Exposure of human keratinocytes to ultraviolet A (UVA) radiation at physiological doses leads to a biphasic activation of transcription factor activator protein-2 (AP-2) and subsequently to a biphasic increase in gene expression of, e.g., intercellular adhesion molecule-1 (ICAM-1). Both kinetics follow a pattern with a first peak between 0.5 and 2 h and a second, more sustained activation between 16 and 48 h. We have previously reported on a non-enzymatic triggering of the ceramide signaling cascade as the initiating step in UVA radiation-induced signaling. In this study, we report that this early (0.5–1 h) peak in ceramide content is followed by a second peak that (i) was associated with an increased expression and activity of serine palmitoyltransferase, the key enzyme of ceramide synthesis, (ii) could be prevented by inhibitors of this enzyme, and (iii) was of functional relevance because its inhibition abrogated the second, but not the first peak in UVA radiation-induced ICAM-1 gene expression. We hypothesize that this second peak most likely resulted from a ceramide-mediated autocrine loop, for (i) inhibition of the first ceramide peak resulted in inhibition of the second peak and (ii) cell-permeable ceramides-induced serine palmitoyltransferase expression, activity, and subsequently ceramide content.

Key words: autocrine loop/ceramide/gene expression/signaling/UVA

J Invest Dermatol 125:545–553, 2005
more sustained ceramide production, which is because of de novo synthesis. We also present evidence that the non-enzymatic peak initiates this second peak through activation of serine palmitoyltransferase and thereby mediates the sustained increase in AP-2 activation and gene expression that can be observed up to 48 h post-irradiation.

**Results**

**Time course of UVA radiation-induced ICAM-1 mRNA expression and ceramide content**  UVA radiation induces gene transcription in human keratinocytes in a biphasic manner (Grether-Beck et al., 1996; Gille et al., 2000). Accordingly, ICAM-1 mRNA steady-state levels were upregulated between 2 and 4 h after exposure to 30 J per cm² of UVA radiation, a dose that was previously shown to effectively induce gene expression but not to reduce viability in this cell type (Grether-Beck, 1996; 2000; Warskulat et al., 2004). This first maximum was followed by a second increase after 24 h (Fig 1A). We now report that biphasic ICAM-1 mRNA expression is associated with a biphasic increase in ceramide content in UVA-irradiated cells. Exposure of keratinocytes to an identical dose of UVA radiation resulted in an approximately 5-fold increase in the level of ceramide between 30 min and 2 h after irradiation, thus confirming previous results (Grether-Beck et al., 2000). This early peak in ceramide content was followed by a second, more sustained increase that could be detected 16 h after exposure and that was still 2.5-fold higher than the background level 48 h after irradiation (Fig 1B). A UVA-induced 2.5-fold increase in other lipid components of the cytoplasmic membrane i.e. cholesterol and phosphatidylcholine has been observed during the first 4 h post-treatment (unpublished observation). This is in contrast to observations in mice where 2–3-fold increases of cholesterol and phospholipids had been detected as early as 72 h post-application of 7.5 MED UVB (Holleran et al., 1997).

**UVA radiation-induced ceramide content is mediated by non-enzymatic and enzymatic mechanisms**  Ceramide can be generated by sphingomyelinase, which hydrolyzes sphingomyelin to produce phosphocholine (Kim et al., 1991; Liu et al., 1998; Levade and Jaffrézou, 1999). We had previously shown that UVA radiation-induced ceramide content within the first two h after exposure (= early ceramide response) was not caused by this mechanism, because UVA radiation-induced ceramide content was not associated with an increased activity of neutral or acid sphingomyelinase in irradiated keratinocytes and because it was also observed in a protein-free liposomal system (Grether-Beck et al., 2000). This conclusion is corroborated by the present finding that neither chloroquine, a well-known inhibitor of acid sphingomyelinase (Bonizzi et al., 1997), nor dichloroisocoumarine (DCIC), a specific inhibitor of neutral sphingomyelinase (Mansat et al., 1997), decreased UVA radiation-induced ceramide content 30 min post-exposure, although both inhibitors at identical concentrations significantly prevented ceramide content by interleukin-1β, which is mediated through sphingomyelinase activation (Bonizzi et al., 1997; Grether-Beck et al., 2000) (Fig 2A, B). Similarly, both inhibitors did not affect UVA radiation-induced ceramide content 16 h after irradiation, indicating that neither the early nor the late ceramide response involves sphingomyelinase activation (Fig 2A, B). Identical results were obtained when scyphostatin (Brannt et al., 2002; Czarmy et al., 2003; Numakawa et al., 2003) (Fig 2C) or spiroepoxide (Arenz and Giannis, 2001; Ramer et al., 2003), two inhibitors of neutral sphingomyelinas, were used (data not shown).

