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Ceramides stimulate caspase-14 expression in human keratinocytes

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Abstract

Caspase-14 is an enzyme that is expressed predominantly in cornifying epithelia and catalyses the degradation of profilaggrin. Additionally, caspase-14 plays an important role in the terminal differentiation of keratinocytes. However, how caspase-14 expression is regulated remains largely unknown. Here we demonstrate that ceramides (C_2-Cer and C_6-Cer), but not other sphingolipids (C_8-glucosylceramides, sphinganine, sphingosine-1-phosphate or ceramide-1-phosphate), increase caspase-14 expression (mRNA and protein) in cultured human keratinocytes in a dose- and time-dependent manner. Inhibitors of glucosylceramide synthase and ceramidase increase endogenous ceramide levels and also increase caspase-14 expression, indicating an important regulatory role for ceramides and suggesting that the conversion of ceramides to other metabolites is not required. The increase in caspase-14 expression induced by ceramides is first seen at 16 h and requires new protein synthesis, suggesting that the ceramide-induced increase is likely an indirect effect. Furthermore, ceramides increase caspase-14 gene expression primarily by increasing transcription. Blocking *de novo* synthesis of ceramides does not affect caspase-14 expression, suggesting that basal expression is not dependent on ceramide levels. These studies show that ceramides, an important structural lipid, stimulate caspase-14 expression providing a mechanism for

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Author contribution

Jiang YJ, Kim P and Uchida Y performed the experiments and analysed data; Jiang YJ and Feingold KR designed the experiments and prepared the manuscript; Feingold KR, Elias PM, Bikle DD and Grunfeld C helped in the interpretation of the data and in designing experiments. Additionally, all authors contributed to critical discussion of the data presented in this manuscript.

Conflict of interests

The authors have declared no conflicting interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Oligonucleotide primer sequences used in this study.

coordinately regulating the formation of lipid lamellar membranes with the formation of corneocytes.

Keywords

ceramidase inhibitors; ceramides; glucosyltransferase inhibitors; PPAR/LXR

Introduction

The stratum corneum (SC) consists of terminally differentiated, anucleate keratinocytes (corneocytes) surrounded by lipid-enriched lamellar membranes composed of three major lipids: ceramides, cholesterol and free fatty acids (1,2). Alterations in stratum corneum lipid profiles have been linked with both human and a canine model of atopic eczema (3–5). To form the SC, keratinocytes undergo a complex pathway of differentiation that results in keratinocyte cornification and the formation and secretion of lamellar bodies, which delivers the lipids to the extracellular space of the SC (1,2,6,7). Thus, the SC is the end product of two interdependent pathways: those that lead to the formation of corneocytes (‘the bricks’) and those that lead to the deposition of extracellular lipid membranes (‘the mortar’). The mechanisms that coordinately regulate the formation of these two interdependent structures required for normal SC structure and function need to be fully elucidated.

Recent studies have demonstrated that the lipids required for the formation of the mortar, in addition to playing structural roles, can also act as signalling molecules (8,9). Free fatty acids activate PPARs, and PPAR-α, PPAR-δ and PPAR-γ activators stimulate keratinocyte differentiation (8,9). Similarly, cholesterol can be converted into oxysterols that activate LXR, which also stimulates keratinocyte differentiation (8,9). In addition, by mechanisms that remain to be fully elucidated, ceramides similarly stimulate keratinocyte differentiation (10–12). Thus, cornified envelope formation and the expression of involucrin, loricrin and other differentiation-related proteins are increased by fatty acids, cholesterol and ceramides.

Caspase-14 is a member of the cysteinyl aspartate-specific proteinases subfamily, which is expressed predominantly in cornifying epithelia (13). In the epidermis, caspase-14 is expressed in differentiated keratinocytes (14,15). Caspase-14 has no apoptotic or inflammatory function but is responsible for proteolytic processing of profilaggrin, the precursor of filaggrin (13,16,17). The failure to process profilaggrin results in a decrease in the formation of free hygroscopic amino acids (‘natural moisturization factors’) that play an important role in SC hydration (13,16,17). The importance of caspase-14 in terminal keratinocyte differentiation is further demonstrated in caspase-14-deficient mice which demonstrate shinier skin, reduced epidermal hydration and increased transepidermal water loss (13,16,17). Studies in our laboratory have shown that the terminal differentiation induced by acute barrier disruption does not occur in caspase-14-deficient mice, indicating that caspase-14 plays a key role in terminal keratinocyte differentiation (18).

