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Mice lacking 25OHD 1α-hydroxylase demonstrate decreased epidermal differentiation and barrier function☆

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Abstract

Keratinocytes express high levels of 25OHD 1α-hydroxylase (1OHase). The product of this enzyme, 1,25$(OH)_2$D, promotes the differentiation of keratinocytes in vitro. To test whether 1OHase activity is essential for keratinocyte differentiation in vivo we examined the differentiation process in mice null for the expression of the *1αOHase* gene (*1αOHase*$^{-/-}$) by light and electron microscopy, by immunocytochemistry for markers of differentiation, by ion capture cytochemistry for calcium localization, and by function using transepidermal water loss (TEWL) to assess barrier integrity. Levels of involucrin, filaggrin, and loricrin—markers of differentiation in the keratinocyte and critical for the formation of the cornified envelope—were reduced in the epidermis of *1αOHase*$^{-/-}$ mice. Calcium in the outer epidermis was reduced with loss of the calcium gradient from stratum basale to stratum granulosum. TEWL was normal in the resting state, but following disruption of the barrier, *1αOHase*$^{-/-}$ mice had a markedly prolonged recovery of barrier function associated with a reduction in lamellar body secretion and a failure to reform the calcium gradient. Thus 1,25$(OH)_2$D is essential for normal epidermal differentiation, most likely by inducing the proteins and mediating the calcium signaling in the epidermis required for the generation and maintenance of the barrier.

Keywords: Keratinocyte; Epidermis; Calcium; 25OHD 1α-hydroxylase; 1,25$(OH)_2$D; Differentiation; Permeability barrier

1. Introduction

Keratinocytes are not only capable of producing Vitamin D_3 from endogenous sources of 7-dehydro-cholesterol (7-DHC) in a regulated fashion but of metabolizing Vitamin D via 25 hydroxylation and 1α-hydroxylation to 1,25$(OH)_2$D [1–4]. Keratinocytes are the only cell in the body with the whole pathway from 7-DHC to 1,25$(OH)_2$D.

1,25$(OH)_2$D induces keratinocyte differentiation [5–9]. 1,25$(OH)_2$D is likely to be an autocrine or paracrine factor for epidermal differentiation since it is produced by the keratinocyte, but under normal circumstances keratinocyte production of 1,25$(OH)_2$D does not appear to contribute to circulating levels [1,2]. 1,25$(OH)_2$D increases involucrin, transglutaminase, and cornified envelope formation at subnanomolar concentrations in preconfluent keratinocytes [5–9]. When examined, these effects of 1,25$(OH)_2$D can be reproduced by 25OHD [3,10], presumably because of endogenous conversion of 25OHD to 1,25$(OH)_2$D, but are not observed with the biologically inactive β isomer of 1,25$(OH)_2$D [6] (the natural isomer is lα,25$(OH)_2$D).

Calcium is the best studied prodifferentiating agent for keratinocytes. In vivo, a calcium gradient exists in the epidermis such that in the basal and spinous layers calcium is primarily intracellular and in low amounts, but in the upper granular layers calcium accumulates in large amounts in the cell and the intercellular matrix [11]. This gradient of calcium may provide the driving force for differentiation in intact epidermis [12]. Calcium and 1,25$(OH)_2$D interact in their ability to stimulate differentiation [9]. Both calcium (in the absence of 1,25$(OH)_2$D) and 1,25$(OH)_2$D (at 0.03 mM Ca^{2+}) induce involucrin and transglutaminase in a dose-dependent fashion, but together their actions are synergistic [9].

The recent availability of mice lacking the 1OHase (*1αOHase*$^{-/-}$) [13,14], has provided an opportunity to evaluate the degree to which 1,25$(OH)_2$D and its epidermal production are required for epidermal differentiation in vivo. As will be demonstrated in this report these mice

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show a reduction in levels of proteins forming the cornified envelope. Furthermore, *1αOHase*$^{-/-}$ animals have a retarded recovery of barrier function when this is disrupted, associated with an impaired reestablishment of the calcium gradient in the epidermis. Unlike *VDR*$^{-/-}$ mice, *1αOHase*$^{-/-}$ mice do not have a defect in hair follicle cycling. These observations indicate that 1,25$(OH)_2$D is critical for normal epidermal differentiation, in part by facilitating the formation and maintenance of the calcium gradient.