As ceramides can also be generated by degradation of glycosphingolipids, we next asked whether the increase of ceramide in irradiated cells is because of this mechanism. For this purpose, keratinocytes were preincubated with D,L-threo-(1R,2R)-1-phenyl-2-decanoylamino-3-morpholinopropan-1-ol, which is an inhibitor of glucosylceramide biosynthesis, i.e. the common precursor of all glycosphingolipids formed in vertebrates (Kolter et al., 2002; Sandhoff and Kolter, 2003). As is shown in Fig 2D, preincubation with D,L-threo-PDMP did not affect UVA radiation-induced ceramide content at all time points. In these experiments, the glucosylceramide synthase inhibitor D,L-threo-PDMP was active because it greatly reduced the formation of the ganglioside GM3 (NeuAcα2 → 3Galβ1 →

---

**Figure 1**

Ultraviolet A (UVA) irradiation results in biphasic (A) upregulation of intercellular adhesion molecule-1 (ICAM-1) mRNA and (B) release of ceramides in human normal keratinocyte. Keratinocytes were irradiated with 30 J per cm² UVA and harvested at the indicated time points. (A) ICAM-1 mRNA expression was detected by real-time RT-PCR based on β-actin as the housekeeping gene. (B) Ceramide release was assessed in samples based on 500 μg protein using a Folch extraction by high-performance thin layer chromatography on a CAMAG AMD2 device. Data from irradiated cells (colored symbols) are given in comparison with untreated controls (white symbols) that were set equal to one. Data represent the mean ± SD of three independent experiments.
The third main pathway for ceramide production is de novo synthesis (Bose et al., 1995; Perry et al., 2000). This pathway is initiated by condensation of serine and palmitoyl-CoA, which is catalyzed by serine palmitoyltransferase, to form 3-ketodihydrosphingosine. After reduction to dihydroceramide and subsequent conversion to dihydroceramide by ceramide synthase, ceramide is formed by oxidation of dihydroceramide to introduce the trans-4,5 double bond. We had previously observed that UVA radiation did not increase the activity of ceramide synthase 30 min after UVA irradiation and that addition of fumonisin B1, a natural inhibitor of ceramide synthase (Merrill et al., 1993), did not prevent the early ceramide response (Grether-Beck et al., 2000). This is in line with the present observation that the early ceramide response was not affected by treatment of keratinocytes with L-cycloserine (Fig 3A), which inhibits serine palmitoyltransferase (Sundaram and Lev, 1984a, b). Also, inhibition of serine palmitoyltransferase by the specific inhibitor myriocin (Miyake et al., 1995) did not affect UVA radiation-induced ceramide content at this early time point (Fig 3B). In marked contrast, 16 h after UVA irradiation, both inhibitors prevented UVA radiation-induced ceramide content (Fig 3A, B).