The factors that regulate caspase-14 expression are poorly understood. In keratinocyte cultures, caspase-14 expression is increased by forcing the cells to differentiate by growing the cells postconfluently or in suspension (15,19,20). Vitamin D, which induces

keratinocytes to differentiate, has also been shown to stimulate caspase-14 expression (21). In contrast, adding calcium at high concentrations to the medium, which also induces keratinocytes to differentiate, does not affect caspase-14 expression (19,20). The green tea phenol, epigallocatechin-3-gallate, which is a potent activator of AP-1, increases caspase-14 expression but other potent activators of AP-1, such as TNF and phorbol esters, do not induce caspase-14 expression (21–23). Retinoids, which inhibit keratinocyte differentiation, decrease caspase-14 expression (21,24). Additionally, T (H) 2-associated cytokines reduce caspase-14 mRNA levels (22). Like vitamin D, ceramides, as well as PPAR and LXR activators, readily induce keratinocyte differentiation (5–9). Hence, the aim of the present study was to determine whether ceramides or PPAR/LXR activators also regulate caspase-14 expression in cultured human keratinocytes (CHKs).

Materials and methods

Materials

Fumonisin B1 (FB1) and *β*-Chloro-D-alanine hydrochloride (*β*CA) were purchased from Sigma (St. Louis, MO, USA). Myriocin was from Calbiochem (San Diego, CA, USA). D-MAPP, L-MAPP and sphinganine were from EMD Biosciences, Inc (La Jolla, CA, USA). Synthetic sphingolipids, *N*-Hexanoyl-D-erythro-sphingosine (C_6-Cer) and *N*-Acetyl-D-erythro-sphingosine (C_2-Cer), D-thero-P4 (P4), *N*-Hexanoyl-sphingosylphosphorycholine (C_6-SM) and D-threo-1-Phenyl-2-hexadecanoylamino-3-morpholino-1-propanol.HCl (D-PPMP) were purchased from Matreya Inc. (Pleasant Gap, PA, USA). C_8-*β*-D-Glucosyl Ceramide (C_8-GlcCer) was purchased from Avanti Polar lipids (Alabaster, AL, USA). Molecular-grade chemicals, such as TRI Reagent, were obtained from either Sigma or Fisher Scientific (Fairlawn, NJ, USA). The iScript™cDNA Synthesis Kit for first-strand cDNA synthesis was purchased from BIO-RAD Laboratories (Hercules, CA, USA). All reagents and supplies for real-time PCR were purchased from Applied Biosystems (Foster City, CA, USA). All other reagents for Western blot, including NuPAGE® Novex Pre-cast gradient gels (3–8% Tris-Acetate), buffers, protein standards and detection kits were purchased from Invitrogen (Carlsbad, CA, USA). 22(R)-OH-cholesterol (22R) and WY14643 (WY) were purchased from Sigma. Ciglitazone (Cig) and troglitazone were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Synthetic PPAR-δ activator GW610742X (GW) was a generous gift from Dr. Tim Willson (GlaxoSmithKline). 1a, 25-Dihydroxyvitamin D3 (VitD3) was purchased from BIOMOL International (Plymouth Meeting, PA, USA).

Keratinocyte culture

The second passage of human neonatal foreskin keratinocytes was seeded and maintained in 0.07 mM calcium chloride, serum-free 154CF with growth supplement (Cascade Biologies, Inc., Portland, OR, USA). Once the cells were attached, the culture was switched to medium containing 0.03 mM calcium chloride as described in (25).

Real-time PCR

Total RNA was isolated using TRI reagent, and first-strand cDNA for PCR was synthesized using an iScript™cDNA Synthesis Kit, following the manufacturer's protocol. Relative

mRNA levels of caspase-14 and cyclophilin (internal control) were determined as previously described. The primers used are listed in Table S1. The expression levels of caspase-14 were normalized against cyclophilin using the comparative C_T method and expressed as percentage of control, with the control as 100%.