2. Materials and methods

2.1. Animals

Mice heterozygous for the *1αOHase* null mutation, *1αOHase*$^{-/+}$, outbred to C57BL/6 as previously described [13], were bred to provide wildtype (*1αOHase*$^{+/+}$) and homozygous mutant *1αOHase*$^{-/-}$ littermates used for these studies. Genotyping was performed by PCR. Primers used for PCR include an upper primer (5′-CCCATCCCGAGAACTCTA-3′) and a lower primer (5′-GTGCCGTGATAAATGCTT-3′) that encompass the deleted exon 8 in the mutant *1αOHase* allele. After weaning the mice were raised on a 1.3% calcium, 1.03% phosphorus diet (Teklad diet 8656, Harlan Teklad, Madison, WI) or on a 2% calcium, 1.25% phosphorus, 20% lactose diet (rescue diet) (TD96348, Teklad, Madison, WI) shown previously to normalize mineral ion homeostasis and prevent rickets in these mice [15].

2.2. Assessment of barrier function and its recovery after perturbation

The integrity of the permeability barrier was assessed by transepidermal water loss (TEWL) as previously described [16]. The barrier was disrupted by cellophane tape stripping until TEWL levels were 4 mg/cm^2 per h, and measurements of TEWL repeated at 3 and 6 h following tape stripping. Samples of skin were also obtained for electron microscopy and calcium gradient determinations prior to and immediately following barrier disruption as well as 3 and 6 h later to evaluate recovery. All studies were approved by the Animal Use Committee at the Veterans Affairs Medical Center, San Francisco.

2.3. Immunohistochemistry

The skin from the upper portion of the back was excised and fixed with 4% formaldehyde (freshly prepared from paraformaldehyde) in phosphate-buffered saline at 4 °C for 12 h, embedded in paraffin, cut in 5 μm sections, deparaffinized and rehydrated, boiled in 10 mM citrate buffer for 20 min, and endogenous peroxidase activity quenched. After blocking with 4% bovine serum albumin, the sections were incubated with specific antibodies for PCNA and the differentiation markers involucrin, loricrin, and filaggrin as we have previously described [17].

2.4. Electron microscopy

Skin samples from 6-week-old wildtype (*1αOHase*$^{+/+}$) and homozygous mutant *1αOHase*$^{-/-}$ littermates were prepared according to previously reported procedures [18]. Briefly, they were fixed in 2.5% glutaraldehyde, osmicated with 1% osmium tetroxide, dehydrated in ethanol, and embedded in epoxy medium. Ultra-thin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined with a Zeiss electron microscope operated at 60 kV. In each section, the entire area of the granular layer was examined, and representative images were photographed for analysis.

2.5. Assessment of epidermal calcium gradient

Skin samples were removed and processed for ion capture cytochemistry, as previously described [11]. Samples were minced and immediately immersed in an ice-cold fixative containing 2% glutaraldehyde, 2% formaldehyde, 90 mM potassium oxalate, and 1.4% sucrose, pH 7.4. After overnight fixation at 4 °C in the dark, samples were postfixed in 1% osmium tetroxide containing 2% potassium pyroantimonate and routinely processed and embedded in an epon–epoxy resin mixture. Ultra-thin sections were double-stained with uranyl acetate/lead citrate, and examined with a Zeiss electron microscope operating at 60 kV.

2.6. Statistical analysis

Statistical comparisons were made using Student's *t*-test.

3. Results

3.1. General appearance is little affected

The *1αOHase*$^{-/-}$ mice demonstrated little difference in gross phenotype over the first 6 weeks of life. Body size and weight gain were not significantly different from their wildtype littermates during this period regardless of whether the mice were maintained on the 1.3% calcium diet or the 2.4% calcium rescue diet. Only as the mice grew older were differences in body size observed in the *1αOHase*$^{-/-}$ animals on the 1.3% diet, although the 2% calcium rescue diet prevented any decline in growth rate. At no time was alopecia observed.