At the 16 h time point, UVA radiation was found to 2.5-fold upregulate the activity of serine palmitoyltransferase, whereas no such increase could be detected 1 h after UVA irradiation (Fig 3C). This late increase in enzyme activity was associated with an upregulation of the mRNA expression of the 2 subunits encoding this enzyme (Fig 3D). The SPTLC1 subunit was induced 14-fold 16 h post-UVA, and the SPTLC2 subunit showed a 7-fold induction after 24 h. Another possibility to consider is that the second ceramide release is due to induction of de novo ceramide synthesis. Human normal keratinocytes were preincubated with inhibitors of ceramide de novo synthesis, i.e. (A) L-cycloserine (2 mM, rising left hatched) or (B) Myriocin (5 μM, diagonally crosshatched) for 0.5 h or left untreated prior to UVA irradiation (30 J per cm²). Cells were harvested 0.5 and 16 h after UVA treatment (light gray bar). Ceramide release was assessed as described in the legend of Fig 1. Data are given in comparison with untreated controls (white bars). Stimulation using 50 U per mL IL-1β for 0.5 h (dark gray bar) was used as a positive control, as IL-1β activates both types of sphingomyelinases. (E) Quantification of GM3, the main ganglioside in keratinocytes, was used as a positive control for the function of Δ1-threo-PDMP Data represent the mean ± SD of three independent experiments.
peak might be produced because UVA radiation disrupts the utilization of ceramide for sphingomyelin or glycosylceramide synthesis. This is, however, very unlikely, because we have observed that at these later time points sphingomyelin and glycosphinoglipid formation did not decrease in UVA-irradiated cells (data not shown).

Role of the late ceramide response in UVA radiation-induced gene expression The observation that the late, but not the early, ceramide response could be prevented by addition of myriocin to UVA-irradiated keratinocytes allowed us to assess the functional relevance of the second ceramide peak for UVA radiation-induced gene expression. As is shown in Fig 4A, B, addition of myriocin at a concentration that inhibited UVA radiation-induced serine palmitoyltransferase activation (Fig 3C) and ceramide content (Fig 3B) inhibited UVA radiation-induced upregulation of ICAM-1 mRNA after 24 h and protein expression 16 and 24 h after exposure. In contrast, 3 h after UVA irradiation, increased ICAM-1 mRNA expression was not inhibited. Moreover, myriocin—at the indicated concentrations—inhibited UVA-induced activation of transcription factor AP-2 as detected by gel electrophoresis mobility shift assays in nuclear extracts from keratinocytes 16 and 24 h after irradiation (Fig 4C).

Effects of ceramide on ceramide synthesis and serine palmitoyltransferase activation In order to assess whether in UVA-irradiated keratinocytes the first and the second ceramide response were connected to each other, keratinocytes were left unirradiated and instead, cell-permeable C6 ceramides were added as a surrogate for the first, UVA radiation-induced ceramide peak. Subsequently, cells were analyzed for ceramide content, serine palmitoyltransferase mRNA expression, and activity. Addition of cell-permeable ceramide, but not vehicle control, increased ceramide content in a time-dependent manner with an approximately 4-fold maximum after 8 h (Fig 5A). This response was long lasting, because ceramide levels were still increased 3-fold compared with background levels 48 h after stimulation. Ceramide-induced ceramide content was associated with a concomitant increase in mRNA expression of the SPTLC1 (2-fold) and the SPTLC2 (4-fold) subunit of serine palmitoyltransferase between 4 and 48 h with a maximum 24 h after stimulation (Fig 5B). In addition, a 2.5-fold increase in serine palmitoyltransferase activity 16 h after addition of cell-permeable ceramide was found (Fig 5C). We therefore next assessed the effect of myriocin on ceramide-induced ceramide content. As is shown in Fig 5D, addition of myriocin completely abrogated ceramide-induced ceramide content 16 h after ceramide stimulation. In aggregate, these observations indicate that stimulation of keratinocytes with exogenous ceramides induced de novo ceramide synthesis in human keratinocytes through induction and activation of serine palmitoyltransferase. In this regard, addition of exogenous ceramide induced a ceramide response, which closely resembled the late ceramide response observed in UVA-irradiated keratinocytes. We therefore next assessed whether in UVA-irradiated keratinocytes the early ceramide peak was a prerequisite for the formation of the later ceramide response.