Construction of caspase-14 promoter luciferase reporter genes

The 2500-bp human caspase-14 promoter was obtained by amplifying human Hep3B cell genomic DNA with primers (Table S1). This 2500-bp fragment was then subcloned into pGL3.1 (Promega, Madison, WI). The additional 5′-terminal deletion variants (1325, 405 and 165 bp) were generated by subcloning into the multicloning site of pGL3 basic with similar approaches. The final constructs were sequenced to ascertain the orientation and fidelity of promoter sequence.

Transient transfection and reporter gene assay

Cultured human keratinocytes cells were seeded on six-well plates at a density of 2×10^5/well, and 1 μg promoter-driven luciferase construct and 0.2 μg RSV-β-gal (a reporter plasmid) with Fugene 6 reagent (Roche, South San Francisco, CA, USA) were added in the media following manufacturer's instruction. After incubation for 5–12 h at 37°C, transfection medium was removed and the cells were treated with either vehicle (ethanol), ceramide or D-MAPP for 24 h. At the end of the treatment, cells were harvested for determining the luciferase activity (Promega). β-Galactosidase activity was used to normalize transfection efficiencies.

Western blot analysis

Western blots were carried out according to the manufacturer's protocol. Briefly, for caspase-14, 50–60 μg protein prepared from keratinocytes was fractionated on precast gels (4–20%) and transferred to PVDF membranes, overnight at 4°C. The proteins on the membrane were subsequently probed with polyclonal primary antibodies against human caspase-14 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then visualized by secondary antibody using the Enhanced Chemiluminescence (ECL) Western Blotting Detection System Kit. Membranes were then exposed to CL-XPosure film.

Statistical analysis

All data are expressed as mean ± SEM. Comparison between two groups is undertaken using two-tail and unpaired *t*-test. Differences in values are considered significant if $P < 0.05$.

Results

PPAR and LXR activators do not alter caspase-14 expression

We initially determined the effect of PPAR and LXR activators on caspase-14 expression. Neither PPAR-α, PPAR-δ, PPAR-γ nor LXR activators induced caspase-14 expression (data not shown). However, 1α, 25-OH vitamin D3 stimulated while retinoids inhibited caspase-14 expression (data not shown), consistent with results previously reported by other groups (21,24).

Exogenous ceramides but not other sphingolipids stimulate caspase-14 mRNA

To determine whether sphingolipids regulate caspase-14 gene expression, we examined the effect of exogenously added sphingolipids, including C_6-Cer, C_2-Cer, C_8-GlcCer, sphinganine, sphingosine-1-phosphate (S1P) or ceramide-1-phosphate (C1P) on caspase-14 mRNA levels in CHKs (Fig. 1a). C_6-Cer, a synthetic ceramide which is cell permeable, increased caspase-14 mRNA levels ~5.2-fold. Similarly, C_2-Cer, another synthetic ceramide, also increased caspase-14 mRNA levels ~5.4-fold (Fig. 1a). In contrast, neither synthetic C_8-GlcCer, sphinganine, S1P, C1P nor DC_6 (D-erythro-Dihydroceramide), an inactive control for C_6-Cer or LC_6 (L-threo-ceramide), a non-active enantiomer of C_6-Cer, induces caspase-14 mRNA expression (Fig. 1a). These results indicate that ceramides but not other major metabolites of ceramide increase caspase-14 mRNA levels in human keratinocytes.

Ceramide stimulates caspase-14 expression in a time- and dose-dependent manner

C_6-Cer increases caspase-14 mRNA levels in a time-dependent fashion, with a large increase first seen at 16 h, and a further increase by 24 h (Fig. 1b). Further, C_6-Cer also increases caspase-14 mRNA levels in a dose-dependent manner, with a half maximal effect at ~5 μM (Fig. 1c). Higher doses ($\geq$ 12.5 μM) of C_6-Cer were toxic to the cells (data not shown). Similarly, C_2-Cer, another synthetic and cell-permeable ceramide, also increased caspase-14 mRNA in a similar dose- and time-dependent manner (data not shown). Interestingly, the stimulatory effect of C_6-Cer is specific for caspase-14, as C_6-Cer did not induce the expression of other caspases, such as caspase-3 or caspase-8 (data not shown). Finally, consistent with our mRNA data, an increase in caspase-14 protein mass (~2.7-fold) was evident following C_6-Cer treatment (Fig. 2a,b). Together, these results indicate that exogenous ceramide induces caspase-14 expression in human keratinocytes.