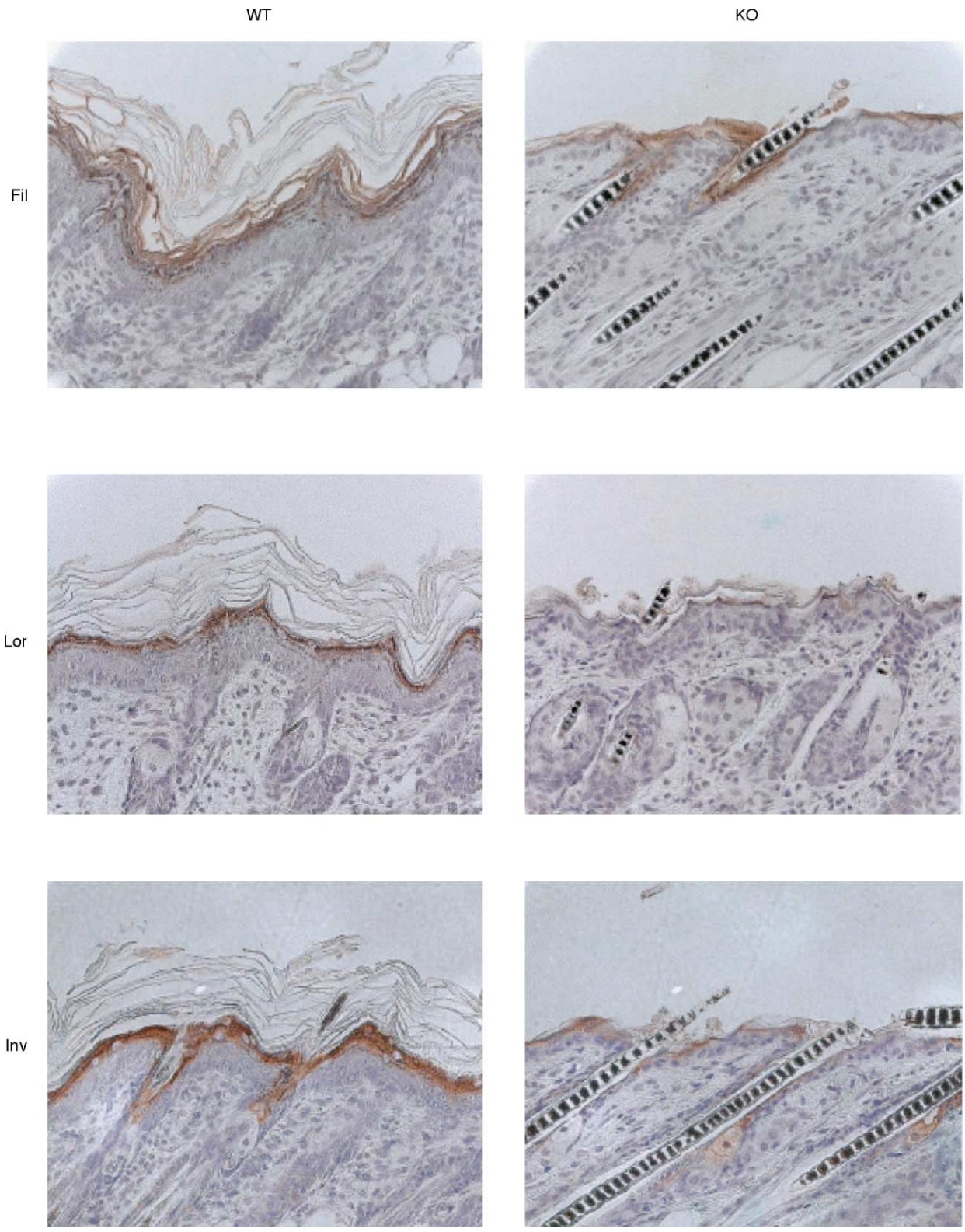

Fig. 1. Decreased levels of filaggrin, loricrin, and involucrin in the *1αOHase*$^{-/-}$ mouse. Representative sections of the skin from the backs of 2-week-old wildtype and *1αOHase*$^{-/-}$ mice were assessed by immunocytochemistry for the expression of filaggrin (Fil), loricrin (Lor), and involucrin (Inv). Expression was substantially lower in the epidermis of the *1αOHase*$^{-/-}$ mice. Note also the marked reduction in granules in the outer epidermis of the *1αOHase*$^{-/-}$ mice.

