In skin, the physiological consequence of an epithelial sodium channel (ENaC) deficiency is not obvious directly at birth. Nevertheless, within hours after birth, mice deficient for the α-subunit of the highly amiloride-sensitive epithelial sodium channel (αENaC/Scnn1a) suffer from a significant increased dehydration. This is characterized by a loss of body weight (by 6% in 6 h) and an increased transepidermal water loss, which is accompanied by a higher skin surface pH in 1-day-old pups. Although early and late differentiation markers, as well as tight junction protein distribution and function, seem unaffected, deficiency of αENaC severely disturbs the stratum corneum lipid composition with decreased ceramide and cholesterol levels, and increased pro-barrier lipids, whereas covalently bound lipids are drastically reduced. Ultrastructural analysis revealed morphological changes in the formation of intercellular lamellar lipids and the lamellar body secretion. Extracellular formation of the lamellar lipids proved to be abnormal in the knockouts. In conclusion, ENaC deficiency results in progressive dehydration and, consequently, weight loss due to severe impairment of lipid formation and secretion. Our data demonstrate that ENaC expression is required for the postnatal maintenance of the epithelial barrier function but not for its generation.

The highly amiloride-sensitive epithelial sodium channel (ENaC) is a membrane constituent of many salt-absorbing epithelia. It facilitates Na⁺ movement across tight epithelia and is therefore involved in whole net sodium ion balance of the body (1). ENaC is a member of the superfamily of ion channels implicated in mechanoperception and acid sensing (2). It is composed of three highly homologous subunits (α-, β-, and γENaC) encoding Scnn1a, Scnn1b, and Scnn1g and characterized in several species, including man and mouse (3–5). In vitro experiments clearly demonstrate that, in the absence of the α-subunit, channels made of β- and γ-subunits alone do not confer ENaC activity (4, 6). In vivo, the constitutive inactivation of the α-subunit of ENaC leads to complete abolishment of ENaC activity, and αENaC knockout animals die within 40 h after birth due to lung clearance failure (7). Further gene targeting experiments clearly demonstrated that β- and γENaC (Scnn1b and Scnn1g) subunits are important for survival with the channel composed of the remaining subunits (αβ or αγ) conferring less channel activity (4, 6, 8). This may also explain the distinct clinical phenotypes observed (9, 10). In human, mutations in all three ENaC subunits are reported to result in either ENaC hyper- or hypo-function associated with hypertension (Liddle syndrome) or salt-wasting syndrome (pseudohypoaldosteronism type-I, see for review Ref. 1).

ENaC expression has been demonstrated in skin of amphibians and in hair follicles, interfollicular epidermis, and sweat glands of mammals (11–13). All three subunits (Scnn1a, Scnn1b, and Scnn1g) are found in mouse and human keratinocytes (12, 13). Interestingly, their expression level is increased in more differentiated keratinocytes and only found in the later stages of fetal epidermal development. In human epidermis, ENaC mRNA is expressed throughout adulthood, but is absent in 10-week-old fetal epidermis (12, 13). Patch clamp recordings of human keratinocytes reveal a sodium channel conductance that is blocked by benzamil with similar affinity and voltage dependence of the amiloride block as previously described for ENaC (13). Further evidence that ENaC-mediated Na⁺ transport may be implicated in keratinocyte and epidermal differentiation comes from the previous analysis of newborn αENaC knockout mice, which exhibit epidermal thickening and premature lipid secretion in the upper epidermis, suggesting that ENaC-mediated sodium ion fluxes control selective aspects of keratinocyte differentiation (14).

The skin is a physical barrier at the interface between an organism and its environment preventing water loss and withstand-
undergoes keratinization, a process in which epidermal cells progressively mature from basal cells with proliferative potential to lifeless flattened squames of the stratum corneum. This differentiation is accompanied by alterations in gene expression, affecting structural proteins, expression, and activation of enzymes that control post-translational modifications, metabolic changes, and lipid synthesis (15). A defect in any one of these structural components or enzymatic processes has therefore the potential to impair the barrier function of the skin and to cause diseases.

Given the important role played by ENaC in the whole body sodium ion homeostasis, we sought to reveal its physiological role in skin. At birth, no differences in physiological parameters could be identified, but a few hours later, αENaC knockout mice show a severe life-threatening transepidermal water loss, and major impairment of the barrier function due to highly disturbed lipid secretion and formation. This dehydration is accompanied by impaired acidification of the skin surface pH. Our data demonstrate that this sodium channel is not required for establishment of the epidermal barrier function but rather for maintaining this barrier function in postnatal life.