Effects of vitamin E, ectoin, and cholesterol on UVA radiation- and ceramide-induced ceramide content Because of the non-enzymatic nature of the early (Grether-Beck et al, 2000, Figs 2 and 3) and the enzymatic nature of the late ceramide response (Fig 3), we used a strategy that allowed us to inhibit the early ceramide peak, but did not prevent ceramide de novo synthesis. In this regard, we had previously shown that the early UVA radiation-induced ceramide response was initiated through the generation of singlet oxygen, and that it could be effectively prevented by means of singlet oxygen quenchers including vitamin E (Grether-Beck et al, 2000). In addition, in independent studies we have recently observed that preincubation of keratinocytes with the osmolyte ectoin (Bünger et al, 2001) or the lipid cholesterol prevented UVA radiation-induced gene expression through inhibition of the early ceramide response in UVA-irradiated keratinocytes. Accordingly, in UVA-irradiated keratinocytes, pretreatment of cells with vitamin E,
ectoin, and cholesterol prevented the early, non-enzymatic response (Table I). In all three cases, abrogation of the early ceramide response was associated with the complete inhibition of the late ceramide response. This was not because of inhibition of ceramide de novo synthesis, because vitamin E, ectoin, and cholesterol did not inhibit ceramide synthesis in human keratinocytes induced after stimulation of cells with cell-permeable C2 ceramide (data are only shown for C2−, but were identical for C6-ceramide stimulation) (Table I).

Table I. Effects of vitamin E, ectoin, and cholesterol on ultraviolet A (UVA) radiation- and ceramide-induced ceramide content

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Time point (h)</th>
<th>Ceramide increase (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>UVA</td>
<td>1</td>
<td>3.0</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>Ectoin</td>
<td>1</td>
<td>0.9</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1</td>
<td>1.2</td>
</tr>
<tr>
<td>Vitamin E/UVA</td>
<td>1</td>
<td>1.3</td>
</tr>
<tr>
<td>Ectoin/UVA</td>
<td>1</td>
<td>1.1</td>
</tr>
<tr>
<td>Cholesterol/UVA</td>
<td>1</td>
<td>0.9</td>
</tr>
<tr>
<td>None</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>UVA</td>
<td>16</td>
<td>2.5</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>16</td>
<td>1.0</td>
</tr>
<tr>
<td>Ectoin</td>
<td>16</td>
<td>0.9</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>16</td>
<td>1.3</td>
</tr>
<tr>
<td>Vitamin E/UVA</td>
<td>16</td>
<td>1.3</td>
</tr>
<tr>
<td>Ectoin/UVA</td>
<td>16</td>
<td>0.7</td>
</tr>
<tr>
<td>Cholesterol/UVA</td>
<td>16</td>
<td>1.4</td>
</tr>
<tr>
<td>C2 ceramide</td>
<td>16</td>
<td>2.0</td>
</tr>
<tr>
<td>Vitamin E/C2 ceramide</td>
<td>16</td>
<td>2.3</td>
</tr>
<tr>
<td>Ectoin/C2 ceramide</td>
<td>16</td>
<td>2.6</td>
</tr>
<tr>
<td>Cholesterol/C2 ceramide</td>
<td>16</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Keratinocytes were either preincubated for 1 h with vitamin E (25 μM), ectoin (1 mM), or cholesterol (30 μM) or left untreated prior to stimulation with UVA (30 J per cm²) or with C2 ceramide (10 μM). Cells were harvested after 1 or 16 h. The ceramide content was analyzed as described in Materials and methods. The ceramide content is given as fold increase compared with untreated time matched controls. Results are from one of four essentially identical experiments.

Discussion

In this study, we demonstrate that UVA radiation causes a biphasic ceramide response in human keratinocytes with an early maximum between 0.5 and 2 h and a late response that can be detected 16 h after irradiation and that lasts up to 48 h. In previous studies, the early ceramide response was shown to be mediated by a non-enzymatic mechanism, because UVA radiation-induced ceramide content was not associated with an increased activity of neutral or acid sphingomyelinase in irradiated keratinocytes and because it was also observed in a protein-free liposomal system (Grether-Beck et al, 2000). This is consistent with the present observation that neither inhibitors of neutral and acid sphingomyelinase (Fig 2), nor inhibitors of serine palmitoyltransferase (Fig 3A, B) affected UVA radiation-induced ceramide content at these early time points. In the same experiments, however, inhibition of serine palmitoyltransferase activity by means of l-cycloserine or myriocin significantly decreased the late ceramide response (Fig 3A, B). Also, at these later time points, UVA radiation-induced
ceramide content was associated with an increase in serine palmitoyltransferase activity (Fig 3C). In addition, the late phase of ceramide content was not because of UVA-induced degradation of glycosphingolipids (Fig 2D, E).