3.2. Reduction in the expression of markers of differentiation in 1αOHase$^{-/-}$ mice

In *1αOHase*$^{-/-}$ mouse epidermis, no evidence of hyperplasia was observed, and no consistent difference in the expression of the proliferating cell nuclear antigen (PCNA) was observed at any age evaluated (data not shown). However, when the expression of differentiation markers in the epidermis was evaluated by immunocytochemistry, a decrease in the levels of filaggrin, loricrin, and involucrin was observed in *1αOHase*$^{-/-}$ mice (Fig. 1). These differences were apparent by 1 week of age and persisted throughout the 6-week study. The reduction in expression of these differentiation markers was accompanied by a reduction in the granules in the upper layers (stratum granulosum) of the epidermis (Fig. 1). Electron microscopic examination of the epidermis of the *1αOHase*$^{-/-}$ mice confirmed the marked reduction in both the keratohyalin and loricrin granules (Fig. 3). Raising mice on the 2% calcium rescue diet did not correct these differences.

3.3. Barrier function is abnormal in 1αOHase$^{-/-}$ mice

In the resting state transepidermal water loss was comparable in the *1αOHase*$^{-/-}$ mice and their wildtype littermates. However, the *1αOHase*$^{-/-}$ mice showed a marked retardation in the recovery of the barrier following its disruption (Fig. 2). In particular, 3 h after barrier disruption the wildtype mice had recovered 55% of the baseline value, with a further recovery to 65% by 6 h, whereas the *1αOHase*$^{-/-}$ mice had recovered only 27% of the baseline value by 3 h, with no further improvement by 6 h. The dietary calcium level had little effect on barrier recovery in either *1αOHase*$^{-/-}$ mice or their wildtype littermates.

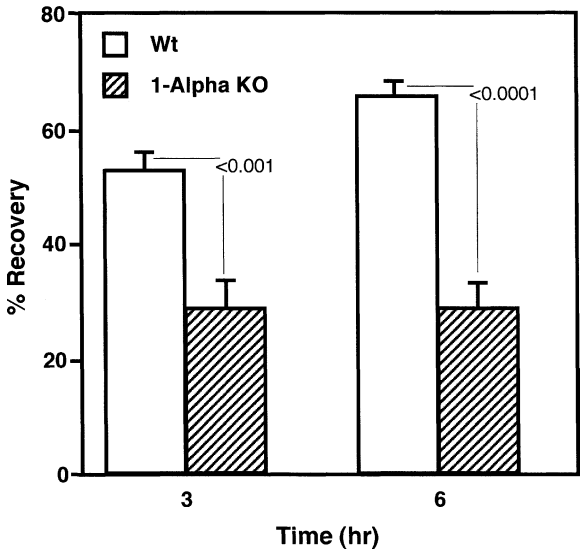

Fig. 2. Barrier recovery is delayed in *1αOHase*$^{-/-}$ mice. Transepidermal water loss (TEWL) was measured prior to and 3 and 6 h after tape stripping. The degree to which recovery of the barrier after tape stripping is achieved at these time points is recorded. Eleven *1αOHase*$^{-/-}$ mice and twelve wildtype mice, age 6–7 weeks, were used in this experiment. All mice were raised on the 2% calcium rescue diet. At both the 3 and 6 h time points, recovery of barrier function was significantly delayed in *1αOHase*$^{-/-}$ mice. Similar results were observed in mice raised on the 1.3% calcium diet. The error bars enclose mean ± S.D.

3.4. Lamellar body secretion is abnormal in the epidermis of 1αOHase$^{-/-}$ mice following barrier disruption

Following barrier disruption, the cells in the stratum granulosum secrete the contents of the lamellar bodies into the intercellular space between the stratum granulosum and stratum corneum, contents which are used to restore the barrier. As shown in Fig. 3, very little lamellar body contents can be found in the junction between the stratum corneum and stratum granulosum in the epidermis of *1αOHase*$^{-/-}$ mice compared to that of wildtype mice 3 h after barrier disruption by tape stripping.

