Hydrolysis of sphingomyelin to ceramide induced by heat-killed *Lactoplantibacillus plantarum* APsulloc 331261 via neutral sphingomyelinase 2 activation protects against Staphylococcal α-toxin–induced cytotoxicity in keratinocytes

Minjung Chae[1*], Kyung-mi Joo[1], Donghyun Cho[1], Jonghwa Roh[1], Wangi Kim[1]

[1] AMOREPACIFIC Research and Innovation Center, AMOREPACIFIC, Yongin, Korea, Republic of (South)

* Minjung Chae, AMOREPACIFIC, Yongin 17074, Korea, Tel: 82-31-280-2694, Fax: 82-31-899-2595, E-mail: minjungc@amorepacific.com

Abstract

Background: Oral or topical application of probiotics has shown to provide incredible health benefits to atopic dermatitis (AD). In the present study, we investigated the effect of heat-killed *Lactoplantibacillus plantarum* APsulloc 331261 (APsulloc 331261) on the mechanism of ceramide synthesis and *S. aureus* α-toxin-induced toxicity.

Methods: Heat-killed APsulloc 331261 lysates were prepared by heating cell suspension (10^9 CFU/mL) to 80 ℃/10 min. Normal human epidermal keratinocytes (NHEKs) were incubated with heat-killed APsulloc 331261. The trasncript levels of neutral phingomyelinase 2 (SMPD3) were analyzed by quantatative real-time PCR (qRT-PCR) and the SMPD3 activity was measured by neutral sphingomyelinase activity assay kit. Lipids level was accessed by lipid chromatography-mass spectrometry. Finally, cytotoxicity was measured by lactate delyrogenenase release.

Results: Heat-killed APsulloc 331261 stimulated SMPD3 mRNA expression and neutral sphingomyelinase activity under both low and high calcium conditions in NHEKs. SMPD3 expression was increased during keratinocyte differentiation and the upregulation of SMPD3 expression by heat-killed APsulloc 331261 was mediated by peroxisome proliferator-activated receptor delta (PPAR delta). As a result of SMPD3 activation, ceramide, especially ceramide NS, synthesis was increased. Heat-killed APsulloc 331261 protected staphylococcal alpha-toxin-induced cell death, presumably due to the reduction of sphingomyelin, which is known to a receptor of alpha-toxin.

Conclusion: Heat-killed APsulloc 331261 are potential bioactive supplement to provide health benefits to AD treatment.

Keywords: *Lactoplantibacillus plantarum* APsulloc 331261; SMPD3; sphingomyelin; ceramide; alpha-toxin; atopic dermatitis

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease associated with skin barrier dysfunction as the initial step of the development of AD. The function of skin barrier is based on lipid matrix, which are composed of ceramides, cholesterol and free fatty acids in the outermost layer of the skin, the stratum corneum (SC). In particularly, reduction of ceramides in the SC is involved in barrier impairment in AD [1]. In addition, the colonization of *Staphylococcus aureus* (*S. aureus*) in the skin have an important role in the pathogenesis of AD [2]. Recently, oral or topical application of probiotics has shown to provide incredible health benefits to AD treatment [3]. Although the effect of a selected probiotic extract in increasing ceramide levels on the SC in AD patients is reported [3], there was no direct evidence how probiotic extract can modulate ceramide production and *S. aureus* α-toxin sensitivity in human keratinocytes.

Materials and Methods

APsulloc 331261 adjusted to 10^9 cfu/ml was heat-killed at 80℃ for 10 min in a water bath. Normal human epidermal keratinocytes (NHEKs) were incubated with heat-killed APsulloc 331261 at various concentrations (0, 10^7, and 10^8 cfu/ml) under either low calcium (50 μM) or high calcium (1.2 mM). mRNA expression responsible for ceramide synthesis was determined using quantitative real time PCR (qRT-PCR) and SMPD3 activity was measured by neutral sphingomyelinase activity assay kit. Lipids of NHEKs were extracted by Bligh Dyer method and analyzed ceramide species (NDS, NS, NP, AP, and AS) by liquid chromatography-mass spectrometry. Alpha-toxin-induced cytotoxicity was measured by lactate dehydrogenase release. Statistical comparisons were performed using Student's *t*-test between two groups or one-way ANOVA test within multiple groups, followed by Turkey's post hoc test.

