Sphingoid Base-Upregulated Caspase-14 Expression Involves MAPK

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Received November 18, 2017; accepted February 3, 2018

Sphingolipids are putative intracellular signal mediators in cell differentiation, growth inhibition, and apoptosis. Especially, sphingoid base-backbones of sphingolipids (sphingosine, sphinganine, and phytosphingosine) and their metabolites N-acyl-sphingoid bases (ceramides) are highly bioactive. In skin, one of the caspases, caspase-14, is expressed predominantly in cornifying epithelia, and caspase-14 plays an important role in keratinocyte differentiation. As ceramides were surrounding lipids in the keratinocytes and ceramides stimulate keratinocyte differentiation, we therefore examined the upregulation of caspase-14 by various sphingoid bases and ceramide. Sphingosine, sphinganine, phytosphingosine, and C2-ceramide treatment at the doses not damaging cells significantly increased caspase-14 mRNA and protein expression in dose-dependent manner on human keratinocyte HaCaT cells. These results indicated that sphingoid bases and ceramide upregulated caspase-14 mRNA to increase intracellular caspase-14 protein level. We next examined the caspase-14 upregulation mechanism by sphingoid bases. We used the most effective sphingoid base, phytosphingosine, and revealed that specific inhibitors of the mitogen-activated protein kinase, p38 and c-jun N-terminal protein kinase (JNK), blocked caspase-14 expression. This indicates that phytosphingosine upregulation of caspase-14 is involved of p38 and JNK activation. Moreover, phytosphingosine induced caspase-14 upregulation in vivo, suggesting that sphingoid bases were involved in keratinocyte differentiation by affecting caspase-14.

Key words sphingoid base; caspase-14; keratinocyte; mitogen-activated protein kinase

The stratum corneum of skin consists of terminally differentiated keratinocytes (corneocytes), surrounded by lipids such as ceramides (CER), to protect from invasion of outside foreign bodies as a barrier. Water retention is another major role of the skin and is governed by lipids as well as by the cornified envelope. Cornified envelopes, which replace the plasma membrane in corneocytes, consist so-called natural moisturizing factors (NMFs). NMFs are made of amino acids and retain the water. These NMFs are derived as a result of filaggrin degradation. Profilaggrin, which consists several filaggrin repeat units, are processed in the transitional layer between stratum granulosum and stratum corneum, and further degraded to NMFs. In the recent study, one of the proteases, caspase-14, is responsible for degrading profilaggrin. Unlike other caspases have a major role in proceeding apoptosis, caspase-14 has no apoptotic or inflammatory functions. Caspase-14 is expressed predominantly in cornifying epithelia, and caspase-14-deficient mice had a defect of the terminal filaggrin degradation and resulting in a substantial reduction of the amount of NMFs. This evidence indicates that caspase-14 plays an important role in keratinocyte differentiation. A few factors are reported to upregulate caspase-14. Vitamin D, which induces keratinocytes to differentiate, has also been shown to stimulate caspase-14 expression. The green tea phenol, epigallocatechin-3-gallate also had reported the caspase-14 stimulation activity. Moreover, CER, but not other sphingolipids, was also reported to upregulated caspase-14 expression.

Sphingomyelin and its metabolites, sphingolipids, are known to be involved in diverse types of signal transduction, including cell proliferation, differentiation, and apoptosis. Especially, sphingoid base-backbones of sphingolipids and their metabolites CER induces intracellular signal transduction by inhibiting a variety of pro-growth signal kinases, including Akt and protein kinase C (PKC), with direct activation of protein phosphatases. In contrast, CER also activates kinases such as c-jun N-terminal protein kinase (JNK), and the so-called stress-activated protein kinases (SAPK), which is one of the mitogen-activated protein kinases (MAPKs). CER is further metabolized to sphingosine (SPH). Like CER, SPH and sphinganine (SPG) inhibit PKC, and Akt. Recent reports have revealed that SPH and its derivatives SPG and phytosphingosine (PHS) mediate dephosphorylation of pro-growth signal kinase, extracellular signal-regulated kinase (ERK)1 and ERK2 (one of the MAPKs), with involvement of PKC. PHS is abundant in yeasts and plants. In animals, PHS is distributed in keratinocytes, microvillus membranes in the small intestine, and leukocytes, where there is large amount of cell-turnover activity, suggesting that PHS involvement to cell-turnover.

As CER were surrounding lipids in the keratinocytes and CER stimulate keratinocyte differentiation, we therefore examined the upregulation of caspase-14 by various sphingoid bases and CER. There was a controversial effect of sphingolipids that CER differentiates keratinocyte, but SPH promotes proliferation of keratinocyte. In this study, we found that PHS induces caspase-14 expression in mRNA and protein level, indicating that PHS upregulated caspase-14 transcription. Moreover, we determined whether MAPK is involved in PHS-induced caspase-14 upregulation.

