

## INVESTIGATIVE REPORT

# Induction of a Hardening Phenomenon by Repeated Application of SLS: Analysis of Lipid Changes in the Stratum Corneum

Christian HEINEMANN<sup>1</sup>, Christiane PASCHOLD<sup>1</sup>, Joachim FLUHR<sup>1</sup>, Walter WIGGER-ALBERTI<sup>1</sup>, Sibylle SCHLIEMANN-WILLERS<sup>1</sup>, Hany FARWANAH<sup>2</sup>, Klaus RAITH<sup>2</sup>, Reinhard NEUBERT<sup>2</sup> and Peter ELSNER<sup>1</sup>

<sup>1</sup>Department of Dermatology, Friedrich-Schiller-University Jena, Germany, and <sup>2</sup>Institute of Pharmaceutics and Biopharmaceutics, Martin-Luther-University Halle, Germany

**Adaptation of the skin to repeated influence of exogenous irritants is called the hardening phenomenon. We investigated the stratum corneum lipid composition before and after induction of a hardening phenomenon. Irritant contact dermatitis was induced in 23 non-atopic volunteers by repeated occlusive application of 0.5% sodium lauryl sulfate (SLS) over 3 weeks. At 3, 6 and 9 weeks after irritation, the SLS responses of pre-irritated skin and normal skin were compared. The horny layer lipid composition (ceramides 1–7, cholesterol and free fatty acids) was assessed before irritation and 3, 6 and 9 weeks after irritation. During the first 2 weeks of irritation the transepidermal water loss increased continuously and seemed to decrease during the third week (effect of adaptation). The barrier function of pre-irritated sites was more stable to SLS challenge. Three weeks after irritation, there was a significant increase of ceramide 1 ( $p < 0.001$ ). The only volunteer without hardening phenomenon showed no increase of ceramide 1. Ceramide 1 seems to play a key role as a protection mechanism against repeated irritation. Key words: hardening phenomenon; irritant contact dermatitis; stratum corneum lipids; ceramides; transepidermal water loss.**

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Christian Heinemann MD, Department of Dermatology, Friedrich Schiller University Jena, Erfurter Str. 35, DE-07740 Jena, Germany. E-mail: christian.heinemann@derma.uni-jena.de

Chronic irritant contact dermatitis (ICD), the most frequent occupational disease of the skin, represents an important socio-medical problem. Additionally, ICD is known to be a risk factor for the development of the less frequent allergic contact dermatitis (1).

Over the last decades, the epidemiology and pathogenesis of ICD have been subject to intense investigation (2, 3). It is well known that the hands are most frequently concerned due to daily exposure to a wide range of irritants at home or in the workplace (4). However, only a few employees develop hand eczema, while most employees, doing the same work and exposed to the same irritants, do not. In the latter

group, transient dermatitis is often followed by a long-lasting hyporeactivity to irritants. This is called the hardening phenomenon and describes the adaptation of the skin to repeated exogenous irritative noxes. Widmer et al. (5) have induced hardening by repeated irritation with sodium lauryl sulfate (SLS), but the mechanisms of this phenomenon are still not fully understood. However, for prevention of ICD and early recognition of a predisposition, further knowledge about the hardening phenomenon is of utmost importance.

The intact stratum corneum is the most important structure for the skin barrier function. The bricks and mortar model describes a two-compartment system of lipid-depleted cells embedded in a uniquely organized intercellular complex of multilamellar non-polar lipids (6). During differentiation, keratinocytes synthesize lipids which accumulate in lamellar granules. At the interface of stratum granulosum and stratum corneum the lipids are transported into the intercellular spaces. After enzymatic processing, bilayers consisting of ceramides, cholesterol and fatty acids are formed (7). The lipid composition of the stratum corneum has been described in detail (8–12).

There is some evidence that ceramides are particularly important for the barrier function of the stratum corneum (13). Exogenous application of ceramides led to accelerated barrier regeneration (14). Imokawa et al. (15) reported a decreased amount of ceramides in atopic individuals. This study focused on the lipid composition of the stratum corneum before and after induction of a hardening phenomenon with special attention to the different types of ceramides.

## MATERIALS AND METHODS

### *Study population*

The study was performed under standardized laboratory conditions between December 2001 and May 2002.

