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Effects of Glycogen on Ceramide Production in Cultured Human Keratinocytes via Acid Sphingomyelinase Activation

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Abstract: Glycogen is a highly branched storage polysaccharide found mainly in the liver and the muscles. Glycogen is also present in the skin, but its functional role is poorly understood. Recently, it has been reported that glycogen plays an important role in intracellular signal transduction. In the epidermis of the skin, keratinocytes are the predominant cells that produce ceramide. Ceramides are lipids composed of sphingosine, and prevent water loss, as well as protecting the skin against environmental stressors. In this study, we investigated the effects of glycogen on ceramide production in cultured keratinocytes. Thin-layer chromatography revealed that incubation of keratinocytes with 2 % glycogen enhanced the cellular amount of ceramide NS (ceramide 2) by 3.4-fold compared to the control. We also found that glycogen regulated the mRNA expression levels of signaling molecules of the sphingomyelin-ceramide pathway by quantitative real-time PCR. The activity of sphingomyelinase was also significantly enhanced by 2.5-fold in cultures with 1 % glycogen compared to the control. Moreover, glycogen increased the ATP production by 1.5-fold compared to the control, while glucose did not affect the production. Western blotting showed that phosphorylation of Akt, a cellular signaling molecule, was inhibited in the presence of glycogen in cultured keratinocytes. This study shows that glycogen upregulates the ceramide production pathway from sphingomyelin in epidermal keratinocytes, and provides new insights into the role of glycogen in cellular signal transduction.

Key words: glycogen, ceramide, sphingomyelin, akt phosphorylation, keratinocytes

INTRODUCTION

Glycogen is a highly branched polymer of glucose that exists in various human tissues, including the liver, muscles, brain, skin, and so on, even in mother's milk.[1)2)] Liver glycogen acts as an energy store and supplies glucose to maintain a normal blood glucose level, while glycogen in the skeletal muscles provides energy for muscle contraction. Glycogen has been also reported to retain a large amount of water in muscles.[3)] It has previously been shown that the distribution of glycogen in the skin changes with exposure to stresses such as ultraviolet light or hypoxia.[4)] These changes of its distribution might be related to biochemical change of glycogen, but the role of glycogen in the skin is poorly understood.

Recently, it has been reported that glycogen exists inside and outside cells and plays an important role in intracellular signal transduction.[5)6)7)8)] Kakutani *et al*. have shown that cytokine production in cultured macrophages is enhanced in the presence of glycogen. The study suggested that glycogen is recognized by the toll-like receptor 2 (TLR2) which then triggers the mitogen-activated protein kinase signaling cascade, resulting in the increase in cytokine level.[9)] The digestion of glycogen has also been reported to increase the level of heme oxygenase-1 in RAW 264.7 cells through the activation of NF-E2-related factor-2 transcription factor. As the result, glycogen increases antioxidant activity of the cells.[10)]

Keratinocytes are the predominant cells in the epidermis, the outmost layer of the skin. Extracellular space surrounding them has lipids such as ceramide, cholesterol and free fatty acid. Ceramide is an important component of the stratum corneum that prevents loss of water, and protects skin against the damage of sunlight and chemicals. Ceramide is produced within the cells by three pathways: de novo synthesis, sphingomyelin hydrolysis, and the salvage pathway. De novo ceramide synthesis involves several enzymes such as serine palmitoyltransferase (SPT) and ceramide synthase. In the hydrolysis of sphingomyelin, ceramide is produced by activation of neutral or acid sphingomyelinase (SMase) and suppression of sphingomyelin synthase (SMS). In the salvage pathway, ceramide is synthesized from sphingosine. However, it is known that the ceramide content of the skin decreases with age,[11)] and reduction of the ceramide content results in dry skin or relates to severe

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Abbreviations: SPTLC, serine palmitoyltransferase long chain base subunit; a-ceramidase, acid ceramidase; SMS, sphingomyelin synthase; a-SMase, acid sphingomyelinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ATP, adenosine triphosphate.

skin diseases such as atopic dermatitis and psoriasis.[12)13)] Therefore, many efforts have been made to increase the ceramide content to keep the skin younger and healthier. Topical application including ceramide or a ceramide analogue increases the ceramide content in the skin, leading to improvement of the skin barrier function, but there are few agents that are effective enough to improve healthy skin.[14)] In addition, there is concern about the risk of inducing allergy by continued use of these agents.

