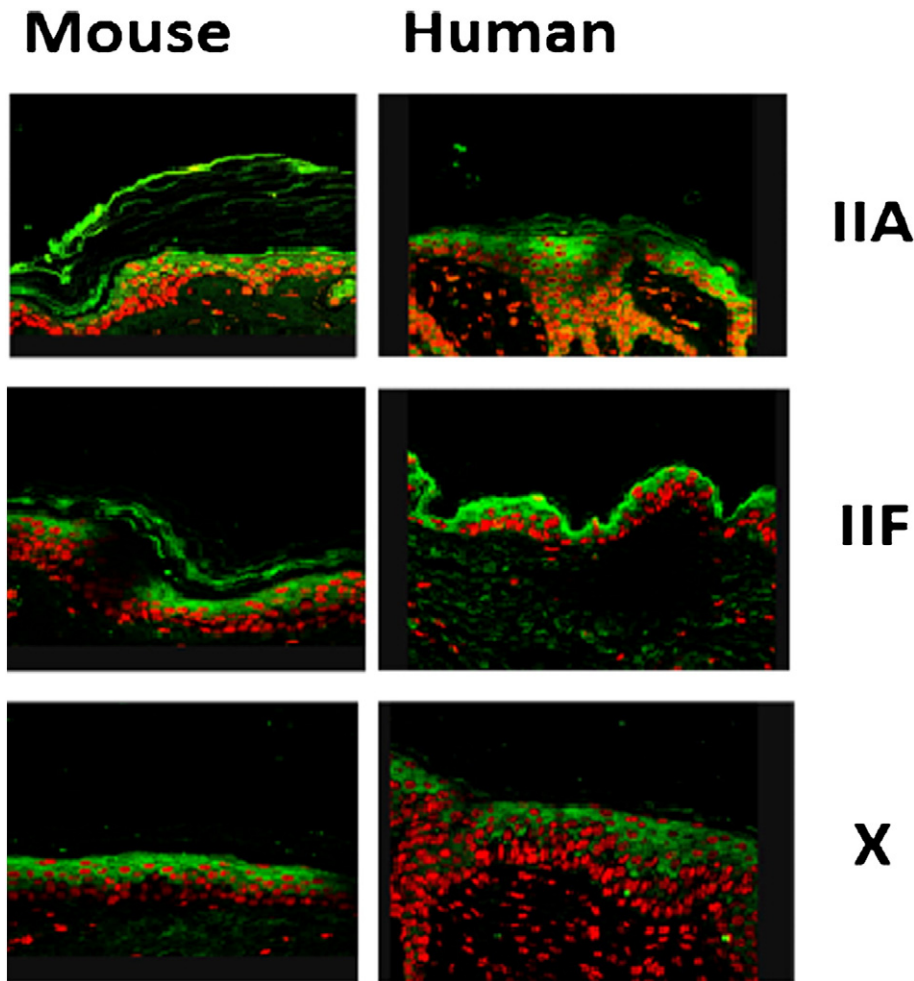


Fig. 1. Localization of sPLA2 isoforms to the Epidermis. Normal hairless mice and normal human adult skin samples were immunostained with antibodies specific to sPLA2 IIA, IIF and X. All animal procedures were approved by the Animal Studies Subcommittee (IACUC) of the San Francisco Veterans Administration Medical Center and performed in accordance with their guidelines. Skin samples were fixed in Formalde-Fresh solution (Fisher), then paraffin-embedded. Antibodies directed against specific sPLA2 isoforms were a gift of Dr. Gelb [49]. Immunohistochemical staining for assessing changes in epidermal differentiation was performed as described earlier [50,51]. IIA and IIF localized to the SG and SC of the epidermis, while X was found in the suprabasal levels.

eccrine glands, or colonizing bacteria [33,52,53]. However, a normal acidic SC pH is found in mice lacking sebaceous glands (asebia) mice [10]. Further, acidification begins at the deeper SC levels, not at the

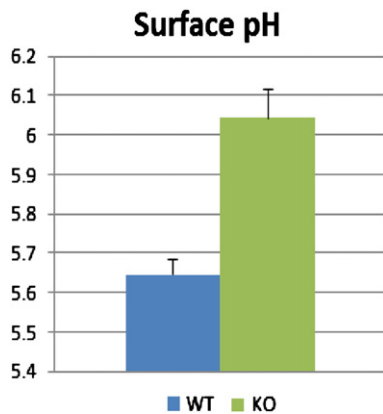


Fig. 2. Surface pH is Less Acidic in Mice Lacking Epidermal sPLA2 IIF. Surface pH was measured using in 3 month old sPLA2 IIF^{-/-} mice vs. their normal littermates. SC surface pH was measured with a flat, glass surface-electrode from Mettler-Toledo (Giessen, FRG), attached to a pH meter (pH 900; Courage & Khazaka, Cologne, FRG). Surface pH was measured from flanks in all mice. sPLA2 IIF^{-/-} mice were a gift from Michael Gelb, PhD. The sPLA2 IIF^{-/-} genotype was verified by PCR. Normal mice displayed an acidic pH. In contrast, sPLA2 IIF^{-/-} mice were unable to acidify their SC normally.

surface, as would be expected, were these exogenous primary mechanisms [15]. Besides sPLA2, other endogenous sources, such as the sodium proton pump (NHE1) or urocanic acid, also appear to contribute to SC acidity.