Twenty-three healthy volunteers (11 women, 12 men; mean age 26.7 ( $\pm 6.9$ ) years; range 18–44 years) without any skin disease participated in this study after obtaining a written informed consent in accordance with the Helsinki declaration. During the study period, the volunteers were allowed to shower as usual but they had to avoid bathing, application of detergents, moisturizers or emollients on their forearms and

upper arms. They were also asked to avoid sunbeds and solar radiation. The study had passed review by the Ethical Commission of the Friedrich Schiller University.

### Procedures

**Induction of cumulative irritation.** Cumulative ICD was induced at three sites (f2–f4) on the medial one-third of one forearm and at three sites (u1–u3) on the medial side of one upper arm by application of 230  $\mu$ l of 0.5% aqueous solution of SLS (Serva Feinbiochemie, Heidelberg, Germany) in large Finn chambers (inner diameter 1.2 cm, Epitest Ltd, Hyrlä, Finland) for 1 h on weekdays over a period of 3 weeks (Fig. 1).

**Challenge phase.** Three, 6 and 9 weeks after the end of the induction phase (on days 43, 64 and 85), an SLS irritation test was performed. Each time a 1% aqueous solution of SLS was applied for 24 h to one of the sites on the pre-treated upper arm and to a previously untreated site on the opposite upper arm (at the same distance from the elbow). Thus it was possible to compare the reactivity of a pre-irritated skin site to the reactivity of normal, previously non-irritated skin. The pre-treated sites were identified for challenge by characteristic anatomical features (such as veins and naevi), which were marked for each volunteer on a separate plastic sheet for each forearm and each upper arm.

**Clinical evaluation and biophysical measurements.** The measurements were performed after the volunteers had rested for at least 15 min and after acclimatization to the standardized laboratory conditions (temperature 20–22°C, humidity 20–40%). They were made by the same observer at the same time ( $\pm 1$  h), 10 min before irritation. The following methods were used. Visual scoring was performed according to Frosch & Kligman (16). Transepidermal water loss (TEWL) as a valid indicator of epidermal barrier function disruption by SLS (17) was measured using the Tewameter TM 210<sup>®</sup> (Courage & Khazaka, Cologne, Germany). The widely used TEWL measurement using an open chamber system is based on assessing the vapour pressure built up inside the probe tunnel after application of the probe to the skin surface. The authors are aware of possible errors of this method due to changes in relative humidity and skin surface temperature (18). In order to minimize errors, standard procedures were followed (19, 20). The intensity of erythema was quantified by using the chromameter CR-300<sup>®</sup> (Minolta GmbH, Ahrensburg, Germany) according to the recommendations of Elsner (21) by exclusively considering the a\*-value which represents the red-green axis of the three-dimensional CIE L\*-a\*-b\*-assay system.

On day 1 of the induction phase all parameters were assessed on fields f1–f4 and u1–u3. In the following the visual score was

assessed daily, whereas the TEWL and the chromametry a\* value were assessed on days 5, 12 and 19.

During the challenge phase (days 43, 64 and 85) all parameters were assessed 10 min before and 10 min after irritation with SLS on one of the fields u1–u3 and on the corresponding field from u4–u6 (Fig. 1).

**Extraction of the stratum corneum lipids.** The lipid content (ceramides 1–7, total ceramides, cholesterol, free fatty acids) of the stratum corneum was assessed at four time points. The first extraction was conducted on day 1 on the inner side of one forearm (test side f1, not pre-irritated).

The other extractions took place 3, 6 and 9 weeks after induction of the ICD on pre-irritated test sides (f2–f4). An open glass ring with an inner diameter of 18 mm was pressed to the skin on the test site and 6 ml of the extraction fluid (acetone and diethylether 1:1) was pipetted into the lumen (15, 22). After 30 seconds the first fraction was removed (sample 1). The procedure was repeated on the same test site and the second fraction (sample 2) was removed 20 min after application. The solution was mixed every 5 min using the pipette. Superficial lipids (sebum lipids) were assumed to be washed off with the first fraction, whereas the stratum corneum lipids should be represented mainly in the second fraction. Both fractions were stored for analysis in a glass tube at –20°C. The analysis was performed using automated multiple development high performance thin-layer chromatography (HPTLC) (23).

**Chemicals for AMD-high performance thin-layer chromatography.** Cholesterol, cholesteryl oleate and palmitic acid (Sigma-Aldrich, Taufkirchen, Germany) and ceramide 3 (Cosmoferm, now Goldschmidt, Essen, Germany) were used as reference substances to which the lipid quantifications were related.

