Lysophosphatidic acid inhibits proliferation of *Cutibacterium acnes* by promoting human beta-defensin-3 expression via LPA1 receptor

Natsuki Toda* [1], Mizuki Kobayashi[1], Koji Kurihara[1], Yoko Niki[2], Yuki Katsumata[1]

1 Nippon Fine Chemical CO., LTD. Takasago, Japan

2 Pharm. Sci., Mukogawa Women's Univ., Nishinomiya, Japan

1. Introduction

Lysophosphatidic acid (LPA) is a phospholipid mediator present in the body that activates signal transduction through six specific receptors (LPA1-6) [1] and is known to regulate various physiological functions such as cell proliferation, migration, and differentiation [2,3]. In cosmetics, LPA has been reported to improve the barrier function of the epidermis by enhancing tight junctions and promoting the maturation of the cornified envelope [4,5]. Furthermore, it has been confirmed that LPA induces pore shrinkage by promoting the contractile force of cells through the polymerization of actin, a component of the cytoskeleton [6].

Acne vulgaris is a skin disorder predominantly observed in adolescents and young adults [7]. It is characterized by non-inflammatory comedones and inflammatory papules, pustules, nodules, and cysts, which can lead to scarring and skin pigmentation. The primary factors contributing to this condition include excessive sebum production, abnormal follicular keratinization, overgrowth of *Cutibacterium acnes* (*C.acnes*), abnormal immune responses, and epigenetic modifications, such as DNA methylation. The relationship between acne vulgaris and *C.acnes* is well documented. *C.acnes* secrete lipase, which metabolizes sebaceous triglycerides into glycerol and fatty acids, leading to comedones formation and skin inflammation [8].

The skin possesses innate defense mechanisms against microbes, including antimicrobial peptides. Human beta-defensins (hBDs) are representative examples of these peptides. hBDs are cationic peptides composed of 18-45 amino acids. They form multimers on the bacterial cell membrane through electrostatic interactions, creating pores that exhibit broad-spectrum antimicrobial activities against bacteria, fungi, and viruses [9]. In the skin, the expression of hBD-1, hBD-2, hBD-3, and hBD-4 has been identified, with hBD-3 exhibiting antimicrobial activity against *C.acnes* [10]. Additionally, the pathway for hBD-3 induction involves the activation of cyclooxygenase-2 mediated by Toll-like receptors, leading to prostaglandin D_2 (PGD_2) production [11].

In our study, we discovered that LPA significantly increases hBD-3 levels. We investigated the impact of LPA on the growth of *C.acnes* and the mechanism by which LPA promotes hBD-3.

2. Materials and Methods

2.1. Materials

Normal human epidermal keratinocytes (NHEKs) HuMedia-KB2 and HuMedia-KG2 were purchased from Kurabo (Osaka, Japan). Reconstructed human epidermis (RHE) model was obtained from J-TEC (Aichi, Japan). ONO-7400234 was obtained from Selleck Biotechnology (Kanagawa, Japan). Bovine serum albumin (BSA) and primers for hBD-1, hBD-2, hBD-3, hBD-4, and GAPDH were purchased from Merck (Darmstadt, Germany). BCA protein assay kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). The RNeasy Mini Kit was purchased from Qiagen (Hilden, Germany). PrimeScript RT Master Mix was acquired from Takara Bio (Shiga, Japan). KAPA SYBR FAST was purchased from Kapa Biosystems (Wilmington, MA, USA). Anti-hBD-3 antibody was obtained from Novus Biologicals (Littleton, CO, USA). Peroxidase-labeled anti-mouse IgG polyclonal antibody (Fab') was purchased from Nichirei (Tokyo, Japan). Recombinant human BD-3 was purchased from PeproTech (Rocky Hill, NJ, USA). ELISA POD Substrate TMB Kit was procured from NACALAI TESQUE (Kyoto, Japan). Prostaglandin D_2 (Competitive EIA) ELISA Kit was procured from LifeSpan BioSciences (Seattle, WA, USA). 4% paraformaldehyde phosphate-buffered solution (PFA) and Triton X-100 were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). 4′,6-diamidino-2-phenylindole (DAPI) was procured from Dojindo Laboratories (Kumamoto, Japan). Alexa Fluor 546-conjugated goat anti-rabbit IgG (H+L) secondary antibody was purchased from Invitrogen (Carlsbad, CA, USA). RIPA Lysis Buffer was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

2.2. Cell culture and treatment of LPA and LPA1 receptor inhibitor

NHEKs were cultured in HuMedia-KG2, a medium supplemented with growth factors at 37°C and 5% CO2 atmosphere. The culture media were replaced with HuMedia-KB2, a basal medium without growth factors, and the NHEKs were incubated overnight. After the NHEKs were pre-cultured, they were treated with 0.01% LPA glycerin solution in HuMedia-KB2. In the mechanistic analysis, NHEKs were pretreated with 10 μmol/L ONO-7400234, which is the inhibitor of the LPA1 receptor, for 1 h before LPA treatment and additionally during LPA treatment.