Glucosyltransferase inhibitors induce caspase-14 expression

To determine whether endogenous ceramides would also induce caspase-14 expression, we next examined caspase-14 mRNA levels after raising endogenous ceramide levels by inhibiting glucosylceramide synthase activity with three different inhibitors. Treatment of human keratinocytes with D-PPMP for 24 h increased total intracellular ceramide levels by 90% and decreased glucosylceramide levels by ~58% (26). As shown in Fig. 3a, the treatment of cells with D-PPMP increased caspase-14 mRNA levels. Additionally, we also treated cells with P4 or D-PDMP, other inhibitors of glucosylceramide synthase and observed similar increases in caspase-14 mRNA levels (data not shown). Moreover, co-treatment of keratinocytes with D-PPMP and C_6-Cer, which blocks the conversion of ceramides to glucosylceramides, did not affect the ceramide-induced increase in caspase-14 mRNA levels (Fig. 3a). These results indicate that the conversion of ceramide to glucosylceramide cannot be the basis for the C_6-Cer-induced increase in caspase-14 expression. Together, these data indicate that raising endogenous ceramide levels using glucosylceramide synthase inhibitors increases caspase-14 mRNA levels and that the conversion of ceramide to glucosylceramide is not required for the stimulation of caspase-14 expression.

Ceramidase inhibitors induce caspase-14 mRNA

In addition to serving as a substrate for the formation of complex sphingolipids, ceramides can be further hydrolysed into sphingo-sine, a reaction catalysed by a family of ceramidases. Four of the five known ceramidase isoforms are widely expressed in cutaneous and extracutaneous tissues, including alkaline and acidic ceramidases, which can be blocked by D-MAPP and B13, respectively (27). D-MAPP, an alkaline ceramidase inhibitor, has been shown to increase endogenous ceramide levels in CHK (26). Here, we demonstrated that D-MAPP increases caspase-14 mRNA levels (Fig. 3b). In contrast, L-MAPP, a non-biological enantiomer of D-MAPP that does not inhibit alkaline ceramidase activity, had no effect on caspase-14 expression (Fig. 3b). Of note, D-MAPP does not inhibit the ability of C_6-Cer to stimulate caspase-14 expression (Fig. 3b). B13, a compound that specifically inhibits acidic ceramidase activity, resulting in the intracellular accumulation of ceramide (28), also induces caspase-14 mRNA levels (Fig. 3c). Similar to D-MAPP, B13 does not inhibit the ability of C_6-Cer to stimulate caspase-14 expression. Together, these results indicate that increasing endogenous ceramide levels by inhibiting ceramidase activity leads to a stimulation of caspase-14 expression. Additionally, these results suggest that the metabolism of C_6-Cer via the ceramidase pathway is not required for C_6-Cer-induced increase in caspase-14 gene expression. As reported previously, C_6-Cer, C_2-Cer, D-PPMP, D-MAPP, B13 or C_6-Cer plus D-PPMP/D-MAPP treatment does not induce apoptosis or toxicity in keratinocytes under these culture conditions (26).

Blocking de novo synthesis of ceramide does not affect caspase-14 mRNA expression

Previous studies have shown that *de novo* ceramide synthesis is robust in epidermis/keratinocytes (2,29). As exogenously added ceramide induces caspase-14 expression, we next examined whether blocking ceramide biosynthesis would decrease caspase-14 expression. Myriosin and βCA are inhibitors of serine-palmitoyl transfer-ase, the key enzyme for initiating *de novo* biosynthesis of ceramides (2,29). In previous studies, we have shown that treatment with either myriosin or βCA decreases intracellular ceramide levels in CHKs (30). However, treatment with neither myriocin nor βCA altered caspase-14 expression (data not shown). Furthermore, FB1, a mycotoxin that inhibits the activity of ceramide synthase (sphingosine *N*-acyltransferase), another key enzyme required for ceramide synthesis, and decreases intracellular ceramide levels in CHKs (30), also did not alter caspase-14 expression (data not shown). Thus, caspase-14 expression is not affected by decreasing ceramide levels by inhibiting *de novo* ceramide biosynthesis.