In the present study, we investigated the effects of glycogen on ceramide production. We showed that glycogen enhances ceramide production in cultured keratinocyte cells. We also determined that glycogen induces the expression of molecules in the pathway for production of ceramide from sphingomyelin. These findings offer new insights into the role of glycogen in cellular signal transduction.

MATERIALS AND METHODS

Cell culture. Normal human epidermal keratinocyte cells were obtained from Kurabo Industries Ltd. (Osaka, Japan). Keratinocytes were cultured in EpiLife™ medium with 60 μM calcium (MEPI500CA, Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with or without growth factors of Humedia-KG (KK6150, Kurabo Industries Ltd.). The cells were seeded in 6-well dishes at a density of 1×10^5 cells/well and culture was carried out according to the manufacturer's instructions, with exchange of the culture medium every 2 days. Sub-confluent cells were treated with or without 0.2, 1, or 2 % glycogen for 24 h or 7 days.

Three-dimensional model. Three-dimensional epidermal model was also used. This multilayered culture model contained highly differentiated skin epidermal cells with intracellular lipids including various ceramides (types 1-7). EPI-200 and specific medium (Kurabo Industries Ltd.) were used for culture, which was carried out according to the manufacturer's instructions. Cells were treated with or without 0.2 or 2 % glycogen for 24 h.

Glycogen. Glycogen was synthesized enzymatically from corn starch by the method described previously.[15)] We previously confirmed that the physicochemical properties of synthetic glycogen were the same as those of natural glycogen extracted from oysters, scallops, bovine liver or sweet corn.[16)]

Thin-layer chromatography (TLC) and quantitative analysis. Cultured keratinocytes and three-dimensional model were washed with PBS and collected by using a scraper after the stimulation with glycogen. Intracellular lipids were extracted by the method of Bligh & Dyer,[17)] dissolved in chloroform and methanol (2:1), and spotted onto a thin-layer silica gel plate (10 × 10 cm HPTLC plate, Merck KGaA, Darmstadt, Germany). Ceramides were isolated from the lipids by developing the plate in chloroform/methanol/acetic acid solution (190:9:1) for 30 min × 2, after which the plate was air dried. Then a mixture of aqueous copper sulfate solution (10 %) and aqueous phosphoric acid (8 %) was sprayed onto the plate, followed by heating at 180 ℃ for 15 min. A mixture of ceramide NS (ceramide 2, S - sphingosine, N - nonhydroxy), ceramide NP (ceramide 3, P - phyto-sphingosine, N - nonhydroxy), and ceramide AP (ceramide 6, P - phytosphingosine, A - α-hydroxy) (each at 3.3 mg/mL in chloroform and methanol [2:1] was also spotted onto the plate as standards. Photographs of the developed thin layer plates were taken and spots were analyzed by using an Image Reader LAS-4000 (FUJIFILM Corporation, Tokyo, Japan).