Taken together, these data demonstrate that UVA radiation-induced ceramide content is initiated by a non-enzymatic mechanism, which is followed by ceramide de novo synthesis as a result of an increased activation of serine palmitoyltransferase. Thus, the ceramide response induced by UVA radiation involves both non-enzymatic and enzymatic mechanisms.

UVA radiation-induced ceramide content and ICAM-1 gene expression show essentially identical biphasic kinetics (Fig 1). Also, inhibition of the late ceramide response abolished the late, but not the early, phase of increased gene transcription (Fig 3), whereas inhibition of the first ceramide peak prevented the first increase in gene expression (Grether-Beck et al, 2000, Table I). We therefore propose that the capacity of UVA radiation to elicit a biphasic ceramide response in human keratinocytes is responsible for the biphasic nature of UVA radiation-induced gene expression and transcription factor activation. The in vivo relevance of UVA-induced ceramide synthesis is not known. In this regard, it is worth mentioning that previous studies by Lehmann et al (1991) and Wefers et al (1991) indicate, that UVB, but also UVA radiation improve skin barrier function. In addition, animal studies have revealed that UVB radiation is capable of inducing SPT expression and ceramide synthesis. The kinetics of the UVB effects, which are monophasic (Holleran et al, 1997), differ from the biphasic pattern observed in our study for UVA. This further emphasizes the specificity of the biphasic activation pattern for UVA radiation.

Activation of serine palmitoyltransferase and a concomitant increase in ceramide content was not only observed in UVA-irradiated keratinocytes but also in cells that had been left unirradiated but were stimulated with cell-permeable C2 or C6 ceramide. Stimulation of keratinocytes with exogenous ceramide can thus be regarded as a substitute for the early ceramide response in UVA-irradiated cells, indicating that the early and the late ceramide response are not independent from each other, but are possibly connected in an autocrine manner. In keeping with this hypothesis, we have observed that abrogation of the first ceramide peak through strategies that did not interfere with ceramide de novo synthesis always prevented the second ceramide response as well. Based on these observations, we would like to propose a model (Fig 6) in which ceramide that is formed upon UVA irradiation through a non-enzymatic mechanism induces—in an autocrine manner—the expression and activation of serine palmitoyltransferase and thereby a second increase in ceramide production. This model would also help to explain the long-lasting nature of the late ceramide response, because newly synthesized ceramide could lead to a perpetuation of serine palmitoyltransferase activity and ceramide synthesis. Accordingly, ceramide content (Fig 1B) and serine palmitoyltransferase expression (Fig 3D) were still increased above background levels 48 h after UVA irradiation. Lipid-mediated autocrine/paracrine signaling has previously been reported for sphingosine-1-phosphate (Maceyka et al, 2002). In these studies, platelet-derived growth factor-induced cell motility was found to be mediated via stimulation of sphingosine kinase and the subsequent production of intracellular sphingosine-1-phosphate that can act in an autocrine or paracrine manner and thereby may activate a family of cell surface sphingosine-1-phosphate specific G-protein-coupled receptors, which subsequently leads to an activation of prosurvival pathways (Liu et al, 2000; Hobson et al, 2001). To the best of our knowledge, this is a report that suggests that ceramide may be involved in an autocrine signaling loop as well.

Generation of endogenous ceramide in response to exogenously added, cell-permeable ceramide has previously been observed in the human lung carcinoma cell line A549 (Ogretmen et al, 2002). In this study, ceramide-induced ceramide content was found to be because of recycling of the C6-ceramide sphingosine backbone of via deacylation/ reacylation, and not to ceramide de novo synthesis. Contrary to these findings, we demonstrate in this study that, in non-transformed, primary human epidermal keratinocytes exogenously added ceramide can induce endogenous ceramide content through de novo synthesis via activation of serine palmitoyltransferase.