Ceramide trans-activates caspase-14 promoter luciferase reporter activity

The up-regulation of caspase-14 mRNA can result from increased transcription of its gene and/or decreased degradation (stability). To test these possibilities, we first examined the effect of C_6-Cer on caspase-14 mRNA stability. As shown in Fig. 4a, C_6-Cer treatment does not significantly increase caspase-14 mRNA stability in CHKs (half-life ~9.7 h) compared with vehicle control (~7.4 h). In addition, the C_6-Cer-induced increase in caspase-14 mRNA requires new protein synthesis, as inhibition of protein synthesis with cycloheximide (CHX) completely blocks the C_6-Cer-induced increase in caspase-14 mRNA (Fig. 4b). These results suggest (i) C_6-Cer stimulates caspase-14 gene transcription (rather than increasing its

stability) and (ii) C_6-Cer-induced increase in caspase-14 mRNA level requires new protein biosyn-thesis.

Next, we determined whether the increase in caspase-14 mRNA levels induced by exogenously added or endogenously produced ceramide was due to an increase in transcription by generating human caspase-14 promoter constructs (2500, 1325, 405 and 165 bp) linked with a luciferase reporter gene. Following the transient transfection of CHKs with these constructs, luciferase activity was determined, and our results demonstrate that the luciferase activities of these caspase-14 promoter constructs were significantly increased by either C_6-Cer or D-MAPP treatment (Fig. 4c, d). These results confirm that the increase in caspase-14 mRNA levels by C_6-Cer or D-MAPP is primarily due to an increase in gene transcription.

Discussion

Caspase-14 is an important enzyme that plays a key role in filaggrin processing and in the terminal differentiation of keratinocytes into corneocytes (13,16–18). Here we demonstrate that the expression of caspase-14 is regulated by ceramides. In contrast, activators of PPARs and LXR do not alter caspase-14 expression. These results suggest that as keratinocytes differentiate and produce increasing quantities of lipids to form lamellar bodies that the increase in ceramides may signal the keratinocyte to simultaneously increase the expression of caspase-14, an enzyme that is required for the formation of a normal functional SC.

The present study demonstrated that exogenous, synthetic, cell-permeable ceramides, C_6-Cer and C_2-Cer, when incubated with human keratinocytes increase the mRNA and protein levels of caspase-14. The increase in caspase-14 mRNA levels is primarily due to increased transcription and not to alterations in RNA stability. In contrast, other sphingolipids, including sphinganine, sphingo-sine, glucosylceramide, S1P or ceramide-1-phosphate did not increase caspase-14 gene expression. Additionally, increasing endogenous ceramide levels also stimulated caspase-14 expression. Blocking the conversion of ceramide to glucosylceramide by glucosylceramide synthase inhibitors and blocking the hydrolysis of ceramides to sphingosine by ceramidase inhibitors increase cellular ceramide levels and concomitantly increase caspase-14 mRNA levels. Thus, both short-chain, exogenous added synthetic ceramides and long-chain, endogenously produced ceramides stimulate caspase-14 gene expression. However, blocking *de novo* ceramide synthesis with a number of different inhibitors did not decrease caspase-14 expression. This may simply indicate that the basal expression of caspase-14 is regulated by other factors and is not dependent on ceramide levels. Alternatively, it is possible that inhibiting *de novo* ceramide synthesis diminishes a cellular pool that does not regulate caspase-14 expression.

Our studies further suggest that ceramides per se and not metabolites derived from ceramides regulate caspase-14 gene expression. Blocking the conversion of ceramides into glucosylceramides by inhibiting glucosylceramide synthase activity or blocking the metabolism of ceramides into sphingosine and sphingosine-1-phosphate by inhibiting ceramidase activity does not decrease the ability of C_6-Cer to increase caspase-14 mRNA levels. Additionally, as noted previously, sphinganine, ceramide-1-phosphate and

glucosylceramide did not alter caspase-14 gene expression. Together, these results suggest that ceramides directly regulate caspase-14 expression. However, as the specific molecular pathway by which ceramides increase caspase-14 expression has not been fully elucidated, one cannot rule out the formation of an alternative ceramide metabolite accounting for the effect.

