Original Article

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Electrolytic-reduction ion water induces ceramide synthesis in human skin keratinocytes

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SUMMARY Ceramides play a critical role in the skin barrier. We previously demonstrated that electrolytic-reduction ion water (ERI) improves skin integrity and enhances the protective barrier function of the epidermis. Here, we first examine the effect of ERI on the expression of ceramide synthesis–related enzymes in human skin keratinocytes. The expression of enzymes involved in the elongation of very-long-chain fatty acids protein 4 (ELOVL4) was increased after treatment with ERI-containing media. The expression of ceramide synthase 3 (CerS3), which binds ultra-long-chain fatty acids to sphingosine to produce ceramides found in the skin, was also increased. Subsequently, we examined the expression of ceramides in keratinocytes treated with ERI using thin-layer chromatography. The results showed that ERI increased the ceramide content, and these ceramides were more hydrophobic than those extracted from untreated keratinocytes. These results suggest that ERI enhances the expression of enzymes involved in the synthesis of ceramides containing ultra-long-chain fatty acid residues, which have a protective function in the skin.

Keywords elongation of very-long-chain fatty acids protein 4, ceramide synthase 3, skin barrier

1. Introduction

Skin is the tissue located on the external surface of the body. The epidermis, in particular, is responsible for defense against various environmental stimuli. For this reason, the skin has a physically resistant structure. One of the elements of the skin's physical barrier is a lamellar structure composed of ceramides (*1*). This structure is also known as the intercellular lipids and consists of repeating oil and water phases, constituting a functional barrier that is responsible for maintaining the moisture of the skin (*2*). In addition to ceramides, this structure is also composed of other lipids, such as free cholesterol and free fatty acids. The molecular species of the ceramides constituting the lamellar structure in the epidermis greatly influence the barrier function and moisture retention capacity of the skin.

There are more than one thousand molecular species of ceramides, which are distinguished by differences in the length of the carbon chain between the fatty acid and sphingosine (*3*). Ceramides have a structure in which the sphingosine and fatty acid residue are ester-bonded and are biosynthesized from serine with palmitic acid as a precursor through the pathway shown in Figure 1.

Ceramides in the skin are biosynthesized primarily by keratinocytes and, unlike in other organs, contain very long chain fatty acids specific to skin (*4*). These are bound to sphingosine by ceramide synthase 3 (CerS3) to produce the ceramides (*5*).

Mutations in CerS3 are detrimental to the barrier function of the skin and maintenance of moisture. CerS3-deficient mice die due to epidermal water loss immediately after birth (*6*). In humans, ichthyosis is an inherited skin condition characterized by deterioration of the barrier function of the skin caused by mutations in the enzymes involved in the biosynthesis of ceramides with very long chain fatty acids (*7,8*). In addition, the stratum corneum of patients with atopic dermatitis contains fewer ceramides of high molecular weight and more ceramides of low molecular weight than the proportions that are found in healthy individuals (*9,10*).

Our research group has been investigating the effects of electrolytic-reduction ion water (ERI) on the skin. ERI is an aqueous solution with surfactant activity and skin moisturizing properties that has been used as a base component for cosmetics. We previously reported that ERI promotes wound healing in a mouse model of burns (*11*), promotes healing of scalds in children while preventing keloid scarring (*12,13*), and

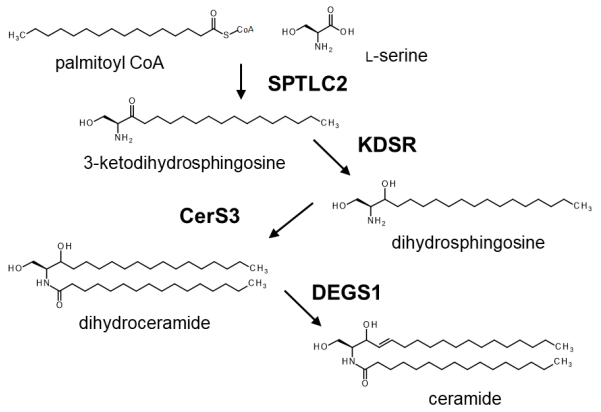

Figure 1. Ceramide synthesis pathway. SPTLC2: serine palmitoyltransferase long-chain base subunit 2; KDSR: 3-ketodihydrosphingosine reductase; CerS3: ceramide synthase 3; DEGS1: delta 4-desaturase, sphingolipid 1.