Solvents for TLC purposes were of analytical grade and purchased from Merck (Darmstadt, Germany), Baker (Deventer, The Netherlands) and Roth (Karlsruhe, Germany). Silica TLC plates (Kieselgel 60 F245, 20  $\times$  10 cm), were supplied by Merck.

**Application of the samples.** The dried lipid extracts were first dissolved in 500  $\mu$ l of chloroform:methanol 1:1 (v/v); 50  $\mu$ l of each sample were applied automatically using the TLC Sampler 4 (CAMAG, Muttenz, Switzerland). Band length, first application position and distance from the bottom of the plate were 8 mm, 16 mm and 8 mm, respectively.

**Development of the plates and postchromatographic derivatization.** The lipid separation was carried out by means of an AMD-2 apparatus (CAMAG, Muttenz, Switzerland) as described previously (23). Briefly, an automated multiple development procedure including 17 steps and based on

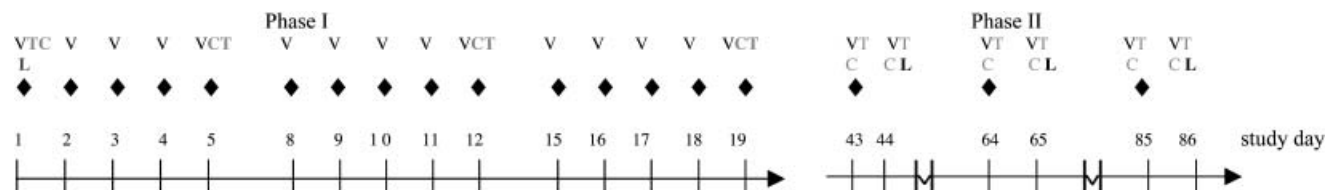


Fig. 1. Scheme of phase I (induction of irritant contact dermatitis (ICD)) and phase II (provocation and lipid extraction (L)). Test sites f1–f4: forearms (f1 ceramide extraction on day 1, no irritation), test sites u1–u3: upper arm (phase I). Test sites f2–f4: forearms, preceding ICD; test sites u1–u3: upper arm, preceding ICD; test sites u4–u6: opposite upper arm, without preceding ICD (phase II). V, visual score; T, trans-epidermal water loss; C, chromametry,  $\blacklozenge$ , irritation using 0.5% SLS for 1 h and 1% SLS for 24 h (phase I and II, respectively). All measurements were assessed before irritation.

mixtures of chloroform, ethanol, acetone, n-hexane and ethylacetate under acidic conditions was used to separate the ceramide classes and the other lipids in the same run. After drying, the plates were dipped into an aqueous solution of 10% CuSO<sub>4</sub>, 8% H<sub>3</sub>PO<sub>4</sub> (v/v) and 5% methanol for 20 s and charred in a drying oven at 150°C for 30 min.

The visualized lipid bands were scanned using a TLC Scanner 3 (CAMAG). The measurements were performed in reflectance mode at a wavelength of 546 nm. The software winCats was used to carry out the peak-based integration and quantification. To avoid experimental errors, individual curves of each reference lipid were set up for each HPTLC plate. As standards are not commercially available for each ceramide class, ceramide 3 was used as a standard for all ceramide classes because of its average intensity as described previously (24).

### Statistics

Data were analysed using the statistical software program SPSS 12.0 for Windows. For each test field, the mean values of the chromametry and TEWL data points were calculated from three and two measurements, respectively. The comparisons of the test and control data were performed with the non-parametric Wilcoxon test. Alpha correction has been conducted as measurements were made at three time points and compared to the baseline values.

## RESULTS

### Induction of cumulative irritation

During the first 2 weeks of irritation a continuous increase of TEWL, chromametry values and visual score was observed ( $p < 0.001$ ). At the end of week 2, the values reached a maximum (Fig. 2). The parameters on the upper arm test sites reached higher values (especially the TEWL), but generally showed a similar pattern (data not shown).

### Challenge phase

Three weeks after the induction phase the visual score had returned to normal. There was no difference in the TEWL between pre-irritated test sites and non-irritated test sites before re-challenge at days 43, 64 and 85. After re-challenge, the increase of TEWL was higher on non-irritated test sites compared with pre-irritated sites at day 65 ( $p < 0.01$ ; Fig. 3). The chromametry values seemed to be higher on pre-irritated test sites at all time points (Fig. 4).