**EXPERIMENTAL PROCEDURES**

**Animals (Source and Genotyping)**

Transgenic αENaC homozygous mutant (−/−, knockout), heterozygous mutant (+/−), and wild-type (+/+), and littermates obtained by interbreeding mice heterozygous mutant for the αENaC allele (Scnn1a<sup>tm1</sup> (16)) and analyzed after birth. Genotyping was performed by PCR essentially as described (17). All experiments were performed coded. Animals were kept on a 14:10-h light-dark cycle. Experimental procedures and animal maintenance followed federal guidelines and were approved by local authorities. At birth, (α)ENaC knockout animals present a metabolic acidosis as found by Hummler and coworkers (17), but for our present skin analysis, only animals without apparent respiratory distress syndrome have been considered for further analysis.

**Functional Analyses of the Epidermal Barrier**

**Skin Permeability Assay**—Newborn mice were killed and subjected to methanol dehydration and subsequent rehydration as described previously (18). They were further washed in PBS, stained overnight at 4 °C in 0.1% toluidine blue/PBS (Merck), destained in PBS, and photographed with a digital camera (Coolpix 950, Nikon) (19).

**Measurement of the Transepidermal Water Loss**—The rate of transepidermal water loss (TEWL) from the ventral skin of newborns from five independent litters were separated from their mother to prevent fluid intake, and the rate of epithelial water loss was calculated by measuring the reduction of body weight as a function of time.

**Dehydration Assay**—To determine the rate of fluid loss (19), newborns from five independent litters were separated from their mother to prevent fluid intake, and the rate of epithelial water loss was calculated by measuring the reduction of body weight as a function of time.

**Tight Junction Permeability Assay**—The tight junction functional test was performed as described (21, 22). Following subcutaneous injection of biotin (∼300 Dalton, Pierce), skin samples were snap frozen. Cryosections (10 μm thick) were prepared, fixed in 4% paraformaldehyde/PBS buffer, and incubated with streptavidin FC630 overnight at 4 °C (Fluorobees, Interchim, Montluçon, France). Fluorescence was visualized using laser scanning confocal microscopy (model LSM510 Meta, Carl Zeiss MicroImaging, Inc.). For each genotype (wild type and knockout), three pups were independently analyzed. Nuclei were counterstained with 0.2 μg/ml DAPI (Roche Applied Science) in mounting medium (Dako Schweiz AG, Switzerland, Baar).

**Skin Surface pH**—Skin surface pH of littermates was determined with a pH meter (PH900, Courage and Khazaka, Cologne, Germany). Briefly, a few microliters of bi-distilled water was applied on the skin surface, and a flat glass surface electrode (Mettler-Toledo, Giessen, Germany) was put on the top to equilibrate. The skin surface pH was then measured according to the manufacturer’s guidelines.

**Immunohistochemistry**

Immunohistochemistry was performed on littermates. Tissue from knockout and wild-type mice was fixed overnight in 4.5% phosphate-buffered formalin (pH 7) and embedded in paraffin. Following deparaffinization in xylene and progressive rehydration, 4-μm sagittal sections were incubated with primary antibodies for 1 h at room temperature after treatment with unmasking solution (10 mM Tris, 0.5 mM EGTA, pH 9, boiling for 10 min). Staining was visualized by laser scanning microscopy after 1 h of incubation at room temperature with Alexa633-conjugated anti-rabbit IgG for involucrin, loricrin, and filaggrin or Alexa488-conjugated anti-rabbit IgG (Molecular Probes, Invitrogen) for K1, K6, and K14. All primary rabbit antibodies were purchased from Covance and were used at a dilution of 1:1000 except for K14 (1:4000). Nuclei were counterstained with DAPI. To analyze tight junction proteins, skin was dissected and frozen in optimum cutting temperature compound (OCT, Tissue-Tek, Sakura, Zoeterwoude, Netherlands). Cryosections (10 μm thick) were processed to further fixation (30 min in 95% ethanol at 4 °C and 1 min in acetone at room temperature) and permeabilization (10 min in 0.2% Triton X-100/PBS). Following 30 min in PBS/0.3% bovine serum albumin blocking solution, sections were incubated with primary antibody at room temperature for 1 h. Rabbit anti-claudin-1 polyclonal antibody (Zymed Laboratories) was used (1:100 dilution) in combination with Alexa633-conjugated anti-rabbit IgG (Molecular Probes, Invitrogen) for paraffin sections (as described above). Rat anti-occludin (MOC37) and mouse anti-ZO-1 (T8–754) polyclonal antibody (kindly provided by M. Furuse, Kyoto University, Kyoto, Japan) were combined with anti-rat FC488-conjugated (Fluoprobes) and anti-mouse Alexa546-conjugated antibodies (Molecular Probes, Invitrogen), respectively. Nuclei were counterstained with DAPI. Immunofluorescence labeling was visualized by laser scanning microscopy.