Results

We found that, among ten genes responsible for ceramide synthesis, SMPD3 which catalyzes the hydrolysis of sphingomyelin to form ceramide and phosphocholine, was the only gene

that was upregulated by heat-killed APsulloc 331261 under both low and high calcium conditions in NHEKs (Fig. 1A, B). Sphingomyelinase activity also showed that heat-killed APsulloc 331261 increased SMPD3 activity in both low and high calcium conditions (Fig 1C).

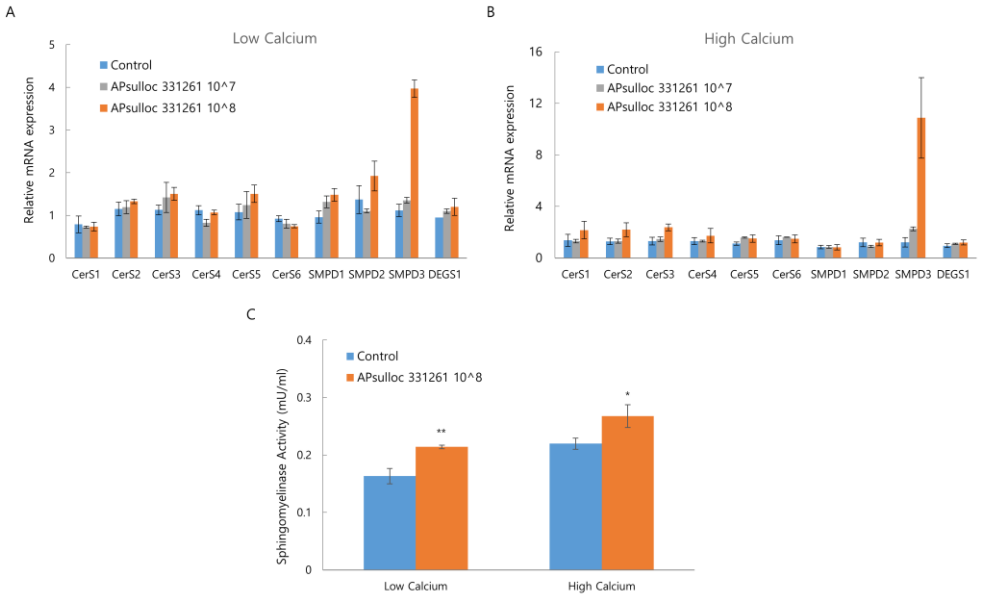

Figure 1. Heat-killed APsullloc 331261 stimulates SMPD3 expression in NHEKs under both low and high calcium conditions. NHEKs were treated wtih different concentrations (0, 10^7, and 10^8 cfu/ml) of heat-killed APsulloc 331261 under either low calcium (50 μM) (A) or high calcium (1.2 mM) (B) and cultured for 24 h. Total RNA was extracted from the cells, and relative mRNA levels of ceramide synthesis genes were measured via qRT-PCR. C. NHEKs treated with heat-killed APsulloc 331261 at 10^8 cfu/ml were cultured for 5 days. The cells were lysed and analyzed biochemically for the determination of neutral sphingomyelinase activity via sphingomyelinase activity assay kit. All data represent the mean±SD of three independent experiments. Significant differences: **P*<0.05, ***P*<0.01 compared with the control

Unlike SMPD1 and SMPD2, mRNA expression of SMPD3 was drastically upregulated during NHEK differentiation (Fig, 2A). Since peroxisome proliferator-activated receptors (PPARs) are known to govern epidermal lipid synthesis and metabosim [4], the expression

level of PPARs were measured. Heat-killed APsulloc 331261 at 10^8 cfu/ml upregulated PPAR delta expression regardless of calcium concentration (Fig. 2B) and the increase of SMPD3 expression induced by heat-killed APsulloc 331261 was blunted PPAR delta antagonist (GSK3787) (Fig. 2C), suggesting PPAR delta dependent mechanism of action of heat-killed APsulloc 331261.