leads to improvement of atopic dermatitis (*14*). In the present study, we aimed to clarify whether the effect of ERI on the skin is through improvement in ceramide synthesis or biosynthesis of very long chain fatty acids and associated improvement of the barrier function. For this purpose, we investigated changes in the expression of enzymes related to the synthesis of very long chain fatty acids and ceramides in keratinocytes treated with ERI.

2. Materials and Methods

2.1. Materials

ERI was obtain from AI system products (Aichi, Japan). Normal human epidermal keratinocyte (NHEK) cells were purchased from Promo Cell (Heidelberg, Germany). All other chemicals were of reagent grade.

2.2. Cellular activity

NHEK were cultured in Keratinocyte Growth Medium 2 (Promo Cell, Germany) under humidified air containing 5% CO_2 at 37°C. The cells were seeded into a 96-well plate (10^4 cells/well) and cultured overnight at 37°C in a humidified atmosphere with 5% CO_2. Next, various concentrations of ERI were added to the culture medium, and the cells were incubated for a further 24 h. Cellular activity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Dojindo, Kumamoto, Japan) assay. Briefly, the cells were treated with 110 µL of Dulbecco's modified Eagle's medium containing MTT (0.5 mg/mL) and incubated at 37°C for 4 h. Subsequently, 100 µL of 10% sodium dodecyl sulfate in 10 mM hydrochloric acid was added to dissolve the formazan crystals that formed in metabolically active cells. Spectrophotometric absorbance was determined at 560 nm using a microplate reader (VersaMAX, Molecular device, San Jose, CA, USA). The absorbance was calculated and expressed as percentage of cellular activity.

2.3. Expression of ceramide synthesis–related genes

The effects of ERI on the expression of ceramide synthesis–related genes were assessed using real time-polymerase chain reaction (RT-PCR). NHEK cells were seeded in a six-well tissue culture plate (10^5 cells/well) and incubated overnight. Subsequently, the cells were treated with various concentrations of ERI and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 72 h. Total RNA was extracted from the NHEK cells using TriPure Isolation Reagent (Roche Life Science, Indianapolis, IN, USA), and cDNA was synthesized using ReverTra Ace (Toyobo, Osaka, Japan) according to the instructions provided by the manufacturer. Specific primers were designed to amplify human serine palmitoyltransferase long chain base subunit 2 (*SPTLC2*) (5'-CCAGACTGTCAGGAGCAACCATTA-3' and 5'-CGTGTCCGAGGCTGACCATA-3'), human 3-ketodihydrosphingosine reductase (*KDSR*) (5'-GTGGTGGTTACAGGAGGTTC-3' and 5'-AATTTACCAGCATGTCCACT-3'), human *CerS3* (5'-ACATTCCACAAGGCAACCATTG-3' and 5'-CTCTTGATTCCGCCGACTCC-3'), human delta 4-desaturase, sphingolipid 1 (*DEGS*1) (5'- GCGTTTGGCAGTTGCATTAA -3' and 5'-CATTGTGGGCAATCTCATGAA -3'), human elongation of very long chain fatty acids protein (*ELOVL*) 1 (5'-ATTCTCCTGACCTACGTGTACTT-3' and 5'-TTCCGATTAGCCATGATGCGA-3'), human *ELOVL4* (5'-CATGTGTATCATCACTGTACG-3' and 5'-AAAGGAATTCAACTGGGCTC-3'), human *ELOVL7* (5'-TTCCATCATACCATCATGCC-3' and 5'-CCCAATGCAGAAAGTCCATA-3'), and human *β-actin* (5'-AGTCCTGTGGCATCCACGAAAC-3' and 5'-GCAGTGATCTCCTTCTGCATCC-3'). RT-PCR was performed using StepOne (Applied Biosystems, Foster City, CA, USA). Thermocycling was performed under the following conditions: one cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. The cycle threshold (C_t) values for each sample were normalized to that of *β-actin*, and the relative expression was calculated using the comparative C_t method.