Serine palmitoyltransferase is a heterodimer consisting of the 53 kDa SPTLC1 subunit and of the 63 kDa SPTLC2 subunit, both of which are bound to the endoplasmatic reticulum as integral membrane proteins (Hanada, 2003). In mammalian cells, SPTLC2 alone was found to be sufficient to achieve serine palmitoyltransferase activity, and it has therefore been proposed that SPTLC2 encodes for the catalytic activity of this enzyme (Weiss and Stoffel, 1997). Accordingly, in this study, UVA radiation/ceramide stimulation increased mRNA expression of the SPTLC2 subunit 6- and 4-fold, respectively. Upregulation of the SPTLC1 subunit was found to be stronger after UVA radiation (up to 14-fold) than after ceramide stimulation (maximal 2-fold). In addition,
SPTLC2 mRNA expression was found to be more sustained in UVA-irradiated compared with ceramide-stimulated keratinocytes. These observations are in line with the concept that the SPTLC1 subunit may have stabilizing functions (Weiss and Stoffel, 1997). UVB-induced upregulation of SPTLC2 mRNA and protein as well as enzyme activity has been observed 48 and 72 h post-application of 230 J per m² (Farrell et al., 1998). Moreover, studies using an apoptogenic dose of UVB (600 J per m²) resulted in a 3.2-fold increase in ceramide content 8 h post-treatment because of an increased ceramide synthase activity (Uchida et al., 2003). Although this increase in ceramide content is comparable with the second ceramide peak detected upon UVA exposure, it is important to note that in this study, UVA-induced ceramide generation occurred without a reduction in cell viability or an increase in cell death (Grether-Beck et al., 1996, 2000; Warskulat et al., 2004). Although the action spectrum for the biphasic ceramide generation has not been determined, it is reasonable to speculate that the observed ceramide formation is because of UVA1 (340–400 nm) radiation, since the light source in the study used emits UVA in the range of 340–400 nm with a peak at 360 nm.

Serine palmitoyltransferase, which catalyzes the initial and committed step in sphingolipid synthesis, is thought to be a housekeeping enzyme that is supposedly regulated, but only relatively few findings demonstrate its regulation and its regulatory mechanisms (Hanada, 2003). Until the regulatory genetic elements within the promoters or introns of SPTLC1 and SPTLC2, respectively, are available an induction by activation of transcription factors such as AP-2 remains speculative. At present, it also cannot be excluded that alterations in mRNA half-life contribute to increased SPTLC1 and SPTLC2 mRNA steady-state levels (Raghavan et al., 2002).

In conclusion, we demonstrate here that the previously observed biphasic nature of the UVA response in human keratinocytes (Grether-Beck et al., 1996; Morita et al., 1997; Gille et al., 2000) results from the sequential triggering of non-enzymatic and enzymatic pathways that lead to the biphasic formation of the second messenger ceramide. We also show that ceramide can act in an autocrine manner to stimulate the de novo synthesis of ceramide.

Materials and Methods

Materials

- 3,4-DCIC, chloroquine, l-cycloserine, myriocin, and vitamin E succinate were purchased from Sigma-Aldrich (Munich, Germany).
- Interleukine-1α was obtained from R&D Systems (Wiesbaden-Norderstadt, Germany).
- dl-three-PDMP (dl-three-phenyl-2-decanoylamino-3-morpholino-1-propanol HCL) was obtained from Matreya (Pleasant Gap, Pennsylvania). Soyphostatin was a kind gift from Sancyo (Tokyo, Japan).
- Serine palmitoyltransferase activity Enzyme activity was assessed in microsomal preparations of keratinocytes prepared as previously described in detail (Liu et al., 1994; Grether-Beck et al., 2000). In brief, cells grown on 10 cm dishes were pelleted, washed once with cold PBS, and resuspended in 300 μL of homogenization buffer free conditions for up to five passages (Grether-Beck et al., 1996, 2000, 2003).
- UV radiation For UV radiation, medium was replaced by phosphate-buffered saline, lids were removed, and cells were exposed to a dose of 30 J per cm² UVA1 using a UVASUN 24.000 system (Sellas GmbH, Dr Sellmeier Gevelsberg, Germany). The UVA1 output was determined with a UVAMETER type II (Waldmann, Villingen-Schwenningen, Germany) and was found to be approximately 150 mW per cm² UVA1 at a tube to target distance of 30 cm (Grether-Beck et al., 1996, 2000).