**Western Blot Analysis**

Whole skin of newborns was homogenized in ice-cold 8 mM urea, 50 mM Tris (pH 8.0), 10 mM EDTA using the tissue lyser, 4 × 30 s (Qiagen). Following 30-min incubation on ice, lysates were centrifuged (13,000 × g for 15 min at 0 °C) and
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![Graphs and images](Image)

FIGURE 1. The postnatal barrier is impaired in αENaC deficient mice. A, barrier-dependent dye exclusion assay. Representative photographs from αENaC (−/−, +/+), and +/+ mutants. B, weight loss in αENaC homozygous (−/−) and heterozygous (+/+), and wild-type (+/+). Data represent the average ± S.E. *p < 0.05, **p < 0.01. C, time-dependent TEWL as measured on ventral skin of αENaC knockout (−/−), heterozygous mutant (+/+), and wild-type (+/+). D-F, time-dependent TEWL as measured on dorsal skin of αENaC knockout (−/−), heterozygous mutant (+/+), and wild-type (+/+). Data represent the average ± S.E.

In accordance with the epidermal permeability barrier function, ENaC expression is postnatally required to quantified using the protein assay (BCA, Pierce). For SDS-PAGE, 50 μg of protein was loaded and separated on a 10% acrylamide gel. Western blot analysis was performed using rabbit antibodies to K14 (1:10,000), K1 (1:10,000), K6 (1:10,000), filaggrin (1:5,000), loricrin (1:5,000), and involucrin (1:5,000, Covance, Berkeley, CA). Signals were revealed with anti-rabbit IgG from donkey (1:2,000) as secondary antibodies and the SuperSignal detection system (West Dura System, Pierce).

Lipid Analysis

Whole skin from newborns was removed at autopsy, frozen, and stored at −20 °C until further treatment. Stratum corneum preparations, lipid analysis, and recovery of covalently bound lipids were performed as described previously (23).

Ultrastructural Analysis

Standard electron microscopy approach consisted of tissue fixation in 2% glutaraldehyde solution in sodium cacodylate buffer, followed by washing and post-fixation in 1% osmium tetroxide (OsO₄, Sigma) in the same buffer. For lamellar lipid visualization, skin biopsies were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.2), washed in PBS, and post-fixed in 0.25% ruthenium tetroxide (RuO₄, Polysciences Inc., Warrington, PA) instead of OsO₄. Thus prepared small tissue fragments were dehydrated in graded ethanol series and embedded in Epon at 60 °C. Ultrathin sections recovered on copper grids were counterstained in uranyl acetate and lead citrate (24).

Calculation and Statistics

All data are expressed as means ± S.E. Values of n refer to the number of mice in each group. Individual groups were compared by using the t test for pair-wise comparisons. To test the independence of groups (dehydration assay), the Kruskal and Wallis statistical test was used. A level of p < 0.05 was accepted as statistically significant for all comparisons.