2.4. Western blotting analysis

Samples (10 µg) of the cell lysates were separated on a 12% (w/v) polyacrylamide gel (*15*). Proteins were blotted onto nitrocellulose membranes (Protran BA85; GE Healthcare, Chicago, IL, USA) in a semi-dry blotting system (NA-1513; Nihon Eidoh Co., Tokyo, Japan) (*16*). Nitrocellulose membranes were blocked with 2% (w/v) skim milk in phosphate-buffered saline. Blocked

membranes were incubated with anti-CerS3 rabbit antibody (1:2,000; Cusabio, Houston, TX, USA), with anti-ELOVL4 rabbit antibody (1:1,000; Cusabio, USA), or with anti-β-actin mouse monoclonal antibody (1:5,000, Wako Chemical, Tokyo, Japan). This was followed by incubation with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) goat antibody (1:5,000; BioSource, Camarillo, CA, USA) for CerS3 or ELOVL4, or horseradish peroxidase-conjugated anti-mouse IgG goat antibody (1:5,000; Wako Chemical) for β-actin. The blots were subsequently developed using an Immunostar LD (Wako Chemical, Japan) or Imobiron (Merck Millipore, Billerica, MA, USA) using LuminoGraph (Atto Corporation, Tokyo, Japan). The bands were densitometrically analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA).

2.5. Ceramide analysis

The NHEK treated with ERI were washed with phosphate-buffered saline; lipids were extracted with chloroform/methanol (1:2, v/v) and maintained at room temperature for 1 h. Next, the liquids were collected, and potassium chloride aqueous solution was added up to a concentration of 0.13% potassium chloride. The samples were centrifuged at 1,000 × g at room temperature for 5 min. The lower phase was transferred to a new tube, while the upper layer was re-extracted with chloroform; this was followed by centrifugation at 1,000 × g at room temperature for 5 min. The extract was dried at 50°C under a stream of nitrogen gas. The residue was dissolved in 100 μL of chloroform/methanol and stored at −20°C until use.

The lipid extracts were analyzed using normal-phase thin-layer chromatography plates (TLC, Silica gel 60; Merck Millipore, Darmstadt, Germany) through the following three solvent systems: (1) chloroform/methanol/water (40:10:1, v/v), developed to 2 cm from the bottom, dried, and developed again to 5 cm from the bottom; (2) chloroform/methanol/acetic acid (47:2:0.5, v/v), developed to 1.5 cm from the top; and (3) hexane/diethylether/acetic acid (65:35:1, v/v) developed to the top. The separated lipids were detected using a copper phosphate reagent (3% $CuSO_4$ in 8% [v/v] phosphoric acid solution) and heating at 180°C.

3. Results

3.1. Confirmation of optimal concentration for cell testing in ERI-containing media

Cellular activity was assessed to allow a correct evaluation of this test using ERI at concentrations ranging from 5.0×10^{-6}% to 50% (v/v). ERI did not influence the activation of NHEK cells at concentrations ≤ 5% (Figure 2). In a study of burn wounds in mice (*11*), atopic dermatitis (*12*) and burn wounds (*13,14)* in humans, the healing effect of ERI was observed at 100% concentration when applied to skin tissue, due to its very low alkali reserve of 0.1 g/100 mL. However, since the direct application of ERI to NHEK cells is affected by pH and surface-active effects, the concentration at which ceramide synthesis can be accurately evaluated in this study was 5% or lower.