MATERIALS AND METHODS

Cells The human keratinocyte cell line HaCaT was pro-

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vided by Professor P. Boukamp and Dr. N. Fusenig (German Cancer Research Center, Heidelberg). The HaCaT cells were maintained at 37°C with 5% CO₂ in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with streptomycin (100 μg/mL), penicillin (100 U/mL), sodium bicarbonate (2 g/L), and heat-inactivated 10% (v/v) fetal bovine serum (FBS).

**Chemicals and Antibodies** PH5, PH6, SP5, and C2-CER were purchased from Sigma (St. Louis, MO, U.S.A.). Anti-caspase-14 antibody and anti-filaggrin antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-β-actin antibody was purchased from Cell Signaling Technologies (Danvers, MA, U.S.A.).

**Preparation of Mice Skin** ICR mice were purchased from Tokyo Laboratory Animals Science (Tokyo, Japan). All mice were 6 to 10 weeks of age. The mice were given *ad libitum* access to standard chow and water, and housed in our departmental animal room under specific pathogen-free conditions. Experimental procedures were approved by the Tokyo Denki University’s ethical and animal experiment regulations. Back skin of the mice was shaved under anesthesia (mixed anesthesia of 0.75 mg/kg body weight (b.w.) medatomidine hydrochloride, 4 mg/kg b.w. midazolam, 5 mg/kg b.w. vecuronium in physiological saline. Antagonist of 0.75 mg/kg b.w. atipamezole hydrochloride in physiological saline). Mice were daily topically treated with 0.5 g of PHS mixed with petroleum jelly—based cream to upper back skin for 3 d. For the control experiment, petroleum jelly—based cream mixed with a PHS-dissolved solvent (methanol) was treated. After 3 d, dorsal skin (1.5 cm square site) was obtained.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl Tetrazolium Bromide (MTT) Assay** The cells were incubated in 96-well plates at 37°C with reagents for 47 h. Then, 10 μL of 5 mg/mL MTT (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added to each well and the plates were incubated at 37°C for 1 h. The media were discarded, and 100 μL of dimethyl sulfoxide was added to dissolve MTT formazan. The absorbance of each well was measured using a microplate reader (Awareness Technology, Palm City, FL, U.S.A.) at 570 nm.

**Real-Time Quantitative (q)RT-PCR** Total RNA from the cells or dorsal mouse skin was extracted with the Trizol reagent from the samples according to the manufacturer’s protocol (Life Technologies, Carlsbad, CA, U.S.A.). cDNAs were obtained from 1 μg RNA by the presence of oligo dT primers and AMV Reverse Transcriptase XL (Life Sciences, Houston, TX, U.S.A.). A qRT-PCR was performed using a Gene Ace SYBR qPCR Mix (Nippon Gene, Tokyo, Japan) with an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, U.S.A.) using the manufacturer’s protocol. The primers used were: for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward: 5′-ATC ATC AGC AAT GCC TTC-3′, and reverse: 5′-CTG CTTC CAC CAC TTT GA-3′; for human caspase-14, forward: 5′-AGG AGG AGC TTT CCT AG-3′, and reverse: 5′-GCT AAG TTT TGG CTG GCT-3′; for mice GAPDH, forward: 5′-TAA CTG TGG GGT TAG GAA-3′, and reverse: 5′-ACA CAT TGG GGG TAG GA-3′; for mice caspase-14, forward: 5′-TGA AGT CCA AAG CAC CCT-3′, and reverse: 5′-AGG GCT GTG ATG AAG GAA-3′. Duplicate cycle threshold (CT) values were analyzed by the comparative CT (ΔΔCT) method. The relative amount of mRNA (2−ΔΔCT) was obtained by normalizing to the endogenous GAPDH reference level in all experiments.