Lipid extraction

Cells were harvested at the indicated time points by scraping the cells off the plate on ice. Pellets were washed and sonicated. Quantification of lipids was carried out using 500 μg protein (Bradford, 1976) for Folch extraction (Folch et al., 1957). Analysis of ceramides included a mild alkaline hydrolysis. The lower phase of Folch extraction was evaporated under nitrogen. The lipids were dissolved in chloroform/methanol (2:1, v/v). As more complex glycolipids and the gangliosides are soluble in water, these substances are lost during washing included in the Folch procedure. Therefore, the aqueous phase of the Folch extraction was collected, desalted by column chromatography using Oasis HLB extraction cartridges (Waters GmbH, Eschborn, Germany) according to the instructions of the manufacturer, and eluted with chloroform methanol (2:1, v/v).

High-performance thin layer chromatography (HPTLC)

Samples and standards were separated on silica gel HPTLC plates (20 × 10 cm) Merck 60 F 254 s (Merck KGaA, Darmstadt, Germany), prewashed for 60 min in 2-propanol, and dried for 30 min at 120 °C.

Samples and standards were applied to the TLC plates using a CAMAG Linomat IV (CAMAG, Berlin, Germany) in bands of 0.8 cm by a spray-on technique (10 μL per sample) in which nitrogen carrier gas atomizes the sample from a syringe onto the plate, which is moving back and forth under the atomizer.

For determination of ceramides, samples were separated using an Automated Multiple Development (AMD) procedure on an AMD2 device (CAMAG). This procedure consisted of seven repeated developments of the chromatogram using a stepwise elution gradient with methanol, dichloromethane, and n-hexane (methanol/dichloromethane/n-hexane: 100:0/0; 90/10/0; 9/91/0; 8/92/0; 3/97/0; 2/98/0; 0/0/100) based on the CAMAG application protocol A 52.3 for separation of phospholipids (Jork et al., 1989) on a CAMAG AMD2 device as described earlier (Grether-Beck et al., 2000).

For determination of glycosphingolipids, e.g., GM3 a 19-step gradient based on methanol, dichloromethane, and n-hexane (methanol/dichloromethane/n-hexane: 100/0/0; 80/20/0; 78/22/0; 74/24/0; 69/31/0; 62/38/0; 54/46/0; 47/53/0; 40/60/0; 33/67/0; 25/75/0; 18/82/0; 11/89/0; 7/93/0; 4/96/0; 2/98/0; 1/99/0; 0/100; 0/0/100) was used.

Visualization of separated bands was carried out by post-chromatic derivatization after dipping in a manganese chloride solution according to Grether-Beck et al. (2000). Therefore, 0.4 g MnCl₂ 4H₂O was solved in 60 mL H₂O under heating, 60 mL methanol, and 4 mL concentrated H₂SO₄ were added, and the solution was cooled down to room temperature. Dervatization was carried out in an automated dipping device (CAMAG) at a vertical speed of 50 mm per s and an immersion time of 1 s. After heating, the plate for 10 min at 120 °C in a temperature-controlled oven, the plate was dried and scanned using a CAMAG TLC Scanner II and CATS software. Quantification was carried out by absorption at 550 nm with a plot of peak area versus weight spotted for a series of standards using a second-order polynomial calibration.