Quantitative real time PCR (qRT-PCR). After keratinocytes were cultured for 7 days with or without glycogen, total RNA was extracted from the cells by using an RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Then the extracted RNA was reverse transcribed into cDNA, and quantitative real-time PCR analysis (qRT-PCR) was performed by using a 7500 Fast Real-Time PCR system (Applied Biosystems Inc., Foster City, CA, USA) as reported previously.[18)] Primers were constructed with Primer Express software for Real-Time PCR version 3.0 (Applied Biosystems), and the primer sequences were as follows: serine palmitoyltransferase long chain base subunit 2 (SPTLC 2): 5′-ccagactgtcaggagcaaccatta-3′ (forward), 5′-cgtgtccgaggctgaccata-3′ (reverse); serine palmitoyltransferase long chain base subunit 3 (SPTLC 3): 5′-gccagcaccaggcatga-3′ (forward), 5′-gccacaaggtcctccaactc-3′ (reverse); acid ceramidase (a-ceramidase): 5′-gcggcctctgagacatgaag-3′ (forward), 5′-aggtcagacagctgcagtgttc-3′ (reverse); sphingomyelin synthase 1 (SMS1): 5′-gccaggacttgatcaacctaacc-3′ (forward), 5′-ccattggcatggccgttcttg-3′ (reverse); sphingomyelin synthase 2 (SMS2): 5′-cacccagtggctgtttctga-3′ (forward), 5′-tgcattccaggcacaggtaga-3′ (reverse); acid sphingomyelinase (a-SMase): 5′-tggctctatgaagcgatggc-3′ (forward), 5′-ttgagagagatgaggcggagac-3′ (reverse); glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5′-gggtgtgaaccatgagaagtatga-3′ (forward), 5′-ggtgcaggaggcattgct-3′ (reverse).

Acid sphingomyelinase activity assay. An acid sphingomyelinase assay kit (Echelon Bioscience, Salt Lake City, UT, USA) was used to measure a-SMase activity. Keratinocytes were cultured with 1 % glycogen for 7 days, and the cells were collected in 1 mM PMSF solution (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) to prevent loss of a-SMase activity. After repeating 3 times of freeze-thaw cycles, the supernatant of the centrifuged cells was used for the analysis.

ATP measurement. Keratinocytes were stimulated for 24 h with 1 % or 2 % of glycogen as well as 1 % glucose, and the cultured cells were collected for ATP measurement with an intracellular ATP analysis kit (Toyo B-Net Co., Ltd., Tokyo, Japan).

Protein measurement. Quick Start™ Bradford 1x Dye Reagent (#500-0205, Bio-Rad Laboratories, Inc., Hercules, CA, USA) were used to measure the amount of proteins. The cultured keratinocytes were washed with PBS and collected in 1 mM PMSF solution. The samples were analyzed by the Bradford method using bovine gamma globulin standard (#500-0209, Bio-Rad Laboratories, Inc.) as a standard.

Western blotting. Proteins in keratinocytes were collected using blue loading buffer with DTT (Cell Signaling Technology, Inc., Danvers, MA, USA). The samples were an-

alyzed by gel electrophoresis using 10 % SDS-polyacrylamide gel (Mini-protein TGX Gel, Bio-Rad Laboratories, Inc., Tokyo, Japan) and were transferred onto polyvinylidene difluoride membranes (iBlot™ 2 Gel Transfer Stacks, Invitrogen Corporation, Carlsbad, CA, USA). The membranes were blocked for 1 h with PBS containing 5 % nonfat dried milk and were incubated overnight at 4 °C with primary antibodies diluted in the blocking solution. The primary antibodies used were against phospho-Akt (Ser473, #4060S, 1:1000 dilution, Cell Signaling Technology, Inc.), total-Akt (#2920S, 1:1000 dilution, Cell Signaling Technology, Inc.) and GAPDH (#2118S, 1:1000 dilution, Cell Signaling Technology, Inc.). Subsequently, the membranes were washed in PBS and incubated for 2 h at room temperature with anti-rabbit IgG, horseradish peroxidase (HRP)-linked secondary antibody (#7074S, 1:1000 dilution, Cell Signaling Technology, Inc.), diluted in the blocking solution. The immunoreactive bands were detected by Amersham ECL Prime Western Blotting Detection Reagents (RPM2232, GE Healthcare, Tokyo, Japan) and analyzed using luminescent image analyzer (ImageQuant LAS 4000 mini, FUJIFILM Corporation, Tokyo, Japan) with Image Reader LAS-4000 software.

Statistical analysis. Results were expressed as means ± SD. Statistical analysis was performed using Student's *t* test or one-way Analysis of Variance (ANOVA) followed by Tukey test; $p < 0.05$ was considered statistically significant ($*p < 0.05$, $**p < 0.01$).