The ability of ceramides to regulate gene expression in keratinocytes has been well documented (10–12). In keratinocytes, C_6-Cer and C_2-Cer have been shown to promote cell differentiation and inhibit cell proliferation (10–12). Ceramides stimulate cornified envelope formation, enhance involucrin expression and increase transglutaminases activity. Additionally, ceramides also enhance glucosylceramide synthase and acid sphingomyelinase expression (31–33). The mechanisms by which ceramides regulate keratinocyte gene expression are not well understood. Ceramides increase AP-1 activity, which could account for the increase in expression of many of the genes required for keratinocyte differentiation as many of these genes contain an AP-1 response element in their promoters (6,34,35). Recent studies by our laboratory have shown that ceramides stimulate the expression of ABCA12 and that this increase is mediated by ceramides increasing the expression of PPARδ (26).

The mechanism by which ceramides increase caspase-14 expression is unknown. Of note, the time course for the activation of caspase-14 expression requires a considerable period of time, and the increase in expression can be blocked by inhibiting protein synthesis. This suggests that the increase in caspase-14 expression induced by ceramides is not a direct effect but rather an indirect effect that likely involves multiple steps. The mechanism is obviously not the same as that for ABCA12 as PPARδ siRNA knockdown did not inhibit the ability of ceramides to stimulate caspase-14 expression (data not shown). Detailed studies of the caspase-14 promoter revealed that only a small segment (165 bp) was required for inducing activity. Within this portion of the caspase-14 promoter, there are two putative AP-2 sites and an NF-κB site. However, simultaneous mutation of both AP-2 sites did not inhibit the ability of ceramides to increase caspase-14 expression (data not shown). Similarly, mutation of the NF-κB site also did not block the increase in caspase-14 expression (data not shown). Thus, at this time, the precise mechanism and pathway by which ceramides increase caspase-14 expression is unknown. It is possible that the well-described ability of ceramides to increase AP-1 activity is involved, as shown by studies from other group that some (green tea phenol (–)-epigallocatechin-3-gallate (EGCG)) but not all compounds (TNFα and TPA) that increase AP-1 activity can enhance caspase-14 expression (21–23).

In summary, our results show that ceramides, an important lipid component required for the formation of lamellar membranes in the SC, stimulate caspase-14 expression providing a mechanism for coordinately regulating the formation of the lipid lamellar membranes with the formation of corneocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

βCAβ	Chloro-L-alanine hydrochloride
FB1	fumonisin B1
Cer-$C_{2:0}N$	Acetyl-D-*erythro*-sphingosine
Cer-$C_{6:0}N$	Hexanoyl-D-*erythro*-sphingosine
DC_6	Cer*N*-Hexanoyl-D-*erythro*-dihydrosphingosine
C_8	GlcCerβ-glucosyl-*N*-octanoyl-sphingosine
C1P*N*	Octanoyl-D-*erythro*-1-phosphate
C_6	SM*N*-Hexanoyl-sphingosylphosphorycholine
haCER1	human alkaline ceramidase 1
D-$MAPP_D$	*erythro*-2-tetradecanoylamino-1-phenyl-1-propanol
L-C_6	Cer*N*-Hexanoyl-L-*erythro*-sphingosine
L-$MAPP_L$	*erythro*-2-tetradecanoylamino-1-phenyl-1-propanol
$P4_D$	*thero*-1-Phenyl-2-palmitoyl-3-pyrrolidinopropanol
$PDMP_{D,\ L}$	threo-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol.HCl
D-$PPMP_D$	*threo*-1-Phenyl-2-hexadecanoylamino-3-morpholino-1-propanol.HCl
LC_6	Cer*N*Hexanoyl-L-*erythro*-sphingosine; Sphingasphinganine

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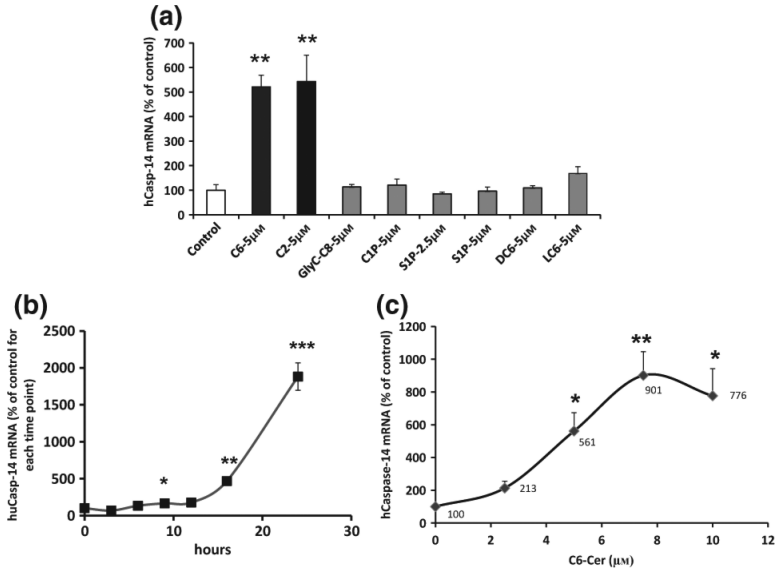