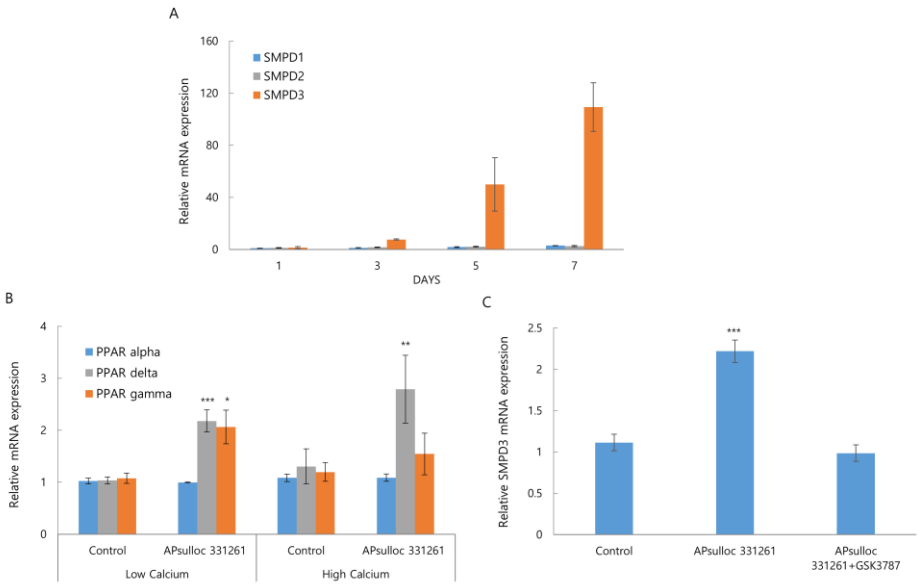

Figure 2. SMPD3 is upregulated during keratinocyte differentiation and the induction of SMPD3 expression by heat-killed APsulloc 331261 is mediated via PPAR delta activation. A. NHEKs were cultured for the indicated days (1, 3, 5, and 7 days) and relative mRNA levels of SMPD1, SMPD2, and SMPD3 were measured via qRT-PCR. B. NHEKs were treated wtih heat-killed APsulloc 331261 at 10^8 cfu/ml under either low calcium (50 μM) or high calcium (1.2 mM) and cultured for 24 h. Total RNA was extracted and anaylzed for relative mRNA levels of PPAR alpha, PPAR delta, and PPAR gamma via qRT-PCR. C. NHEKs were treated with heat-killed APsulloc 331261 at 10^8 cfu/ml in the presence of PPAR delta antagonist (GSK3787, 10 μM) and cultured for 5 days. Relative mRNA levels of SMPD3 were measured via qRT-PCR. All data represent the mean±SD of three independent experiments. Significant differences: **P*<0.05, ***P*<0.01, ****P*<0.001 compared with the control

To confirm ceramide formation by SMPD3 activation in NHEKs, ceramide contents were analyzed in NHEKs treated with heat-killed APsulloc 331261 at 10^8 cfu/ml and cultured for 5 days. Lipid analysis showed that heat-killed APsulloc 331261 resulted in elavated ceramide NS contents at day 5 (Fig. 3A). Because ceramide NS was the most abundant ceramide subclass and other cermide subclass such as NDS, NS, NP, AP, and AS accounted for less than 5% of total ceramides in NHEKs, representative ceramide NS was presented in our data. The upregulation of ceramide NS level was diminished by SMPD3 inhibitor (GW4869) (Fig. 3B). Collectively, Heat-killed APsulloc 331261 contributes to the upregulation of ceramide synthesis, which might be mediated by SMPD3 activation.