RESULTS

Glycogen treatment increased ceramide NS (ceramide 2) production in cultured keratinocytes and three-dimensional epidermal model.

Cultured keratinocytes stimulated with glycogen were collected and extracted lipids for thin-layer chromatography (TLC) analysis, as shown in Fig. 1. TLC identified ceramide NS spots on the upper part of the developed silica gel, revealing that addition of 2 % of glycogen increased the cellular content of ceramide NS (Fig. 1A). Quantitative analysis showed that addition of 2 % glycogen significantly increased the ceramide NS content (24.49±12.15 μg/well) compared with the level in unstimulated control cells (7.17±3.93 μg/well) (Fig. 1B), while there was no significant difference in protein levels between glycogen-stimulated cells and the control. In three-dimensional epidermal model, ceramide NS and other ceramides were detected by TLC (Fig. 1C). Quantitative analysis showed that ceramide NS content after the incubation with 0.2 % and 2 % glycogen were 53.00±10.10 μg/well and 60.90±18.87 μg/well, respectively. Those were higher than the control without glycogen (46.18±28.00 μg/well), although the difference was not significant (Fig. 1D).

Glycogen treatment enhanced the expression of mRNAs involved in the ceramide production pathway from sphingomyelin.

Quantitative real-time PCR was carried out to investigate the expression of mRNAs for molecules related to the ceramide production pathway (Fig. 2). Expression of serine palmitoyltransferase long chain base subunit 2 (SPTLC2) and serine palmitoyltransferase long chain base subunit 3 (SPTLC3), which are enzymes involved in de novo synthesis of ceramides, was not affected by glycogen (Figs. 2A and B). Expression of acid ceramidase (a-ceramidase), which catalyzes the hydrolysis of ceramides to generate sphingosine, was slightly downregulated by glycogen compared to its expression in control cells (Fig. 2C). Sphingomyelin synthase (SMS) is known to downregulate ceramide production,[19] and the result showed that expression of SMS1 was significantly reduced by incubation with glycogen compared to control cultures (Fig. 2D). SMS2 expression was also reduced, but the difference was not significant (Fig. 2E). Acid sphingomyelinase (a-SMase) is a member of a family of enzymes that catalyze degradation of sphingomyelin to produce ceramide, and expression of a-SMase mRNA was significantly increased by addition

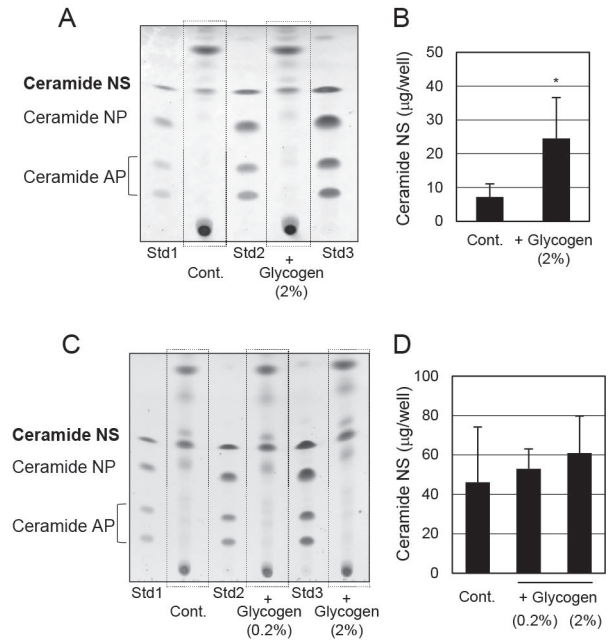

Fig. 1. Glycogen increases the ceramide NS content in cultured keratinocytes.