RESULTS

Time-dependent Impairment of the Epidermal Barrier Function in αENaC-deficient Mice—To study the physiological consequences of ENaC deficiency in the skin, we first tested the functional integrity of the epidermis by a toluidine blue penetration test across the entire epidermal surface. Directly at birth, pups from the knockout group did not show increased blue staining compared with the heterozygous and the wild-type mice (Fig. 1A). We then tested the outward barrier function by a dehydration test, thus determining the reduction in body weight as a function of time. The dehydration rate (milligrams/h/g of body weight) was significantly different in the knockout group (p < 0.001, Fig. 1B). When following one litter over 6.5 h after birth, the dehydration was evident at 2 h after birth (3% water loss versus 1% in the control littermates, p < 0.01) and, at 6 h after birth, the difference in dehydration reached almost 7% of their body weight (compared with 2% in the control (+/+)). In contrast, littermates (p < 0.001, Fig. 1C). Dehydration through the skin was confirmed by TEWL. Although the values were not different within the first 12 h among the three groups (Fig. 1D), knockout neonates measured between 12 and 24 h after birth displayed a significantly increased TEWL value (Fig. 1E, p < 0.05). When measuring TEWL values in pups from the same litter at an early (4 h) and late (24 h) time point after birth, we have clearly seen a reduction in the TEWL values in the heterozygous and wild-type groups with time, whereas the knockout group failed to reach the same basal levels (Fig. 1F, p > 0.05). This indicates that the skin of the knockout mice may not adapt normally to the external environment (Fig. 1F, p < 0.05). These data strongly suggest that αENaC expression is postnatally required to adapt the epidermal permeability barrier function.
αENaC Is Required for Proper Expression of the Differentiation Marker K6, an Unspecific Marker for Proliferation—To unveil defects in epidermal differentiation, we studied the distribution as well as the expression levels of differentiation markers (supplemental Fig. S2).

Immunohistochemistry revealed that keratin 14 (K14), a specific marker for the stratum basale was similarly expressed in epidermal basal cells of wild-type and knockout neonates. Equally, the distribution of an intermediate (keratin 1) and the epidermal terminal differentiation markers (loricrin, involucrin, and filaggrin) was similar in skin from both wild-type and knockout mice (supplemental Fig. S1). Loricrin staining was observed in the top stratum granulosum (SG) and continued in the stratum corneum (SC). Involucrin staining was strong in the SC but was also found in the SG and slightly in the stratum spinosum (SP). In contrast, the expression of keratin 6 (K6), that was normally restricted to the outer root sheath of the hair follicle in wild-type mice, was expressed throughout the interfollicular epidermis in knockout animals indicative for a hyperploration (supplemental Fig. S1). The near-normal expression of the early and late differentiation markers was confirmed by Western blot analysis (supplemental Fig. S2). Additionally, processing of filaggrin, from profilaggrin (300 kDa) to the two-domain intermediate monomers 2DI (50 kDa) and 1DI (25 kDa) was not disturbed (supplemental Fig. S2). In summary, except for keratin 6, none of the analyzed early, intermediate, and late differentiation markers was altered in the knockout group.

Tight Junction Protein Distribution and Functionality Are Not Altered — The impaired skin barrier function may be caused by leakiness of tight junction proteins in the epidermis. However, immunohistochemical analysis shows a normal distribution of tight junction proteins (Fig. 2). Claudin-1 was localized in the whole epidermis except for the SC and the last layer of SG (Fig. 2A). ZO-1 was detected in the mid-SG, and occludin showed a dotted staining, as expected for the presence of tight junctions (Fig. 2A, arrows). We then examined whether the barrier function of the tight junctions was affected. Injection of biotin into the dermis and streptavidin revelation on the sections showed normal diffusion through the paracellular spaces of the epidermis from stratum basale to the supposed localization of the tight junction complexes at the mid-SG (Fig. 2B, arrows). These data clearly indicate that the tight junctions were unchanged in the knockout animals. This experiment has been performed at 4 and 24 h after birth and showed the same results at both time points.