Figure 1.

Exogenous ceramide stimulates caspase-14 expression. Primary cultured human keratinocytes (CHKs) were incubated in the presence or absence of C_6-Cer, C_2-Cer, C_8-glucosylceramide (C_8-GlcC), sphinganine (Sphinga), sphingosine-1-phosphate (S1P), ceramide-1-phosphate (C1P), DC_6 (inactive control) or LC_6 (a non-active inantiomer) for 16 h (a). CHKs were incubated with C_6-Cer (5 μM) for various periods of time (0, 3, 6, 9, 12, 16, 24 h) (b) or cells were incubated with various doses of C_6-Cer (0–10 μM) for 16 h (c). Caspase-14 and cyclophilin mRNA levels were then measured as described in Materials and methods. Data are expressed as percentage of control (100%) and presented as mean ± SEM (n = 3–5). Similar results were obtained when the experiments were repeated with a different batch of cells. *P < 0.05; **P < 0.01; ***P < 0.001.

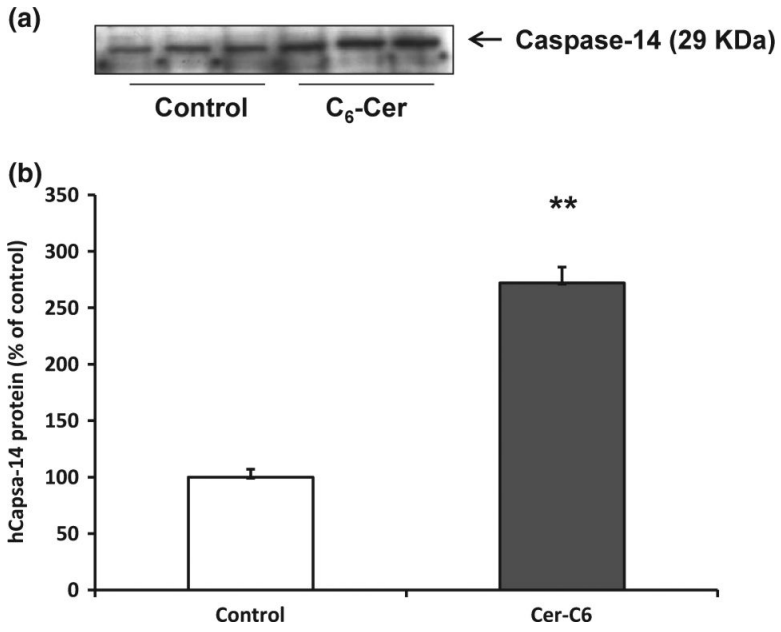

Figure 2.

Ceramide increases caspase-14 protein levels. CHKs were incubated with C_6-Cer (5 μM) for 24 h, and caspase-14 (a) and b-actin protein levels were measured by Western blot. The densitometry ratio of caspase-14 over b-actin was calculated and expressed as percentage of vehicle control (100%) and presented as mean ± SEM (n = 3) (b). Similar results were obtained when the experiment was repeated with a different batch of cells. $^{**}P < 0.01$. CHKs, cultured human keratinocytes.