NHE1 is a ubiquitous transporter that widely regulates intracellular pH [54,55]. NHE1 has been shown to acidify microdomains at the SG/SC interface [37]. These microdomains are the site of initial lipid processing by SM and GCS [37]. An additional mechanism that has been proposed for SC acidification is the generation of urocanic acid from filaggrin via histidine [56]. However, the histidase enzyme responsible for this conversion does not increase with neonatal SC acidification, and histidase deficient mice do not display a defect in SC acidity, possibly due to compensatory NHE1 and sPLA2 upregulation [21]. In contrast, when either the NHE1 or sPLA2 mechanisms are disabled, SC acidity dissipates, indicating that these pathways cannot completely compensate for each other.

4.2. sPLA2 acidification is central to neonatal SC acidity, while NHE1 may be more important in adult/aged skin

Both neonatal and elderly skin, suffer from suboptimal acidification. sPLA2 activity appears to be required to develop an acidic pH after birth, while declining NHE1 activity appears responsible for the impaired SC acidification seen in elderly skin. Thus, each acidification mechanism is developmentally regulated.

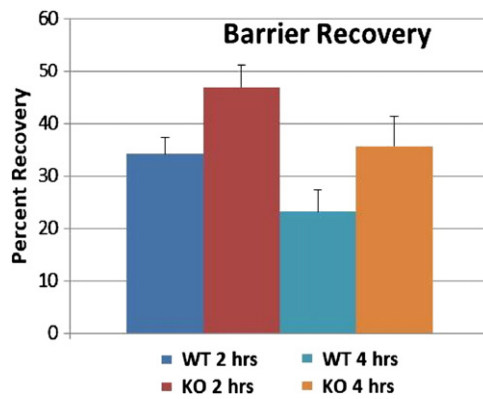


Fig. 3. Barrier Recovery is delayed at 2 but not 4 h in sPLA2 IIF^{-/-} Mice. 3 month old sPLA2^{-/-} mice and their normal littermates underwent barrier perturbation using tape-stripping. Basal epidermal permeability barrier function was assessed by measuring transepidermal water loss (TEWL) using TM300 connected to MPA5 (C&K, Cologne, Germany). For barrier recovery, TEWL was measured using an electrolytic water analyzer (Meeco, Warrington, PA) at 2 and 4 h after tape stripping. A 10-fold increase in TEWL was used as an indicator that the barrier had been perturbed. Percent barrier recovery at 2 and 4 h was calculated as described earlier (Man et al 2011). sPLA2^{-/-} mice and 8 wild-type control mice. Barrier recovery was significantly slower in the sPLA2^{-/-} mice at 2 h ($p = 0.04$). While barrier recovery still lagged at 4 h for sPLA2^{-/-} mice, this difference was not significant ($p = 0.12$). $N = 6$ mice.

SC pH is neutral at birth [38,46,57,58], leading to impaired barrier recovery, even though SC morphology, lipid secretion, and baseline barrier function are normal [59]. While NHE1 expression decreases [15] in the first week after birth, sPLA₂ activity increases substantially, and its distribution broadens to include all SC layers by five days after birth [15]. Pharmacologic inhibition of sPLA₂ delayed development of SC acidity after birth, and suggests that sPLA₂ activity is responsible for a full pH unit of acidity [16]. Nuclear hormone receptor agonists such as LXR and PPAR α activate sPLA₂. PPAR α agonists enhance SC acidity in neonates [18–20] and adult SC, leading to improved barrier recovery after acute disruption [60]. LXR agonists also enhance neonatal SC acidity via sPLA₂ activation leading to improved barrier homeostasis and SC integrity [47].

In contrast, little is known about changes in sPLA₂ activity in aging skin. In ‘aged aged’ (i.e. over 80 years of age), declining barrier function appears to be due to a global decrease in lipid synthesis and secretion [48,61]. In ‘middle age’ (50–80 years), a more specific defect in NHE1 expression is found [62]. In this age group, normal barrier function can be restored by exogenously acidifying the SC [62] suggesting that the decline in NHE1 leads to a defect in SC acidification, and thus causing the decline in barrier function.

sPLA₂ enzymes catalyze essential processes in both the viable cells of the epidermis and in the SC. sPLA₂ activity is required for formation of the lamellar membranes, and acidification of the SC, especially in the post-natal period. Several sPLA₂ isoforms are expressed in the epidermis. Of these, sPLA₂ II F seems to control SC acidity.

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