Depending on the difference in TEWL response between pre-irritated test sites and non-irritated test sites the study population was divided into two groups: those presenting a hardening phenomenon (negative difference) and those without hardening phenomenon (no difference or positive difference). At day 44, 16 volunteers were classified as having a hardening phenomenon, 14 volunteers at day 65 and 15 volunteers at day 86. Only one volunteer had no hardening phenomenon at any time point, whereas eight volunteers showed a hardening phenomenon in all challenges.

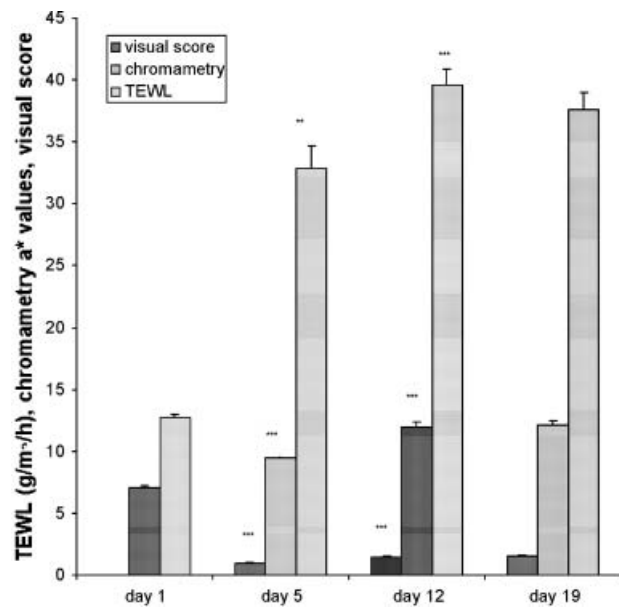


Fig. 2. Irritation phase on forearms, trans-epidermal water loss (TEWL) values. Visual score, chromametry a\* and (g/m<sup>2</sup>/h) from day 5 were compared to values from day 1, from day 12 to day 5 and from day 19 to day 12 for statistically significant differences. \*\*\* $p < 0.001$ . Bars=SEM;  $n=69$ .

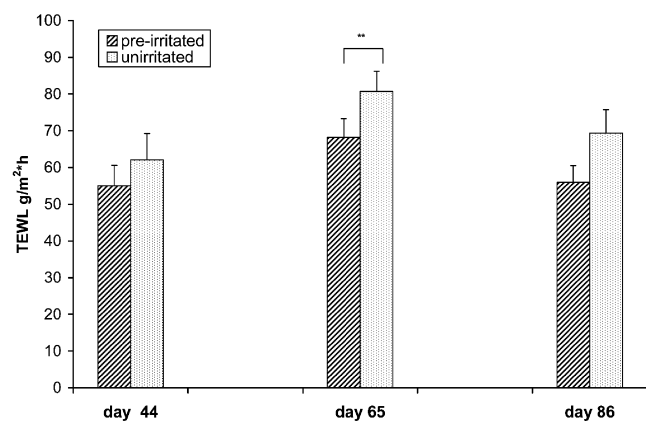


Fig. 3. TEWL (g/m<sup>2</sup>/h) on pre-irritated test sites versus sites not pre-irritated after challenge with 1% SLS occlusive for 24 h (phase II). \*\* $p < 0.01$ . Bars=SEM;  $n=23$ .

### Lipid evaluation

Comparing the baseline stratum corneum lipid composition of volunteers presenting a hardening phenomenon ( $n=21$ ) with the volunteer presenting no hardening phenomenon ( $n=1$ ), ceramide 1 was lower in the volunteers with hardening phenomenon than in the volunteer without hardening phenomenon.

### Lipids in sample 1 at days 44, 65 and 86

In 21 volunteers presenting a hardening phenomenon the relative amount (as a percentage of the rest of the stratum corneum lipids) of free fatty acids in the extracted lipids was lower on challenge days ( $p < 0.01$

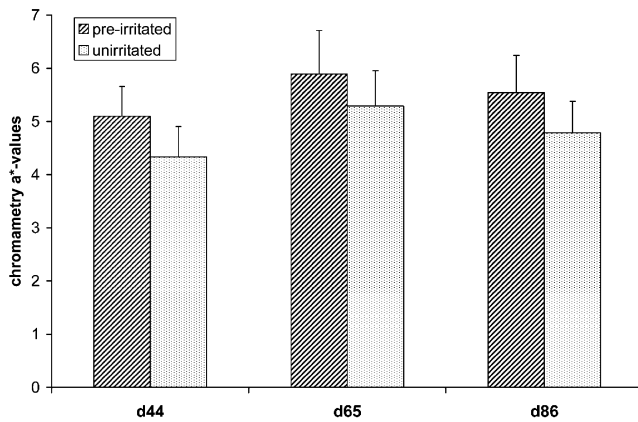


Fig. 4. Chromametry delta a\*-values on pre-irritated test sites versus sites not pre-irritated after challenge with 1% SLS occlusive for 24 h (phase II days 44, 65 and 86 – see Fig. 1). Bars=SEM; n=23.