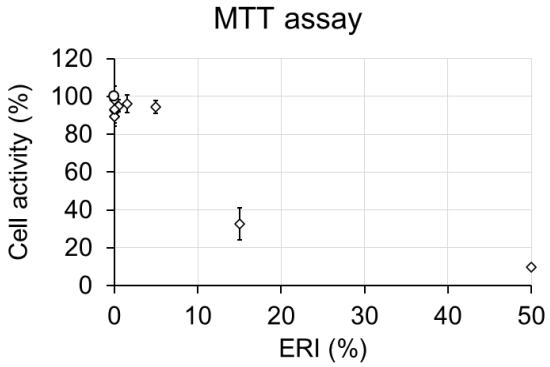

Figure 2. Activity of cells treated with ERI. ERI was added to the cell culture media. Cell activity was measured by MTT assay. Data are shown as the mean ± SD of eight biological replicates. The circle indicates un-treated control. ERI: electrolytic-reduction ion water; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

3.2. Expression levels of ceramide synthesis–related enzyme genes

To examine the expression of ceramide synthesis–related enzymes in keratinocytes treated with ERI, mRNA was extracted from NHEK cells and RT-PCR was performed (Figures 3A-3G). Treatment with ERI increased the expression of *CerS3* and *ELOVL4* mRNA in a dose-dependent manner. In particular, the expression levels of *CerS3* and *ELOVL4* were significantly increased after treatment with 0.5% (v/v) ERI. In contrast, the expression of *SPTLC2*, *KDSR*, *DEGS1*, *ELOVL1*, and *ELOVL7* remained unchanged.

3.3. Levels of CerS3 and ELOVL4 proteins

The levels of CerS3 and ELOVL4 proteins were assessed by western blotting analysis (Figure 4A). CerS3 protein levels were increased after treatment with ERI in a dose-dependent manner (Figure 4B). CerS3 expression in the NHEK by 5% ERI treatment increased more than 3.5-fold. In addition, the expression of ELOVL4 protein was slightly increased after treatment with ERI at concentrations ranging 0.5-5% (v/v) and then ELOVL4 expression induced more than 4.5-fold by 5% ERI treatment (Figure 4C). β-actin levels did not differ among these cells.

3.4. Expression of ceramides in keratinocytes

The ceramide analysis performed using thin-layer

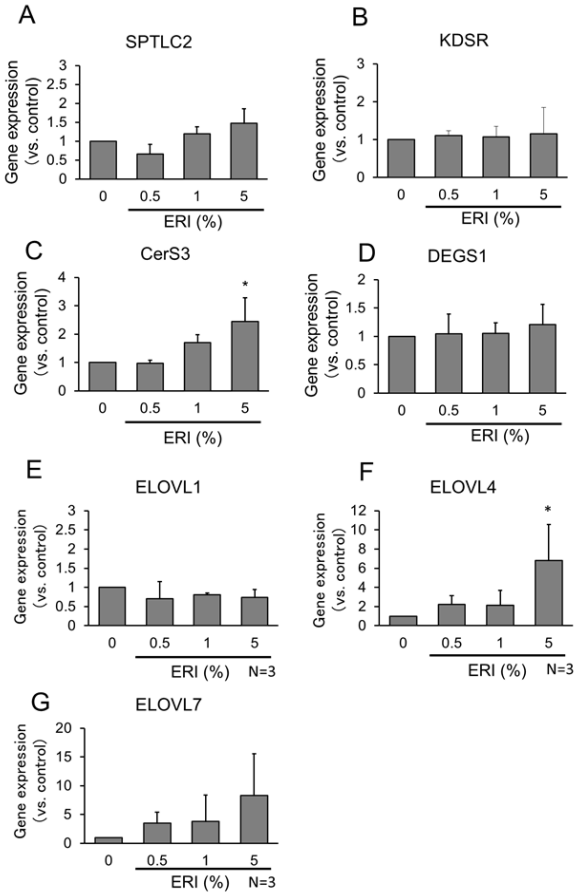

Figure 3. RT-PCR quantification of the expression levels of ceramide synthesis–related enzyme genes. Expression levels of *SPTLC2* **(A)**, *KDS*R **(B)**, *CerS3* **(C)**, *DEGS1* **(D)**, *ELOVL1* **(E)**, *ELOVL4* **(F)**, and *ELOVL7* **(G)**. The mRNA was extracted from NHEK cells treated with 0.5%, 1%, or 5% ERI added to the cell culture medium for 72 h. The expression of mRNA was determined by real time PCR. The expression levels of each enzyme were normalized to those of β-actin. Data are shown as the mean ± SE of three biological replicates. Asterisks: $p < 0.05$ compared with untreated controls. CerS3: ceramide synthase 3; DEGS1: delta 4-desaturase, sphingolipid 1; ELOVL1: elongation of very-long-chain fatty acids protein 1; ERI: electrolytic-reduction ion water; KDSR: 3-ketodihydrosphingosine reductase; NHEK: normal human epidermal keratinocytes; SPTLC2: serine palmitoyltransferase long-chain base subunit 2.