3.5. Epidermal calcium gradient is abnormal, and its recovery after barrier disruption is delayed in 1αOHase$^{-/-}$ mice

The calcium gradient in the epidermis in vivo is associated with the maintenance of barrier function and may play a critical role in the differentiation of the epidermis in vivo. Therefore, we examined the calcium gradient in the epidermis of the *1αOHase*$^{-/-}$ mice compared to that of their wildtype littermates. In the basal state, the calcium gradient in the epidermis of *1αOHase*$^{-/-}$ mice was abnormal. The amount of calcium in the epidermis was reduced overall, and little evidence of a gradient from the stratum basale to the stratum granulosum could be observed, compared to wildtype mice. Immediately after barrier disruption, the amount of calcium in the epidermis of both wildtype and *1αOHase*$^{-/-}$ mice was reduced, and the gradient lost (Fig. 4A). However, by 6 h the calcium gradient was restored in wildtype animals, but was not restored in *1αOHase*$^{-/-}$ mice (Fig. 4B and C).

4. Discussion

In the current study we examined the epidermis of *1αOHase*$^{-/-}$ mice compared to their wildtype littermates to determine whether 1,25(OH)$_2$D production was required for normal differentiation of keratinocytes in vivo. Although there was no striking gross phenotype in *1αOHase*$^{-/-}$ mice, the levels of the differentiation markers involucrin, profilaggrin, and loricrin were reduced. This reduction was associated with a reduction of calcium in the outer layers of the epidermis, consistent with the observations in vitro that 1,25(OH)$_2$D increases intracellular calcium, and increased intracellular calcium is critical for the expression of these genes. Placing the animals on a high calcium diet previously shown to normalize serum calcium and correct the