Sub-confluent keratinocytes were treated with or without 2 % glycogen for 7 days. After washing with PBS, the cells were collected and the intracellular lipids were extracted. The lipids were dissolved in 15 μL of chloroform and methanol mixture. The lipid solution (2 μL) was spotted onto a thin-layer silica gel plate. Ceramides were isolated by thin-layer chromatography (TLC). Standards of ceramide NS, NP, and AP were also spotted onto the plate (Std1: 0.7 μg/spot; Std2: 2.2 μg/spot; Std3: 6.7 μg/spot) (A). Digital analysis from developing the plate showed that ceramide NS in cells incubated with 2 % glycogen was 24.49±12.15 μg/well ($n = 6$), while ceramide NS was 7.17±3.93 μg/well in control cells without glycogen stimulation ($n = 6$) (B). Three-dimensional epidermal keratinocytes were treated with or without 0.2 and 2 % glycogen for 24 h. After washing with PBS, the cells were collected and the intracellular lipids were extracted. Ceramides were isolated by TLC method as described above (C). The digital analysis from developing the plate showed that ceramide NS in three-dimensional epidermal cells incubated with 0.2 and 2 % glycogen was 53.00±10.10 μg/well and 60.90±18.87 μg/well, respectively ($n = 3$), while ceramide NS was 46.18±28.00 μg/well in control cells without glycogen stimulation ($n = 3$) (D). Values are the mean ± SD. $*p < 0.05$, unpaired Student's *t*-test (B) and one-way Analysis of Variance (ANOVA) followed by Tukey test (D).

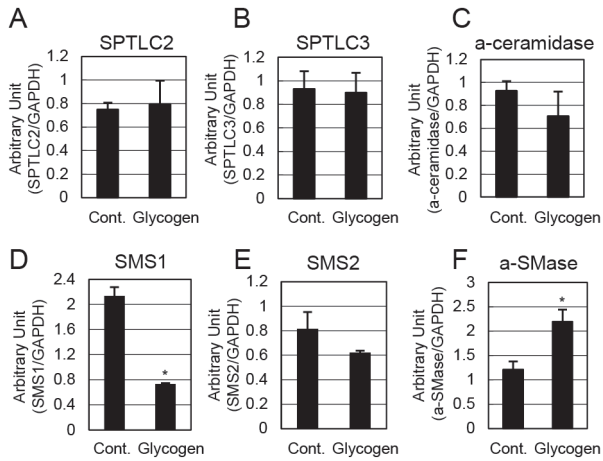

Fig. 2. Glycogen upregulates expression of mRNAs for molecules associated with ceramide production.

Keratinocytes were cultured in the presence of 2 % glycogen for 7 days. The cells were washed by PBS and collected for real-time PCR (n = 3–6). Expression of SPTLC 2 and SPTLC 3 was not affected by glycogen (A, B). Expression of a-ceramidase was slightly decreased by glycogen compared to the control level, but there was no significant difference (C). Expression of SMS1 was significantly downregulated by glycogen compared to the control level (D). Expression of SMS2 expression was also downregulated, but there was no significant difference (E). Expression of a-SMase was 1.8-fold higher in the presence of glycogen than in control cells (F). Values are the mean ± SD. *$p < 0.05$, unpaired Student's t-test.

of glycogen (Fig. 2F). These results suggest that glycogen regulates the sphingomyelin pathway in cultured keratinocytes.

Glycogen treatment enhanced a-SMase activity and ATP production.

Since expression of a-SMase mRNA was upregulated by glycogen, we measured a-SMase activity in keratinocytes after incubation with glycogen, revealing that a-SMase activity was significantly enhanced by 2.5-fold in cultures with 1 % glycogen (47.30±25.86 pmol/h/mg protein) compared to control cultures (18.56±11.47 pmol/h/mg protein) (Fig. 3A). These results suggested that glycogen increased the ceramide level in cultured keratinocytes by activating the pathway for ceramide production from sphingomyelin. It has been reported that a-SMase is activated by adenosine triphosphate (ATP),[20] so we also measured the ATP level in cultured keratinocytes. As shown in Fig. 3B, ATP production was significantly upregulated by culturing keratinocytes with 1 % and 2 % glycogen, but 1 % glucose did not induce ATP production. The results also showed that glycogen did not affect the cell viability of cultured keratinocytes.