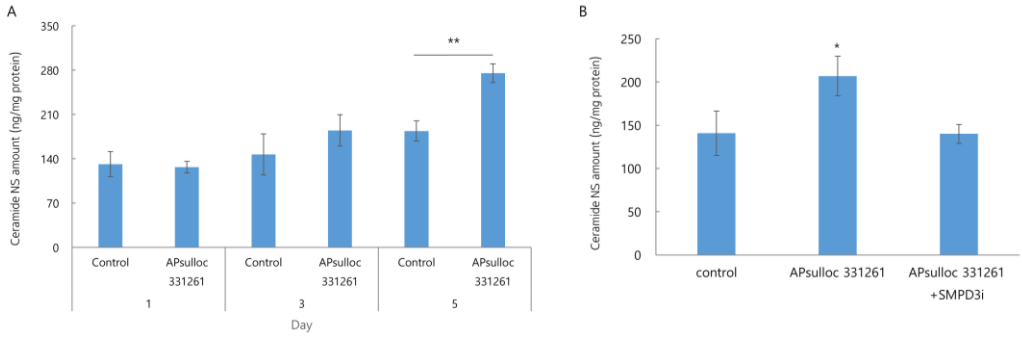

Figure 3. Upregulation of Ceramide NS synthesis is induced by APsulloc 331261 via SMPD3 activation. A. NHEKs treated with heat-killed APsulloc 331261 at 10^8 cfu/ml were cultured for the indicated days (1, 3, and 5 days). Total lipids were extracted from the cells by Bligh Dyer method and analyzed to quantifiy ceramides by liquid chromatography-mass spectrometry. B. NHEKs were treated with heat-killed APsulloc 331261 at 10^8 cfu/ml in the presence of SMPD3 inhibitor (GW4869, 1 μM) and cultured for 5 days. Total lipids were extracted from the cells and analyzed to quantifiy ceramides. All data represent the mean±SD of three independent experiments. Significant differences: *P<0.05, **P<0.01compared with the control

Finally, to determine whether sensitivity to staphylococcal alpha-toxin is affected by keratinocyte differentiation, NHEKs were cultured for 5 days in either low or high calcium conditions. In the presence of 1 nM alpha-toxin, NHEKs cultured in low calcium condition

were more sensitive to the toxic effects of alpha-toxin compared to NHEKs cultured in high calcium condition (Fig. 4A), suggesting differentiation process protects keratinocytes against alpha-toxin-induced cytotoxicity, presumably due to the down-regulation of a receptor for alpha-toxin during differentiation. Because reduction in sphingomyelin, a substrate for SMPD3 and a known receptor for alpha-toxin [5], due to the enzymatic activity of SMPD3 could protect against alpha-toxin-mediated toxicity, we determined the effect heat-killed APsulloc 331261 on alpha-toxin-mediated cytoxicity in NHEKs cultured in both low and high calcium conditions. Our data indicated that heat-killed APsulloc 331261 protected against alpha-toxin-induced cell death and the blockade of alph-toxin cytotoxicity mediated by heat-killed APsulloc 331261 was abrogated by SMPD3 inhibitor (GW4869) (Fig. 4B, C). Taken together, heat-killed APsulloc 331261 protects cells from staphylococcal alph-toxin-induced cytoxicity via SMPD3 activation.