Serine palmitoyltransferase activity

Enzyme activity was assessed in microsomal preparations of keratinocytes prepared as previously described in detail (Liu et al., 1994; Grether-Beck et al., 2000). In brief, cells grown on 10 cm dishes were pelleted, washed once with cold PBS, and resuspended in 300 μL of homogenization...
buffer (25 mM HEPES pH 7.4) followed by the addition of proteinase
inhibitors (Complete, Boehringer Mannheim, Germany). Cells were disrupted using a tissue homogenizer (POTTER, B. Braun
Biotech Int., Goettingen, Germany). Lysates were centrifuged at 800 × g for 5 min. The post-nuclear supernatant was centrifuged at 250,000 × g for 30 min. The microsomal membrane pellet was resuspended in 1.0 mL of homogenization buffer. Membranes were freshly prepared. Serine palmitoyltransferase activity was determined based on the procedure described elsewhere (Williams et al., 1984; Dickson et al., 2000) in aliquots of 50 μg microsomal protein (determined according to Bradford, 1976). The determination of SPT activity was based on the conversion of the water-soluble substrate l-[3-14C] serine to the chloroform-soluble product 3-ketosphinganine. In brief, the final reaction volume of 0.1 mL contained 100 mM HEPES (pH 8.3), 5 mM DTT, 2.5 mM EDTA (pH 7), 50 μM pyridoxal-phosphate, 200 μM palmitoyl-CoA, 1 mM serine l-[3-14C] serine (specific activity 2.11 GBq per mmol), and 50 μg microsomal protein. The reaction was initiated by the addition of palmitoyl-CoA to avoid depletion of this substrate by fatty acyl-CoA hydrolases and incubated for 10 min at 37 °C on a shaker. The reaction was terminated by addition of 1.5 mL chloroform–methanol (1:2, v/v), and the lipophilic product 3-ketosphinganine was isolated by phase separation and quantified by a standard scintillation procedure (Beckman, Beckman Coulter GmbH, Krefeld, Germany) using Quicksafe N as scintillation fluid (Zinsser Analytik GmbH, Frankfurt, Germany). Enzyme activity was expressed as picomoles of 3-ketosphinganine formed per minute per milligram of protein. Background correction to exclude impurities of serine was performed the same way, except that palmitoyl-CoA was omitted. Results are given as fold induction in comparison with unstimulated controls.

**Real-time RT-PCR**

Total RNA was isolated using RNeasy Total RNA Kits (Qiagen, Hilden, Germany). Two-step real-time RT-PCR was performed to detect gene expression in human keratinocytes. SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Karlsruhe, Germany) was used for the reverse transcription step with random hexamers. For each gene, a specific PCR primer pair was designed by Primer Express 2.0 software (Applied Bio-systems, Darmstadt, Germany) based on the cDNA sequence published as indicated: β-actin 5′-CCTGGACCCAGCACAACT-3′/5′-GGCGGATACACCGGATCTA-3′, (Vandekerckhove and Weber, 1978; Ponte et al., 1984); ICAM-1 5′-CCTGGCACCCAGCACAACT-3′/5′-GGCGGATACACCGGATCTA-3′ (Staunton et al., 1988); SPTLC1 5′-GGCGGTCTATGGAAGAAGG-3′/5′-TGTCTCACGCAGAAC-3′, SPTLC2 5′-AGCCGGCAAGATCCTTGAAG-3′/5′-CTGTCGAGCTTGGATTTACC-3′ (Weiss and Stoffel, 1997).

The PCR reactions were carried out on an Option 1 (MJ Research, Waltham, Massachusetts) with SYBR Green PCR Master Mix (Applied Biosystems). Each RT-PCR experiment was performed twice.

**Flow cytometry**

ICAM-1 cell surface expression was assessed by a one-step staining procedure and subsequent flow cytometry (FACS analysis). Cells were detached from the plates by a brief trypsin treatment. After washing, cells were stained with an R-phycocerythrin-coupled murine anti-human ICAM-1 monoclonal antibody CD54 (Leu-54) (Becton Dickinson PharMingen, Heidelberg, Germany). Control stainings were performed with an isotype-matched control monoclonal antibody (mouse IgG1). Stained cells were analyzed in a FACSscan II flow cytometer (BD PharMingen, Heidelberg, Germany). Data are given as mean fluorescence intensity (MFI) based on unstimulated controls that were set equal to one.

**Gel electrophoresis mobility shift assay**

Nuclear extracts were prepared according to the Dignam protocol as described (Dignam et al., 1983). The AP-2 consensus oligonucleotide (top strand, 5’-ACCGCTTCGGCCCGGACCCCT-3’) was deduced from the ICAM-1 promoter (Stade et al., 1990). Specificity controls were performed as before (Grether-Beck et al., 1996).