Glycogen treatment inhibited the phosphorylation of Akt.

Serine-threonine kinase Akt is an intracellular mediator for many cellular processes, and its phosphorylation is known to be suppressed by ceramides, so the protein was analyzed. Western blotting showed that the cultured keratinocytes stimulated with glycogen for 10 min and 24 h inhibited Akt phosphorylation in a dose-dependent manner (Fig. 3C). The result indicates that glycogen affects some

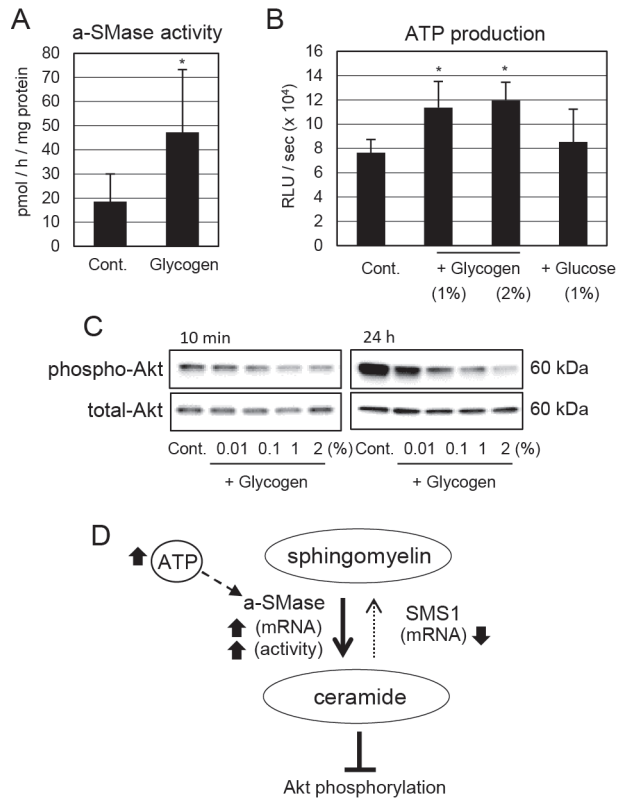

Fig. 3. Glycogen upregulates a-SMase activity and ATP production, and downregulates phosphorylation of Akt.

Keratinocytes were cultured in the presence of 1 % glycogen for 7 days. The cells were collected in PMSF solution, and a-SMase activity was measured. A-SMase activity was significantly increased in the presence of glycogen (n = 6) (A). Keratinocytes were cultured in the presence of 1 % and 2 % glycogen or 1 % glucose for 24 h. The cultured keratinocytes were collected for ATP measurement. ATP production was significantly upregulated by 1 % and 2 % glycogen, but 1 % glucose did not induce ATP production (n = 8) (B). Glycogen at the concentration of 0.01, 0.1, 1, and 2 % was added in sub-confluent keratinocytes and cultured for 10 or 24 h. The cells were collected and analyzed by Western blot. The phosphorylation of Akt (Ser473) was inhibited by the addition of glycogen, while the expression of total Akt did not change (C). Possible mechanism by which glycogen promotes the production of ceramide from sphingomyelin (D). Glycogen upregulates a-SMase mRNA expression and increases a-SMase activity to promote transformation of sphingomyelin into ceramide. Increased a-SMase activity may be mediated by enhanced ATP. Also, expression of SMS1 mRNA is downregulated by glycogen. Glycogen-induced ceramide inhibits Akt phosphorylation. Values are the mean ± SD. *$p < 0.05$, **$p < 0.01$, unpaired Student's t-test (A) and one-way Analysis of Variance (ANOVA) followed by Tukey test (B).

intracellular signal transduction pathways. Fig. 3D displays estimated mechanism by which glycogen may possibly stimulate the production of ceramide from sphingomyelin.