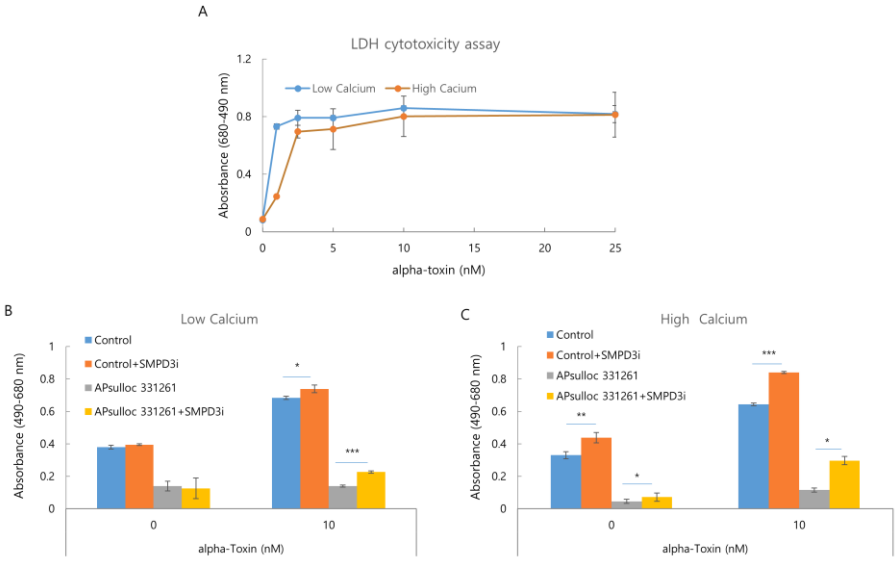

Figure 4. Heat-killed APsulloc 331261 protects against staphylococcal alph-toxin-mediated cell death via SMPD3 activation. A. NHEKs were cultured under either low calcium (50 μM) or high calcium (1.2 mM) and cultured for 4 days. The cells were treated wtih different concentrations (0, 1, 2.5, 5, 10, and 25 nM) of alpha-toxin for additional 24 h. Culture media were harvested and cell death was measured based on lactate dehydrogenase (LDH) release. Absorbance at 490 nm and 680 nm was mesured to determine LDH activity.

B. NHEKs were treated wtih at 10^8 cfu/ml in the presence of SMPD3 inhibitor (GW4869, 1 μM) under either low calcium (50 μM) (B) or high calcium (1.2 mM) (C) and cultured for 4 days. The cells were treated wtih 10 nM alpha-toxin for additional 24 h. Culture media were harvested and cell death was meausred based on lactate dehydrogenase (LDH) release. Absorbance at 490 nm and 680 nm was mesured to determine LDH activity. All data represent the mean±SD of three independent experiments. Significant differences: **P*<0.05, ***P*<0.01, ***<0.001 compared with the control

Discussion

The present study demonstrated that heat-killed APsulloc 331261 stimulated SMPD3 expression, resulting in the increase of ceramide presumably at the expense of sphingomyelin. SMPD3 expression was upregulated during NEHK differentiation and the induction of SMPD3 expression dependent on heat-killed APsulloc 331261 was mediated by PPAR delta activation. PPARs are ligand-activated nuclear receptors, improving epidermal lipid homeostsis and barrier function. In particular, PPAR delta signaling is known to control epidermal lipid processing [4]. Thus, SMPD3 might to be one of PPAR delta target genes and control epidermal lipid synthesis in keratinocytes. Alpha-toxin-produced by *S. aureus* is the main agent that trigger the AD skin lesions [6]. Heat-killed APsulloc 331261 could confer protection against alpha-toxin–induced cell death. The action mechanism underlying the beneficial effects of heat-killed APsulloc 331261 on the alpha-toxin–induced cytotoxicity may be attributed to possibly the reduction of the number of α-toxin binding sites on the cell surface due to enzymatic activity of SMPD3-mediated cleavage of sphingomyelin because sphingomyelin is one of the known α-toxin receptors [5]. Our data that SMPD3 expression is increased during keratinocyte differentiation and keratinocyte differentiation contributes to the blockade of alpha-toxin induced cell death implies that a receptor for alph-toxin especially sphingomyelin, could be down-reugulated by SMPD3 activity during differentiation. Thus, increase in SMPD3 activity during keratinocyte differentiation could contribute to prevent cell death from alpha-toxin. The effect of heat-killed APsulloc 331261 on sphingomyelin level is undergoing in our laboratory. Moreover, because the number of alpha-toxin receptors is reflected by the amount of alpha-toxin heptamer bound to the host cell surface [5, 7], alpha-toxin heptamer binding mediated by heat-killed APsulloc 331261

should be further determined. These results suggest that heat-killed APsulloc 331261 could contribute to the improvement of lipid barrier and a more effective resistance against *S. aureus* infection. Thus, heat-killed APsulloc 331261 are potential bioactive supplement to provide health benefits to skin health.

Conclusion

In current study, heat-killed APsulloc 331261 was shown to significantly upregulate SMPD3 expression, which hydrolyze sphingomyelin to ceramide and subsequently increase the production of ceramides from keratinocyres. Moreover, heat-killed APsulloc 331261 protected against staphylococcal alpha-toxin-induced keratinocytes death. These results suggest that heat-killed APsulloc 331261 are potential supplements for AD treatment.

Acknowledgments

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Conflict of Interest Statement

NONE.

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