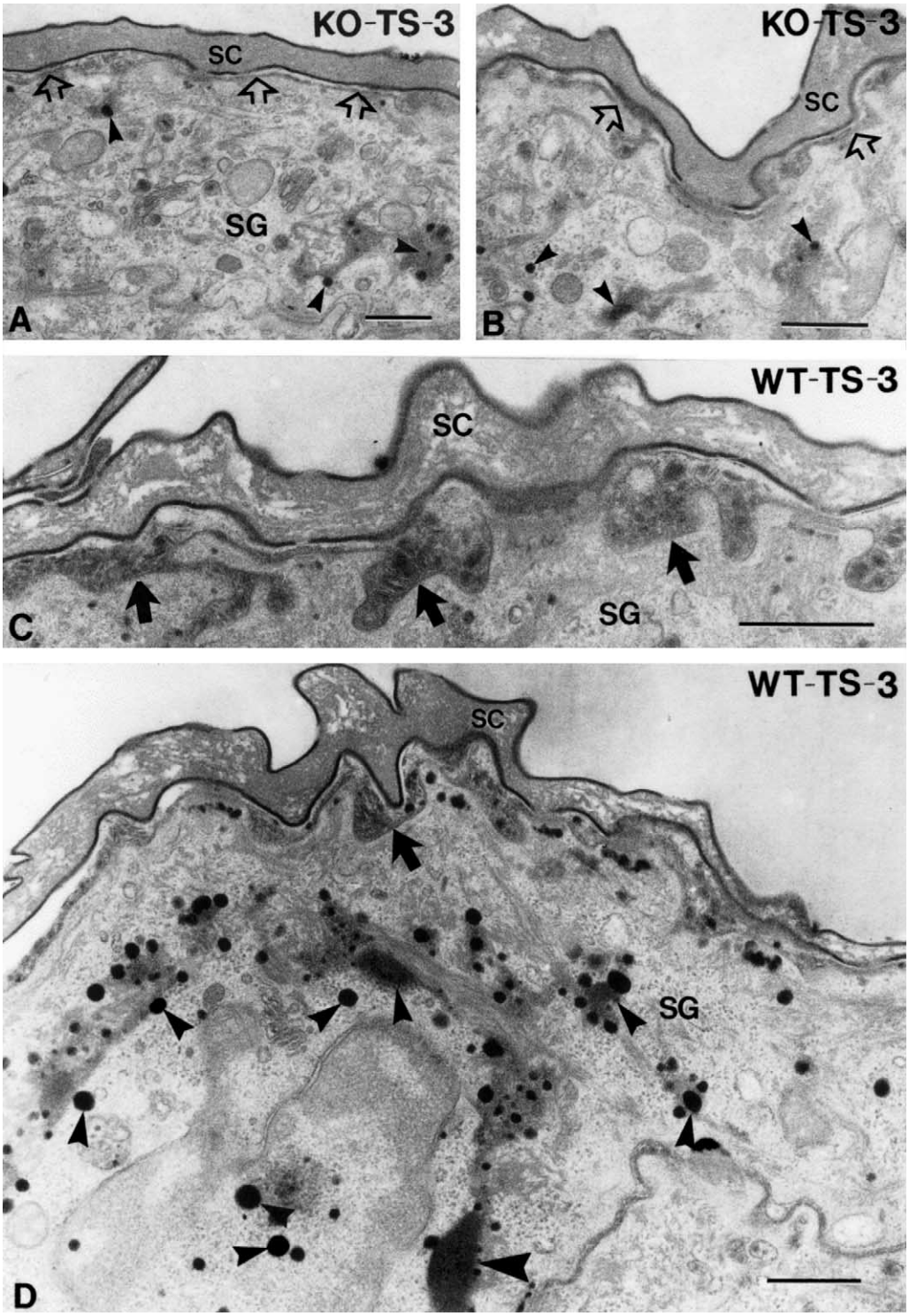

Fig. 3. *1αOHase*$^{-/-}$ mice have a reduced secretion of lamellar body content into the stratum corneum/stratum granulosum junction following barrier disruption. Skin samples from the backs of *1αOHase*$^{-/-}$ mice and their wildtype littermates were processed for electron microscopy 3 h after tape stripping to break the permeability barrier. These mice were raised on the 2% calcium rescue diet, although similar observations were made in mice raised on the 1.3% calcium diet. The epidermis from *1αOHase*$^{-/-}$ mice showed a marked reduction in the amount of osmiphilic material in the intercellular space between the cells of the stratum corneum and stratum granulosum following tape stripping. Note also the reduced number of granules in the epidermis of the *1αOHase*$^{-/-}$ mouse.