αENaC Knockout Mice Exhibit Severe Defects in Lipid Matrix Composition and Highly Impaired Lamellar Lipids — Because content and localization of differentiation markers (see supplemental Fig. S2) seem not to be affected, nor the tight junctions, we focused our analysis on lipid composition. We analyzed the lipid profile (probarrier lipids, lamellar lipids, and covalently bound lipids) at 5 h after birth, and compared it with the lipid profile at 31 h after birth (Fig. 3, A–F). In the αENaC knockout mice, ceramides and cholesterol levels based on dry weight were significantly decreased, whereas the level of the fatty acids was unchanged (Fig. 3, A–D). The ceramides are preferentially formed of probarrier lipids, glucosylceramide (25–27), sphingomyelin (28), and cholesterol sulfate (29). The ceramide precursors glucosylceramide and sphingomyelin are both accumulated in the knockout mice (Fig. 3, B–E). In αENaC-deficient skin, the level of cholesterol sulfate is increased to nearly 2-fold (at 5 h) and 1.6-fold of wild-type mice at 24 h after birth. Interestingly, the heterozygous mutants (+/−) mice display an intermediate phenotype between the wild-type and knockout group, although dehydration and body weight loss are not different from the control (wild-type) group (Figs. 1, B–F, and Fig. 3, A–D). Further densitometric quantification of the ceramides (see supplemental Fig. S2 and Table 1) showed that the decrease observed in total ceramides affected all the ceramides and seemed to be improving with time (31 h compared with 5 h). As the covalently bound lipids are important for the lamellar lipid organization and, therefore, the barrier function, the extractable lipids of the SC samples were removed, and covalent-bound lipids were released by alkaline hydrolysis. The levels of ω-hydroxylated fatty acid, Cer(OS), Cer(OP), and GlcCer(OS) were significantly decreased in the knockout group and this was observed at both time points (Fig. 3, C–F, and Table 2), whereas the Cer(OH) lipid fraction was unchanged (Table 2). Altogether, these data support a disturbed lipid composition with impaired formation of the lamellar lipids of the extracellular lipid matrix in the αENaC knockout mice.

Ultrastructural analysis of (α)ENaC knockout mouse skin (≈24 h after birth) shows morphological changes in the forma-
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FIGURE 3. Severe impairment of SC lipid composition in αENaC-deficient mice. A–F, analysis of SC lipid content and composition of αENaC −/− (white), +/+ (gray), and +/+ littermates (black columns). A and D, levels of free SC lipids at 5 or 31 h. Chol, cholesterol; FFA, free fatty acid; Cer, ceramides. Note that cholesterol and ceramide levels are significantly decreased in knockouts (*, p < 0.05; **, p < 0.01), compared with the corresponding control (+/+ group). B and E, analysis of probarrier lipids at 5 or 31 h. Knockouts show significantly increased levels for all probarrier lipids glucosylceramide (GlcCer), sphingomyelin (SM), and cholesterol sulfate (CSO4). C and F, levels of covalently bound lipids at 5 or 31 h. Note the overall reduction of ω-hydroxylated fatty acid (ω-OH-FA) and the overall reduction in ceramides (Cer(OS)) levels in knockout (−/−) mice compared with the wild-type (+/+ group). Note that heterozygotes (+/−) present an intermediate phenotype for all measured parameters (*, p < 0.05; **, p < 0.01; ***, p < 0.001). Each value is the mean of at least five independent animals ± S.E. (error bars).

Increased Skin Surface pH in αENaC Knockout Mice—When we followed skin acidification in heterozygous and wild-type αENaC mice, we found a gradual decrease of skin surface pH within the first week of life, from almost 7 to 5 (data not shown). The skin surface pH finally reached values as found in adulthood (pH = 5). Interestingly, around 24 h after birth, a time point at which all three genotypes (αENaC +/+; +/−, and −/− mice) were still present within the same litter, the αENaC−/− pups showed a significant delay in acidification (Fig. 5; p < 0.001 versus heterozygotes; p < 0.001 versus wild-types). Here, a skin surface pH of 6.35 was measured in the knockout group compared with 6.03 in the control littermates indicating a failure to complete acidification.

DISCUSSION

ENaC Is Important in the Maintenance of the Epidermal Permeability Barrier Function—Skin as the outermost organ is continuously confronted to the external environment and serves as a primary defense system against infections and primary regulator of body temperature. The epidermal barrier function of the skin impedes the escape of water and electrolytes and thereby presents the physical barrier at the interface between inside and outside.

In this study, gene inactivation of the α-subunit of the highly amiloride-sensitive epithelial sodium channel (αENaC, Scnn1a) leads to distinct perinatal effects on epidermal development and homeostasis, which culminates in a barrier defect within the first 24 h. At birth, αENaC newborns are not distinguishable from their heterozygous mutant and wild-type littermates, and the barrier seemed not yet impaired (see Fig. 1 and Ref. 16). Consistently, at the same time point, the toluidine-blue whole mount assay revealed no impaired skin perme-
ability of αENaC−/− newborns (Fig. 1). Usually, models with barrier defect at birth such as the Klf4−/− mice show an extensive blue staining upon toluidine-blue treatment that penetrate efficiently the skin of the newborns (30). The establishment of the epidermal permeability barrier initiates normally at E16.5 on the dorsal surface and spreads ventrally and was proposed to be terminated before the end of gestation (31). In our model, we could show that αENaC knockout pups lose significantly more weight through dehydration, which becomes life-threatening (Fig. 1). Interestingly, the epidermal barrier function is not different between knockouts and wild types right after birth but seem to evolve during the following hours becoming more efficient in wild types.