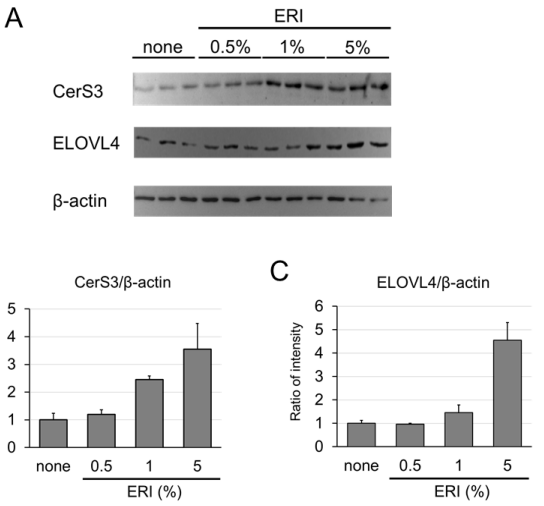

Figure 4. Western blotting analysis of CerS3 and ELOVL4. The protein levels of CerS3, ELOVL4, and β-actin were determined using lysates prepared from NHEK cells treated with 0.5%, 1%, or 5% ERI added to the cell culture medium for 72 h. The expression of CerS3 and ELOVL4 proteins was detected by western blotting analysis **(A)**. The protein content was normalized to the level of β-actin. The intensity of CerS3 **(B)** and ELOVL4 **(C)** in were measured by densitometry. Data represent means ± SD ($n = 3$ *vs*. None). CerS3: ceramide synthase 3; DEGS1: delta 4-desaturase, sphingolipid 1; ELOVL4: elongation of very-long-chain fatty acids protein 4; ERI: electrolytic-reduction ion water; KDSR: 3-ketodihydrosphingosine reductase; NHEK: normal human epidermal keratinocytes; SPTLC2: serine palmitoyltransferase long-chain base subunit 2.

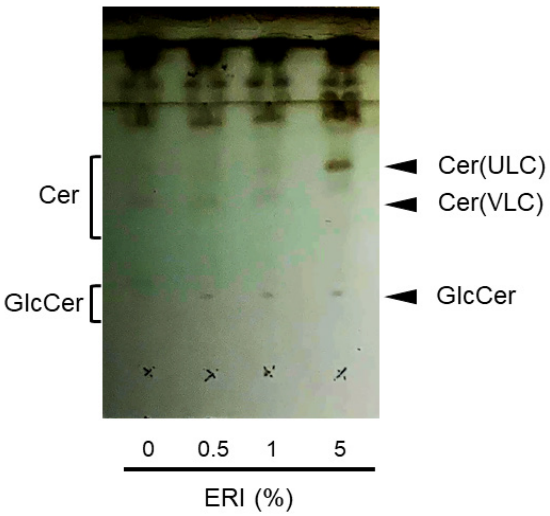

Figure 5. Ceramide-related lipids in the keratinocyte after ERI treatment. The lipids were extracted from NHEK cells treated with 0.5%, 1%, or 5% ERI added to the cell culture medium for 72 h. The extracted lipids were separated by normal-phase TLC and detected by copper sulfate/phosphoric acid solution. Cer: ceramide; GlcCer: glucosylceramide; ERI: electrolytic-reduction ion water; ULC: ultra-long-chain; VLC: very-long-chain.

chromatography (Figure 5) confirmed that higher concentrations of ERI were associated with larger sizes of spots in the glucosylceramide (GlcCer) and ceramide regions and darker color. This result indicated that ERI promoted the synthesis of GlcCer and ceramides in a concentration-dependent manner and increased the intracellular content of GlcCer and ceramides. It was confirmed that higher concentrations of ERI were linked to longer development distances of the spots in the GlcCer and ceramide regions (particularly the ultra long chain ceramide region). These results demonstrated that treatment with higher concentrations of ERI promotes the synthesis of fatty acids with longer carbon chain lengths.