**Western Blotting** Cells were washed with phosphate-buffered saline (PBS) and placed on ice for 20 min in lysis buffer (50 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES)-NaOH, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1 mM ethylene glycol bis(2-aminoethyl ether)-N,N',N'-tetraacetic acid, 1.5 mM MgCl₂, 1% protease inhibitor cocktail [Sigma], pH 7.5, 0.1 mM sodium or-

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**Fig. 1.** Sphingolipids Has Low Toxicity in Micromolar Dose to HaCaT Cells

HaCaT cells were incubated with indicated doses of PHS, SPH, SPG, or C2-CER for 48 h. Cell proliferation was estimated by MTT assay. The data are presented as a comparison to the nontreated control cells. Each bar denotes the standard deviation (S.D.). (n=3). *p<0.05, **p<0.01, and ***p<0.001 vs. nontreated control.
thovanadate). Cell lysates were centrifuged at 4°C for 15 min at 13000×g. The protein concentrations of the supernatant were determined using the bicinchoninic acid (BCA) protein assay (Thermo Scientific, Waltham, MA, U.S.A.). Cell lysates (30 μg) were mixed in the same volume of sodium dodecyl sulfate (SDS) sample buffer (4% SDS, 125 mM Tris, pH 6.8, 10% glycerol, 0.02 mg/mL bromophenol blue, 10% 2-mercaptoethanol) and heated at 100°C for 3 min. Proteins were separated by 12% polyacrylamide gel SDS-electrophoresis and electrically transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, U.S.A.). After the membrane was blocked with the use of 3% skimmed milk, caspase-14, filaggrin, and β-actin were immunodetected using specific antibodies. Thereafter, horseradish peroxidase-conjugated anti-rabbit or -mouse immunoglobulin G was applied as the second antibody, and positive bands were detected by enhanced chemiluminescence (Thermo Scientific). Visualization was done with an Image Quant LAS-4000 digital imaging system (GE Healthcare, Buckinghamshire, U.K.). Relative density of bands was quantified using Image J (National Institutes of Health, Bethesda, MD, U.S.A.).

Detection of Caspase-14 Activity

Activation of caspase-14 was determined by measuring the hydrolyzing activities of caspase-14 substrate, Acetyl-WEHD-p-nitroaniline (Ac-WEHD-pNA; Alexis, San Diego, CA, U.S.A.). Cells were washed with PBS. The pellet was lysed in RIPA buffer (25 mM Tris–HCl, 150 mM KCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.5), and cell extracts were obtained by centrifugation at 14000×g for 5 min at 4°C. Protein concentrations were determined using the BCA protein assay. Cell lysates (0.4 mg/mL) were incubated in caspase buffer (100 mM HEPES, pH 7.4, 0.5 mM EDTA, 20% glycerol, 5 mM dithiothreitol) containing 200 μM substrate Ac-WEHD-pNA for 4 h at 37°C. Caspase activities were assayed by measuring the released pNA at 405 nm using a microplate reader.

Statistical Analysis

All statistical analyses were performed using the Student’s t-test. Differences were established at the p<0.05 level.

RESULTS

Sphingolipids Has Low Toxicity in Submicromolar Dose to HaCaT Cells

Sphingoid base-backbones of sphingolipids, especially SPH and its metabolites are known as a potent inducer of apoptosis.12,20–22) To estimate the potency of the cellular toxicity of the sphingoid bases, MTT assay was performed to determine the cell viability. All of the cell-permeable sphingoid bases and the metabolite CER used in this study (PHS, SPH, SPG, and C2-CER) resulted in only slight HaCaT cell damage up to 15 μM at 48-h treatment (Fig. 1). Treatment at doses over 20 μM sphingoid bases resulted in significant damage to HaCaT cells. Therefore, sphingoid bases and CER used in this study has low toxicity >15 μM to HaCaT cells.

Sphingoid Bases Upregulated Expression of Caspase-14

We next determined whether sphingoid bases and CER regu-
Fig. 4. PHS Activated Caspase-14

HaCaT cells were treated with indicated doses of PHS for 48 h. The cells were lysed and subjected to caspase-14 assay. The results are presented as a comparison to the nontreated control. The data are presented as a comparison to the nontreated control cells. Each bar denotes the S.D. (n=3). *p<0.05 vs. control.

Fig. 5. PHS Degraded Filaggrin

HaCaT cells were treated with indicated doses of PHS for 48 h. Thereafter, filaggrin protein expression was determined by Western blotting. β-Actin was detected as equal protein loading.

Fig. 6. PHS-Induced Caspase-14 Upregulation Involves p38 and JNK

HaCaT cells were treated with 3 µM PHS for 48 h with or without a JNK inhibitor (50 µM SP600125) or p38 inhibitor (8 µM SB203580). Phosphorylated-JNK (JNK1 and 2), JNK (JNK1 and 2), phosphorylated-p38, p38, phosphorylated ERK, ERK, caspase-14, and β-actin protein expression was then determined by Western blotting. β-Actin was detected as equal protein loading. Graphs within each panel are densitometric quantifications of the respective blots of phosphorylated immunoreactivity over total immunoreactivity (in MAPK blots) or caspase-14 immunoreactivity over β-actin immunoreactivity normalized to nontreated control. The data are presented as a comparison to the nontreated control cells. Each bar denotes the S.D. (n=3).
late caspase-14 gene expression. Micromolar (1–3 µM) doses (nontoxic doses for HaCaT cells) of PHS, SPH, SPG, or C2-CER was added to HaCaT cells for 48 h and determined the mRNA level of caspase-14 by qRT-PCR. All of the compound used in this study upregulated the mRNA expression levels of caspase-14 (Fig. 2).