This is the first report that demonstrates the postnatal epidermal barrier adaptation in mice, which evolves in parallel with the skin surface acidification. Moreover, we show that this adaptation requires the participation of the sodium channel ENaC. Recently, Indra and co-workers identified the Taf10 as a gene required for the establishment of skin barrier function in embryonic, but not in adult mouse epidermis (32). We propose that ENaC is not required for the generation of the epidermal barrier, such as the Klf4, or TAF10 (30, 32), but rather in the maintenance of this skin barrier function after birth.

Severely Impaired SC Lipid Composition Is Most Likely Causative for the Barrier Defect—The epidermal barrier function is the result of the combination of three mechanisms: terminal differentiation of keratinocytes forming the corneocytes, an embedding matrix of extracellular lipid membranes mainly consisting of ceramides, cholesterol, and fatty acids, and the intercellular lipid envelope. In wild-type mice, the lipid lamellae are tightly packed and adhere to the well visible corneocyte lipid envelopes. A, A', B, and B': ruthenium tetroxide post-fixation; C and D: osmium tetroxide fixation, A', A'', B', B'', C: αENaC knockout epidermis; B, B', C: wild-type epidermis. SC: stratum corneum; SG: stratum granulosum; N: nucleus; KG: keratohyalin granule; bars = 200 nm in A, 500 nm in C and D. (A' and B' are enlarged versions of A and B.)

![Image](image-url)

**FIGURE 4. Ultrastructural analysis of ENaC wild-type and knockout mice.** Lamellar body secretion and extracellular formation of the lamellar lipids proved abnormal in the knockout epidermis (A and C, asterisks) compared with wild-type pups (B and D). Note that the apical region of the most superficial granular keratinocyte layer (SG, indicated with arrows) in the knockouts contains highly disorganized lipid lamellae (LL; A) and that the lamellar body-derived vesicular structures persist in the intercorneocyte spaces of the lower stratum corneum (D). In contrast, in wild-type animals (B and D), the lipid lamellae are tightly packed and adhere to the well visible corneocyte lipid envelopes. A, A', B, and B': ruthenium tetroxide post-fixation; C and D: osmium tetroxide fixation, A', A'', B', B'', C: αENaC knockout epidermis; B, B', C: wild-type epidermis. SC: stratum corneum; SG: stratum granulosum; N: nucleus; KG: keratohyalin granule; bars = 200 nm in A, 500 nm in C and D. (A' and B' are enlarged versions of A and B.)

**TABLE 1**

| Genotypes | +/− | +/+ |
|-----------|-----|-----|
| Cer(EOS)  | 3.524 ± 0.392 | 5.046 ± 0.739 | 7.412 ± 0.758 |
| Cer(C26-NS) | 15.712 ± 0.894 | 28.824 ± 1.528 | 30.486 ± 1.072 |
| Cer(C18-NS) | 13.256 ± 0.903 | 17.712 ± 1.114 | 21.934 ± 1.588 |
| Cer(C26-H) | 2.276 ± 0.251 | 3.606 ± 0.53 | 3.648 ± 0.609 |
| Cer(C26-AS) | 1.674 ± 0.496 | 2.238 ± 0.545 | 2.454 ± 0.678 |
| Cer(C18-AS) | 0.888 ± 0.143 | 1.594 ± 0.332 | 1.946 ± 0.239 |
| Cer(C26-API) | 0.094 ± 0.052 | 0.138 ± 0.069 | 0.186 ± 0.047 |
| Cer(C26-AH) | 0.120 ± 0.04 | 0.118 ± 0.065 | 0.084 ± 0.035 |
| Cer(C18-AH) | x2 | 0.092 ± 0.0384 | 0.176 ± 0.0648 | 0.142 ± 0.0584 |