DISCUSSION

In this study, we demonstrated that glycogen enhances ceramide NS production in cultured epidermal keratinocytes by transducing the signals, but we could not identify the specific receptors on the cell membrane which recognized exogenous glycogen. It has been reported that glycogen binds directly to the toll-like receptor 2 (TLR2), and the signaling induces the production of nitric oxide and

inflammatory cytokines in macrophages.[9] TLR cascades are deeply associated with lipid rafts which are enriched in sphingolipids including ceramides in keratinocytes. In addition, TLR mRNA is constitutively expressed in keratinocytes.[21][22] Therefore, the first possibility is that glycogen binds to the TLRs and induces ceramide biosynthesis through the existing TLRs and lipid raft interactions. On the other hand, it has been shown that various molecules, including tumor necrosis factor-α, interferon type 1, and interleukin-1, activate signaling for the sphingomyelin cycle in human epidermis.[11] These molecules promote the degradation of sphingomyelin, thereby leading to an increase of the intracellular ceramide level. Then, the second possibility might also be that glycogen acts as a co-factor with them to enhance ceramide production. Our results revealed that glycogen transduces an important signaling to produce ceramide, thus, further studies are required to investigate the mechanism underlying the responses to glycogen and the signaling in the future.

We showed that incubation with glycogen enhanced the intracellular ATP level, while incubation with the same concentration of glucose did not affect ATP (Fig. 3B), suggesting that glycogen itself had this effect without undergoing degradation into glucose. In fact, glucose quantification did not detect extra glucose molecules other than the predominant glucose containing in the medium of cultured keratinocytes stimulated with glycogen for 24 h (data not shown). This means that extracellular glycogen is not degraded into glucose in keratinocyte culture. Therefore, this result suggested that glycogen functions, not only as an energy store, but also as a signal molecule. ATP is involved in many cellular processes, such as internalization of membrane components and intracellular signal transduction as a second messenger. Previous reports have shown that ATP depletion results in a decreased a-SMase activity.[23] Inhibition of a-SMase activity has been reported to attenuate the ATP-mediated hydrolysis of cell membrane sphingomyelin.[16] Both our study and previous observations suggest that ATP is one of the key factors associated with the increase of ceramide production through regulating the a-SMase activity.

Ceramide and its derivatives are involved in many cell signaling pathways, such as cell proliferation, differentiation, and apoptosis in epidermal keratinocytes.[24] It has been reported that ceramide may enhance cell apoptosis by inhibiting Akt activity.[25] Akt phosphorylation is found in keratinocytes of the skin, and its activity increases with age.[26] We showed that Akt phosphorylation was inhibited by glycogen treatment (Fig. 3C). The changing of Akt phosphorylation was occurred after glycogen treatment for 10 min and 24 h in cultured keratinocytes (Fig. 3C). The finding suggests that ceramides may be synthesized by glycogen treatment within 10 min and inhibit Akt phosphorylation. We conclude that glycogen may be a new target for triggering dynamic cellular responses.

We have previously investigated the effect of glycogen in cosmetic products on the human skin. The results showed that glycogen was effective in enhancing the skin moisture (data not shown). Since the amount of ceramide in the epidermis is important for skin moisture, our results support the possibility of glycogen in improving the skin moisture. This highlights the new benefit of glycogen and the possible use of glycogen as a cosmetic ingredient.

In conclusion, glycogen enhances ceramide NS production in keratinocytes. The study showed that glycogen upregulates the expression of mRNAs for molecules involved in the pathway for ceramide production from sphingomyelin. In addition, glycogen enhances a-SMase activity and ATP production related to ceramide production. Accordingly, we propose that glycogen might be effective for maintaining skin health through enhancement of ceramide production. The insights into the role of glycogen may be useful for understanding the cellular signal transduction and activation of skin cells.

CONFLICTS OF INTEREST

Hiroko Yatsuhashi, Takashi Furuyashiki, Phuong Hong Thi Vo, and Hiroshi Kamasaka, are employees of Institute of Health Sciences, Ezaki Glico Co., Ltd.

Takashi Kuriki is Board Director at Ezaki Glico Co., Ltd.

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