Western blotting was performed to estimate the caspase-14 protein level. The 48-h treatment of sphingoid bases and CER at 1–3 µM augmented the caspase-14 protein level (Fig. 3), consistent with the mRNA level (Fig. 2), suggesting that sphingoid bases and CER upregulated the caspase-14 expression at the transcription level. Among the sphingoid bases used in this study, PHS seemed to be most potent inducer of caspase-14 expression (Fig. 2), so we selected PHS for further treatment.

Caspase-14 activity was determined by a specific substrate. As shown in Fig. 4, PHS activated caspase-14, suggesting that PHS induced not only upregulation but also maturation of caspase-14 to cleave caspase-14 substrate (Fig. 4). Filaggrin is cleaved by caspase-14. We determined filaggrin protein after PHS treatment. PHS treatment significantly increased processed form of filaggrin, suggesting that activated caspase-14 enhanced degradation of filaggrin (Fig. 5).

Sphingoid Base-Upregulation of Caspase-14 Was Related to MAPK We further estimated how sphingoid bases upregulated the caspase-14 expression. Sphingolipids induce intracellular signal transduction by activating cell death involving MAPKs, JNK and p38. In contrast, sphingoid bases inhibit the pro-growth MAPK, ERK1 and ERK2 activity.2,3,12,24) In the present study, PHS induced p38 and slight JNK phosphorylation. Similarly, PHS was reported to activate p38 in T-cell lymphoma Jurkat cells,21) and ERK was inhibited by PHS.20) As Hsu et al. revealed by green tea polyphenol treatment that p38 and JNK, but not ERK, are involved in caspase-14 expression,20) PHS-induced caspase-14 upregulation might be related to a PHS-induced p38 and JNK upregulation pathway.

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Sphingoid Base Upregulated Expression of Caspase-14 in Vivo We determined whether sphingoid bases regulate caspase-14 gene expression in vivo. PHS was treated to dorsal skin of ICR mice daily for 3 d, and dorsal skin was obtained. As shown in Fig. 7, PHS treatment upregulated the mRNA expression levels of caspase-14 dose-dependently (Fig. 7).

DISCUSSION

The results of the present study showed that cell-permeable sphingoid bases, especially PHS, induce caspase-14 expression, indicating that cell-permeable sphingoid bases upregulated the caspase-14 transcription. In addition, one of the cell-permeable sphingoid bases, PHS, induced caspase-14 expression that was blocked by a specific inhibitor of one of the MAPKs, p38. This indicates that the PHS upregulation of caspase-14 expression involves p38.

Sphingoid base-backbones of sphingolipids and their metabolites, especially SPH and CER, have often been reported to be involved in cell turnover, affecting the apoptosis of cells, including those of the epidermis.23) However, the results of the present study revealed that the cell-permeable sphingoid bases SPH, SPG, PHS and CER at doses not damaging cells at all (1–3 µM) induce caspase-14 expression. These results demonstrate that cell-permeable sphingoid bases act not only as a barrier in the stratum corneum but also by modulating cellular functions and inducing differentiation. In their recent study, Jiang et al. reported that short-chain CERs but not other sphingoid bases stimulated caspase-14 expression, suggesting that the structure of CER is important.8) In the present study, our findings showed clearly that not only CER but also the other cell-permeable sphingoid bases SPH, SPG and PHS upregulated caspase-14 expression. This phenomenon indicates that the mechanism underlying caspase-14 expression is somewhat a common signal transduction of sphingoid base backbone.

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Sphingoid bases are abundant in yeasts and plants.14) In animals, PHS is distributed in keratinocytes, microvillus membranes in the small intestine, and leukocytes.15–17) We also revealed that PHS treatment to mouse skin significantly upregulated caspase-14 mRNA expression of the epidermis (Fig. 7), however we need to explore the adequate dose not occurring toxicity in vivo in the future. Our present studies suggest that PHS has a possibility to be involved in keratinocyte differentiation.

Acknowledgments This work was partially supported by a Grant-in-Aid for Scientific Research (Grant #26870610) and the Research Institute for Science and Technology of Tokyo
Denki University (Grant #Q13L-02), Japan. We are grateful to Mr. Sulaiman Abulikemu for technical assistance.

Conflict of Interest The authors declare no conflict of interest.

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