**TABLE 2**

| Covalently-bound lipid composition | +/− | +/+ |
|-------------------------------|-----|-----|
| GlcCer(OS) | 0.007 ± 0.001 | 0.0605 ± 0.0546 | 0.0486 ± 0.0315 |
| Cer(OH) | 0.012 ± 0.002 | 0.0487 ± 0.0310 | 0.0051 ± 0.0012 |
| Cer(OP) | 0.005 ± 0.002 | 0.0385 ± 0.0250 | 0.0098 ± 0.0028 |
| Cer(OS) | 0.571 ± 0.056 | 0.7672 ± 0.0742 | 0.9728 ± 0.1461 |
| ω-OH-FA | 0.402 ± 0.057 | 0.4440 ± 0.0512 | 0.6153 ± 0.0962 |

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FIGURE 5. Elevated skin surface pH in αENaC knockout mice. Skin surface pH measurements at 24 h after birth. Note the failure of the knockout group to acidify the surface pH (**, p < 0.001, compared with the +/+ group). The heterozygotes display a phenotype not different from the wild-type (+/+ group), but different from the knockout group (**, p < 0.01).

(hyperkeratosis) (35). Furthermore, analysis of the tight junction proteins (claudin-1, zona occludens-1, and occludin), known to be part of the tight junction complex in the SG, showed no difference in distribution nor in functionality as evidenced by the tight junction functional assay (Fig. 2B).

Stratum corneum intercellular lipids play an important role in the regulation of skin water barrier homeostasis and water-holding capacity, and any modification of intercellular lipid organization and composition may therefore impair these properties. The unique lamellar, bilayer organization of this lipid matrix provides the structural basis for the extraordinary lower permeability to water and other electrolytes (36). In a previous study, electron microscopy in mice deficient for ENaC demonstrated a premature lipid secretion indicative of a disturbed lipid processing (14). We now performed a detailed lipid analysis and found a significant decrease in the major lipid species, namely ceramides and cholesterol, whereas the fatty acid transport protein 4 (Fatp4) show a disturbed fatty acid composition of epidermal ceramides (40). Ceramides derive in large part from hydrolysis of glucosylceramides (GlcCers) of the lamellar bodies mediated by β-glucocerebrosidase (41). Therefore, the significant accumulation of all main three probarrier lipids, namely glucosylceramide, sphingomyelin, and cholesteryl sulfate in the αENaC knockout group explains the reduction of mature lipids observed in knockouts (cholesterol and ceramides) (Fig. 3, B and E).

Similarly, a drastic modification was observed in the composition of the very long chain lipids covalently attached by an ester linkage with their ω-hydroxyl groups to proteins on the surfaces of the corneocytes (Fig. 3, D and F). Taken together, the level of barrier-forming lipids attached to proteins was significantly reduced in knockouts suggesting a potential cause for the observed defect in postnatal epidermal barrier (Fig. 3, D and F, and Table 2). This covalently bound lipidic envelope is thought to be essential for the interaction of highly cross-linked proteins of the corneocytes within the extracellular lipid matrix (42).

To further analyze the epidermal barrier adaptation during the 24 h after birth, we analyzed the lipid profile (probarrier lipids, lamellar lipids, and covalently bound lipids) at 5 h after birth, an early time point (Fig. 3, A–C), where we do see high transepidermal water loss in wild-types and knockouts. Interestingly, the lipid profile as analyzed at 5 h after birth resembles the profile as found at 31 h after birth (Fig. 3, D–F). Here, the transepidermal water loss stays higher in knockouts. It is interesting to note that the ω-hydroxy-fatty acids are important for the stacking of the lamellar lipids around the corneocytes and decrease with time in the knockouts (Fig. 3, C and F). The observed reduction correlates very well with the disorganization observed in the lamellar bodies by electron microscopy, where the ω-hydroxy-fatty acids are attached to the membrane prior to the extrusion (27, 43).

Several mouse models have been described in which distinct lipid defects have been demonstrated (see review in Ref. 44). For example, the mice homozygous mutants for ElovL4 display scaly, wrinkled skin and have severely compromised epidermal barrier function and die within a few hours after birth. Skin histology showed an abnormally compacted outer epidermis while electron microscopy revealed deficient epidermal lamellar body contents and lack of normal stratum corneum lamellar membranes. The lipid analyses of epidermis revealed a global decrease in very long chain fatty acids (45, 46). It is likely that reduced β-glucocerebrosidase activity is causative for the ceramides modifications in the αENaC knockout group. Furthermore, a neutral skin surface pH has been proposed to cause incomplete lipid processing within the early postnatal period (47). Our data indicate, rather, that the lipid abnormalities are the primary defect (Fig. 3).