at day 86), whereas the relative amount of cholesterol had increased ( $p < 0.01$  at day 65,  $p < 0.001$  at day 86) (Table I). The total amount of lipids was significantly higher at day 44 ( $p < 0.001$ ). In the single volunteer without hardening phenomenon a loss of ceramide 1 and total ceramides was obvious on days 44 and 65. Instead, there was an increased amount of free fatty acids as compared with day 1 (data not shown).

#### Lipids in sample 2 at days 44, 65 and 86

In the 21 volunteers with hardening phenomenon the total amount of lipids had significantly increased from baseline to day 44 ( $p < 0.01$ ; data not shown).

The relative amount (as a percentage) of cholesterol ( $p < 0.01$  at day 86) had increased, whereas the amount of free fatty acids had decreased ( $p < 0.05$  at days 44 and 65 and  $p < 0.01$  at day 86). At day 44 there was a significant increase of ceramide 1 ( $p < 0.001$ ; Table I). In the single volunteer without hardening phenomenon there was a decrease of ceramide 1 at days 44 and 65 (data not shown).

Analysis of the total amounts (in  $\mu\text{g}$ ) of lipids showed a significantly increased amount of ceramide 1 ( $p < 0.001$ ), c2 ( $p < 0.05$ ) and c4 ( $p < 0.01$ ) at day 44. c5 and c6 had increased at day 44 ( $p < 0.05$ ) and 65 ( $p < 0.01$ ), c7 at day 44 and 65 ( $p < 0.01$ ). The increase of total ceramides was significant at day 44 ( $p < 0.01$ ) and the increase of cholesterol at days 44 ( $p < 0.001$ ), 65 ( $p < 0.05$ ) and 86 ( $p < 0.001$ ). There were no significant changes in the amounts of free fatty acids (Table I).

#### DISCUSSION

The concept of our study was based on a preceding study by Widmer et al. (5). The question as to the origin of the hyporeactivity of pre-irritated test sites remained unsolved in their study. The authors discussed changes in the lipid composition of the stratum corneum. To further investigate the lipid changes after cumulative irritation we additionally conducted lipid extraction from pre-irritated test sites at different time points.

We were able to reproduce the increase of TEWL during the first 2 weeks of the induction phase with a slight decrease in the third week. Similar observations were made by Gehring et al. (25) performing repetitive washings on the forearms of non-atopic volunteers. At day 65 we observed a significant difference between the TEWL after re-challenge on pre-irritated sites and the normal skin and evident difference at days 44 and 86. This is also in accordance with the results of Widmer et al. (5) and was interpreted as manifestation of a hardening effect. Interestingly, the chromametry values seemed to be higher on pre-irritated test sites after re-challenge. This could be due to changes in the responsiveness in the capillary system of the papillary dermis after induction of irritation. McOsker & Beck (26) hypothesized that a higher permeability of the intercellular space and increased vessel activity might lead to an accelerated wash out of irritating substances in hardened skin.

Table I. Volunteers ( $n=21$ ) with hardening phenomenon, samples 1 (relative amounts) and 2 (relative and total amounts); delta values of the amounts of ceramides 1–7 (c1–c7), total ceramides, cholesterol and free fatty acids in % from day 1 to day 44, 65 and 86