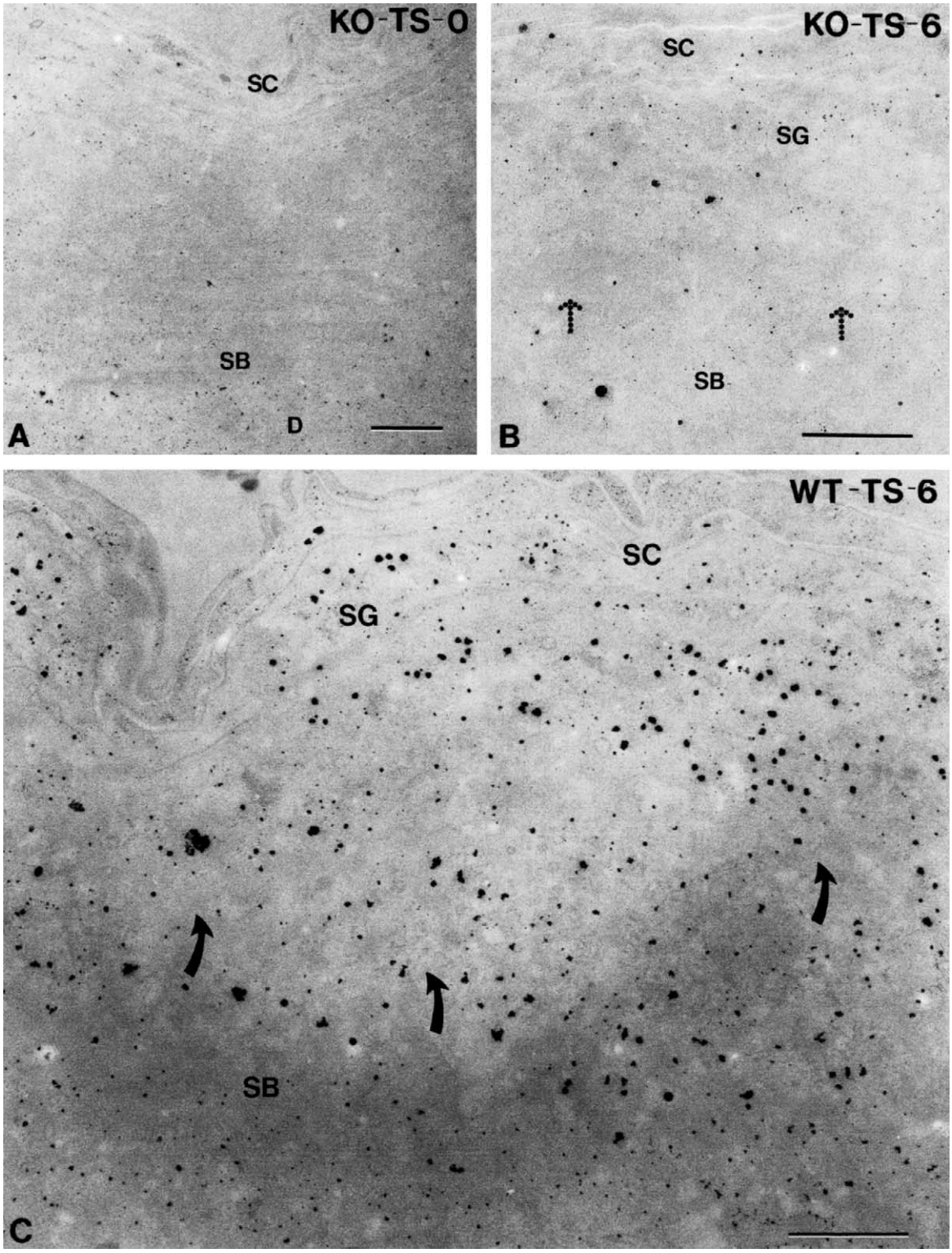

Fig. 4. The calcium gradient is reduced at baseline and fails to reform following barrier disruption in the epidermis of *1αOHase*$^{-/-}$ mice. Skin samples from the backs of *1αOHase*$^{-/-}$ mice and their wildtype littermates were processed by ion capture cytochemistry to detect calcium prior to, immediately after, and 6 h after tape stripping to disrupt the barrier. The results shown are from mice raised on a 1.3% calcium diet. Similar results were seen in mice raised on the 2% calcium rescue diet. The *1αOHase*$^{-/-}$ mice have substantially less calcium in their epidermis at baseline than do wildtype mice, and no obvious gradient of calcium is seen from the stratum basale to the stratum granulosum in *1αOHase*$^{-/-}$ mice unlike their wildtype littermates. Immediately following barrier disruption, calcium was lost from the outer epidermis in all mice (A), however, *1αOHase*$^{-/-}$ mice failed to regain calcium by 6 h (B), whereas wildtype mice did (C).

rickets in these mice [15] did not normalize these findings. Although we did not find a difference in barrier function in the basal state between *1αOHase*$^{-/-}$ mice and their wildtype littermates, we observed a pronounced retardation in the ability of *1αOHase*$^{-/-}$ mice to recover their barrier function following disruption. Again, this was not influenced by dietary calcium. This abnormality identified functionally as increased transepidermal water loss was confirmed morphologically in that lamellar body secretion was reduced and the calcium gradient failed to form after barrier disruption in *1αOHase*$^{-/-}$ mice.

We conclude that 1,25$(OH)_2$D plays an important role in calcium handling by the keratinocyte both in vivo and in vitro. In vivo this role leads to a reduction in calcium in the stratum granulosum, a failure to respond appropriately to barrier disruption with restoration of the barrier, and decreased expression of markers of differentiation. In the basal state compensatory mechanisms appear sufficient to maintain a normal barrier despite a reduced calcium gradient and reduced levels of the differentiation markers. However, one might predict from these observations that disruption of the barrier would have greater pathologic consequences in the human or mouse with *1αOHase* deficiency than in normal animals.

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