αENaC Knockout Mice Show an Impaired Postnatal Skin Surface Acidification—The initial observation that the skin has an acidic surface ("acid mantle") was made a long time ago. Normally, late in gestation, fetal skin develops a permeability barrier competent for life in a terrestrial environment (48). In fetal rat and mouse skin the key steps leading to development of a competent barrier were thought to occur between day E20 and E21 in mice. Both, normal cornified envelope and mature
lamellar bilayers are present by day E21, accounting for barrier competence (49). Yet, certain developmental processes take place after birth in human as well as in rodents, namely stratum corneum hydration, water loss, and skin surface acidification indicating that the stratum corneum barrier function is still in the process of adapting to extra-uterine life. In our present study, we found that wild-type and knockout mice display a near-neutral pH right at birth. Wild-type skin acidifies rapidly within 48 h after birth reaching near-adult levels (data not shown). In contrary, the αENaC knockout mice showed a delay in the acidification of their skin surface pH at 24 h after birth (Fig. 5). This might have a direct consequence on the lipid abnormalities (Fig. 3), because the enzymes responsible for the processing of sphingomyelin and glycosylceramide into ceramides are pH-dependent (50, 51). Thus, our finding of an impaired acidification in αENaC knockout skin coincides well with the impaired lipid maturation. Skin surface acidification is more rapidly achieved in the neonatal mouse (≥2 days), than in the rat (≥7 days) or even human newborns, where this takes several weeks to months (52, 53).

A number of mechanisms have been proposed for the generation of stratum corneum acidity, such as H+/H11350 generated by Na/H exchanger in the stratum granulosum (54) and catabolic processes within the stratum corneum that generate acidic end-products. Intrinsic processes are (i) the breakdown of proteins such as filaggrin resulting in urocanic acid production (55) and (ii) the hydrolysis of lipids through specific phospholipases to yield free fatty acids (55, 56). The epidermal Na/H exchanger antiporter activity certainly contributes to postnatal acidification, although the skin phenotype of the Na/H exchanger knockout mouse is not the primary cause of death (57). Other ion transporters may be implicated as well.

In human infants, the delay in acidification could explain the increased infantile risk for irritant/allergic contact dermatitis, infection, and percutaneous absorption of toxic chemicals (53). We propose here that the absence of ENaC in the epidermis may be crucial for the sodium concentrations in the different strata composing the epidermis and, if absent, interfere with the proton movement needed for the establishment of the pH gradient. This implies that the different strata are functionally separated from each other and that the ions are not moving freely between the layers. The idea that there could be other structures than the “classic” tight junctions has been recently proposed (34), defining compartments in the epidermis that can arise from the presence of many tight junctions proteins all across the epidermis (58).

During the stratification of human skin equivalents, an early and broadened synthesis of tight junction proteins precedes the formation of the stratum corneum, and it has been proposed that tight junctions and tight junction-like proteins are involved in barrier formation, serving as a “rescue system” when the stratum corneum is missing or impaired (33). Transmembrane ionic fluxes might control keratinocyte differentiation, including the synthesis of cornified envelope and other differentiation-specific proteins (14, 59). Expression of ENaC channel subunits is indeed increased in more differentiated keratinocytes (12, 13), although we could not find major changes in the expression profile of the differentiation proteins in the αENaC knockout mice (supplemental Fig. S2). Ionic fluxes further play an important role in skin differentiation processes as shown by identification of mutations in two calcium pumps leading to severe skin pathologies like Hailey-Hailey disease and Darier disease (60, 61). Thus, ENaC activity may play a role in the regulation of diverse cellular processes in the skin like e.g. barrier function, galvanotaxis and wound healing (62, 63).

In conclusion, our results unveil the physiological consequences of the mammalian epithelial sodium channel deficiency in skin that leads to distinct phenotypes in prenatal versus postnatal period. We propose that ENaC plays an important role in the maturation of the postnatal epidermal permeability barrier function. Thereby, the physiological consequences of αENaC deficiency in mouse skin will contribute to a better understanding of the developmental adjustments to achieve optimal epidermal barrier function in the dry ex utero environment.

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