Parameter	Sample 1 (% values)			Sample 2 (% values)			Sample 2 ( $\mu\text{g}$ values)		
	Day 44	Day 65	Day 86	Day 44	Day 65	Day 86	Day 44	Day 65	Day 86
c1	0.938	-0.054	-0.033	2.384***	0.964	0.175	1.874***	0.523	0.450
c2	1.525	0.583	0.069	0.745	1.168	0.806	1.395*	0.767	0.766
c3	-0.385	-0.531	-0.734	-0.915	-0.015	1.065	0.660	0.397	0.164
c4	0.367	-0.017	-0.069	0.426	0.365	-0.043	0.838*	0.319	0.168
c5	-0.664	-0.070	-0.949	-0.666	0.444	0.694	1.525*	1.107**	0.713
c6	-0.296	0.009	-0.579	-0.505	0.328	0.373	0.603*	0.43**	0.140
c7	-0.003	-0.581	-0.234	0.248	1.666	1.094	1.521**	1.149**	0.612
Total ceramides	0.712	-2.884	-4.146	4.274	3.748	8.489	8.416**	4.692	3.013
Cholesterol	4.956	12.943**	11.024***	4.983	5.194	10.218**	7.271***	3.647*	6.333***
Free fatty acids	-6.478	-8.434	-8.434**	-4.717*	-10.673*	-9.026**	7.863	-2.247	-3.478

\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

The onset of hardening after the induction phase may differ inter-individually as well as the duration of the phenomenon (27). Therefore it was not unexpected that some individuals showed a hardening phenomenon 3 weeks and some 6 weeks or 9 weeks after irritation. Eight volunteers showed a hardening phenomenon at all time points. In one volunteer we determined a hardening phenomenon after 3 weeks, none at 6 weeks and again at 9 weeks using our definition. We suppose that in this individual the challenge at 6 weeks on the normal skin was not conducted properly, as the TEWL increased to 85 and 106 g/m<sup>2</sup>/h on normal skin at day 44 and day 86 but only to 49 g/m<sup>2</sup>/h at day 65.

There are few data about the prevalence of hardening phenomenon but an investigation in hairdressing apprentices showed that 90% of those suffering from alkali-eczema at the beginning, cleared during training period. Only 10% finished by presenting the typical hairdressers' hand eczema (28, 29). Schwanitz (30) deducted a prevalence of 90% of hardening phenomenon within this population. Not all individuals are able to adapt to repeated irritation, atopic patients seem to develop hyporeactivity to a lower degree than non-atopics (25, 31). Therefore it is not surprising that only one volunteer in our study population did not show a hardening phenomenon at any time point (atopic individuals were excluded by the study protocol). Onset of hardening later than 9 weeks after irritation seems unlikely, so we compared the lipid profile of this one volunteer without hardening phenomenon to the average of the remaining study population. In the samples at day 1, the baseline ceramide 1 was lower in volunteers presenting a hardening phenomenon. This suggests that it is not possible to predict ability to develop hyporeactivity by measuring baseline ceramide 1. After induction of irritation the production of ceramide 1 was enhanced in volunteers with hardening phenomenon. On the contrary, in the individual without hardening effect, the amount of ceramide 1 had decreased over the time. However, more volunteers without hardening phenomenon would be necessary to deduct conclusions from the comparison of the stratum corneum lipid composition between the two groups.

The stratum corneum lipids were collected in two fractions. In sample 1, which should contain the major part of the superficial lipids (including sebum lipids), the relative amount of total ceramides and free fatty acids in the extracted lipids was lower on days 44, 65 and 86 compared with the baseline, whereas the relative amount of cholesterol had increased. The role of surface lipids for the epidermal permeability barrier function is still not well defined (22) and was not subject to investigation in this study. Sample 2 should reflect more properly the stratum corneum lipids, as the contaminating surface lipids were removed with sample 1. Compared with the baseline, the relative amount of total ceramides

(not significant) and cholesterol in this fraction had increased and the free fatty acids were lower at all challenge days. The total amount of lipids was increased significantly at day 44. This is in accordance with the findings of Proksch et al. (32, 33), who observed an increase in skin lipid synthesis after irritation. They observed an augmented synthesis of cholesterol after acute irritation and an increase in ceramides after chronic irritation. Interestingly, the relative and total amount of ceramide 1 had increased significantly at day 44 and seemed to decline over the following study period. Ceramide 1 is the least polar of the stratum corneum ceramides and probably plays a major role in determining the state of organization of the intercellular bilayers (34, 35). It is responsible for the formation of lipid bilayers and their stability (7). Di Nardo et al. (13) investigated the relationship between the ceramide content of the stratum corneum and the reactivity to SLS. In their findings the basic content of ceramide 1 and the TEWL 24 h after irritation with 3% SLS was correlated negatively in a linear function. Other authors reported reduced amounts of ceramide 1 in atopic individuals and patients suffering from lamellar ichthyosis (15, 36–38). Our findings suggest that there is an upregulation of the production of ceramide 1 in response to chronic irritation. Upregulation of ceramide 1 synthesis seems to play a major role in the development of a hardening effect.

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