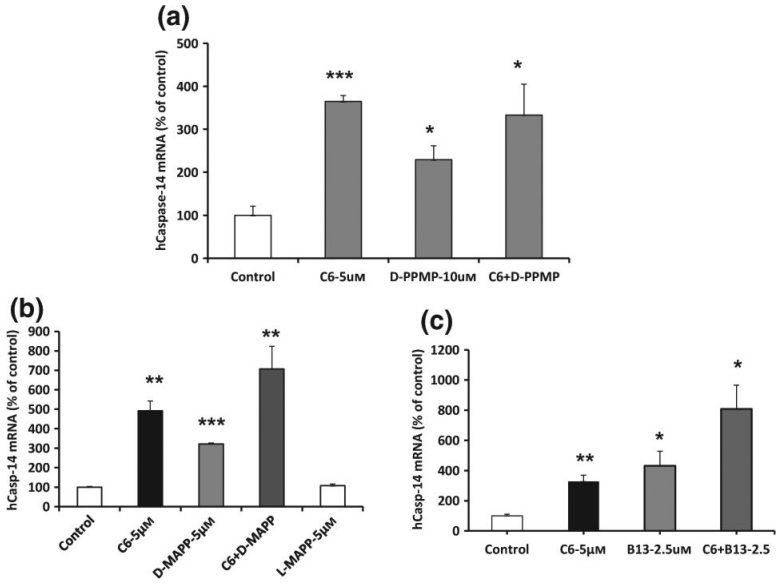

Figure 3.

Glucosyltransferase and ceramidase inhibitors increase caspase-14 mRNA expression. Cultured human keratinocytes (CHKs) were incubated with vehicle control, D-PPMP (10 μM), C_6-Cer (5 μM) (as positive control) or D-PPMP plus C_6-Cer for 16 h (a). CHKs were incubated with vehicle control, C_6-Cer (5 μM), D-MAPP (5 μM), D-MAPP plus C_6-Cer or L-MAPP for 16 h (b). Cells were incubated with vehicle control, C_6-Cer (5 μM), B13 (5 μM) or B13 plus C_6-Cer for 16 h (c). Caspase-14 mRNA levels were measured as described in the Materials and methods. Data are expressed as percentage of control (100%) and presented as mean ± SEM (n = 3–4). Experiments were repeated at least once with similar results. *P < 0.05; **P < 0.01; ***P < 0.001.

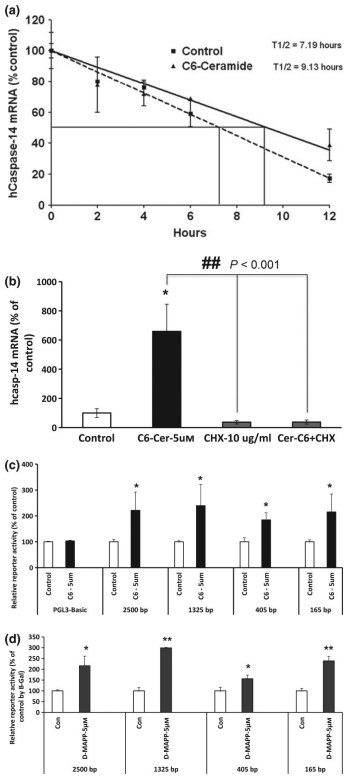

Figure 4.

C_6-Cer increases caspase-14 mRNA levels by increasing transcription. (a) Cultured human keratinocyte (CHK) cells were incubated with either C_6-Cer (5 μM) or vehicle for 24 h and subsequently challenged with 2 μg/ml of actinomycin D for the indicated periods of time (0, 2, 4, 6, 12 h). Caspase-14 mRNA levels were determined by RT-qPCR. Data are expressed as percentage of 0 h and presented as mean ± SEM (n = 3). (b) Cells were incubated with vehicle, C_6-Cer (5 μM) alone, 10 μg/ml cycloheximide (CHX) alone or C_6-Cer plus CHX in low calcium media for 24 h. Caspase-14 mRNA levels were determined by RT-qPCR, and data are expressed as percentage of control (100%) and presented as mean ± SEM (n = 3). CHKs were plated and cultured on six-well plates and luciferase reporter plasmids containing various human caspase-14 promoters were co-transfected with a reporter plasmid into cells, followed by incubation with either vehicle, C_6-Cer (5 μM) (c) or D-MAPP (5 μM) (d) for additional 24 h in low calcium medium. Luciferase activity was determined relative to beta-galactosidase. Data are presented as mean ± SEM (n = 3). Experiments were repeated at least twice with similar results. *P < 0.05; **P < 0.01 (comparing with vehicle control); ##P < 0.01 (comparing with C_6-Cer).