4. Discussion

Studies have shown that ERI of 100% concentration improves thermal wounds in mice (*11*), atopic dermatitis (*12*) and promotes skin healing processes after burns

(*13,14*) in humans. In this study, we hypothesized that ERI may also be involved in the regulation of ceramide synthesis. The epidermis produces ceramides with a characteristic fatty acid structure. Fatty acids with alkyl chain lengths of 11-20, 21-25, and ≥ 26 carbons are considered long-, very-long-, and ultra-long-chain fatty acids, respectively (*16*). In most tissues, the fatty acid residues of ceramides have alkyl chains containing 16-24 carbons (*17,18*), while ceramides of the epidermis additionally include 26-36-carbon ultra-long-chain fatty acid residues. Ceramides synthesized in keratinocytes have fatty acids residues with longer carbon chains than those synthesized in other tissues.

Biosynthesis of ultra-long-chain fatty acid residues is necessary to allow the synthesis of ceramides that incorporate them. Therefore, we evaluated the effects of ERI on the expression of enzymes involved in the synthesis of ultra-long-chain fatty acids in keratinocytes. For this purpose, we evaluated the expression of *ELOVL1*, *ELOVL4*, and *ELOVL7* genes. Addition of ERI to the cell culture medium increased the expression of *ELOVL4*. ELOVL4 is essential for the synthesis of fatty acids with alkyl chain lengths of ≥ 28 carbons, which are characteristically abundant in the epidermis; moreover, recessive mutation of the *ELOVL4* gene is known to cause ichthyosis (*19*). *ELOVL4* knockout in mice is lethal shortly after birth due to failure of skin barrier formation, owing to the inability to synthesize very long chain fatty acids used for the synthesis of ceramides in the epidermis (*20*).

Treatment of keratinocytes with ERI increased the expression of *CerS3*. The CerS family is involved in the amide binding reaction between sphingosine and fatty acids (*21*). CerS enzymes are categorized into molecular types 1-6, and each CerS type is involved in the binding of fatty acids of particular chain lengths to sphingosine. Among the CerS types, only CerS3 is capable of carrying out this action on the ultra-long-chain fatty acids synthesized in the skin. Therefore, similar to ELOVL4-knockout mice, CerS3-knockout mice die shortly after birth due to failure of skin barrier formation (*8*).

While we were unable to determine the ingredients contained in ERI, the liver X receptor alpha (LXRα) pathway is a known regulatory mechanism that alters enzymes involved in ceramide synthesis (*22*). LXR is a transcription factor expressed in tissues (*e.g.*, liver) and regulates the expression of cholesterol and fatty acid metabolic enzymes (*22*). LXRα increases the amount of ceramides in the epidermis of the skin by promoting fatty acid synthesis, thereby enhancing the barrier mechanism of the skin (*22*). It was recently reported that treatment with an LXRα ligand induces the expression of *ELOVL4*, *ELOVL7*, and *CERS3* mRNA in keratinocytes (*23*). In this study, similar to LXRα, treatment with ERI enhanced the expression of *ELOVL4* and *CerS3*. Furthermore, LXRα activation resulted in a non-statistically significant trend towards increased expression of *ELOVL7*, as also observed following treatment with ERI. These results indicate that the mechanism involved in the role of ERI in CerS expression may be related to LXRα.

In summary, this study demonstrated that treatment with ERI induced *ELOVL4* and *CerS3* expression in keratinocytes. ELOVL4 and CerS3 are key enzymes for the synthesis of ceramides containing ultra-long-chain fatty acids. As this type of ceramide is essential in the assembly of the skin barrier, this finding presents a pathway through ceramide synthesis as one of the mechanisms involved in the protective effects of ERI on the skin.

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Conflict of Interest: MI, YO and MO are employees of A.I. System Products Corp.

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