2.3. Comprehensive analysis of gene expression profiles

Microarray analysis was conducted to identify skin-related factors. NHEKs were treated with LPA for 24 h, frozen at -80°C with QIAzol® Lysis Reagent, and RNA was purified using the miRNeasy® Mini Kit (QIAGEN). The collected RNA was used to prepare biotin-labeled targets for DNA microarray analysis using Clariom S. Microarray data were analyzed using Transcriptome Viewer.

2.4. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

The mRNA expression levels of hBD-1, hBD-2, hBD-3, and hBD-4 in NHEKs treated with or without LPA and ONO-7400234 were quantified by RT-qPCR. Total RNAs were extracted by RNase Mini Kit from NHEKs treated with LPA for 4 h, and cDNAs were synthesized using PrimeScript RT Master Mix. RT-qPCR was performed using SYBR Green Gene Expression Assay with a Real-Time PCR System (Takara Bio). The primers used in this study are listed in Table 1.

Table 1. Primer sequences of target genes

Target genes		Sequence (5′-3′)
hBD-1	F	TTGTCTGAGATGGCCTCAGGTGGTAAC
	R	ATACTTCAAAAGCAATTTTCCTTTAT
hBD-2	F	CCAGCCATCAGCCATGAGGGT

hBD-2	R	GGAGCCCTTTCTGAATGAACCGCA
hBD-3	F	CTGTTTTTGGTGCCTGTTCC
	R	CTTTCTTCGGCAGCATTTTC
hBD-4	F	AGCCCCAGCATTATGCAGAGA
	R	GCGACTCTAGGGACCAGCACTAC
GAPDH	F	ACCACAGTCCATGCCATCAC
	R	TCCACCACCCTGTTGCTGTA

2.5. ELISA

NHEKs were treated with or without LPA and ONO-7400234 for 48 h, and the expression of the hBD-3 peptide in the supernatants was measured using ELISA. The supernatants from treated NHEKs were immobilized on an ELISA plate overnight at 4°C. After blocking with PBS(-) containing 1% BSA for 1 h, the samples were incubated with an anti-hBD-3 antibody (1:1000) overnight at 4°C and then with a peroxidase-labeled anti-mouse IgG polyclonal antibody (Fab') (1:100) for 2 h at room temperature. Finally, the ELISA POD Substrate TMB Kit was added and incubated at 37°C for 15 min, and the absorbance at 450 nm was measured. The standards were prepared using recombinant human BD-3.

RHE models were treated with 0.1% LPA from the basal side, and hBD-3 was quantified using the cell lysate obtained by adding RIPA Lysis Buffer and disrupting the cells with ultrasound.

The expression of PGD_2 in the supernatants from NHEKs pretreated with ONO-7400234 for 1 h and then treated with or without LPA and ONO-7400234 for 1 h was quantified using Prostaglandin D_2 (Competitive EIA) ELISA Kit.

NHEKs were lysed with 0.5% Triton X-100, and proteins were quantified using a BCA assay. For the RHE models, the protein content of the cell lysates was quantified. The levels of hBD-3 and PGD_2 were normalized to the protein content.

2.6. hBD-3 immunofluorescence

RHE models were treated with 0.1% LPA for 48 h. After frozen sections were prepared and fixed with PFA for 15 min, the models were blocked using PBS(-) containing 1% BSA. The models were incubated with anti-hBD-3 antibody (1:500) overnight at 4°C and Alexa Fluor 546-conjugated goat anti-rabbit IgG (H+L) secondary antibody (1:1000) for 2 h at room temperature. The nuclei were stained with PBS(-) containing 1 μg/mL DAPI for 30 min at room temperature. The stained cells were observed using a fluorescence microscope (KEYENCE).

2.7. *C.acnes* proliferation inhibition assay

NHEKs were treated with LPA for 48 h, and conditioned media were collected and used for proliferation inhibition assays against *C.acnes*. The conditioned media were inoculated with GAM medium and *C.acnes* (NBRC 107605 (ATCC 6919)) to achieve a concentration of 5×10^3 CFU/mL and were incubated under anaerobic conditions at 37°C. Viable cell counts were measured using the colony counting method at 0, 24, 48, and 72 h.

2.8. Statistical analysis

Data are expressed as the mean ± standard deviation. Statistical significance was analyzed using a one-way analysis of variance with the Tukey-Kramer post hoc test or Student's *t*-test, as appropriate. Statistical significance was set at $p<0.05$.

3. Results

3.1. LPA upregulated several antimicrobial peptides

To explore the new functions of LPA, we performed a comprehensive gene analysis and found that the expression of antimicrobial peptides tended to be promoted (Table 2). Therefore, we focused on representative antimicrobial peptides: hBDs. The expression of *hBD-1*, *hBD-2*, *hBD-3*, and *hBD-4* was examined using RT-qPCR, and the results showed that *hBD-1* and *hBD-3* were significantly upregulated by LPA (Figure 1).

Table 2. mRNA expression levels of antimicrobial peptides in comprehensive gene analysis

Function	Target gene	Change holds	*p* value
Beta-defensin	DEFB1	2.06	0.007
Beta-defensin	DEFB4A	1.35	0.146
Beta-defensin	DEFB103A	13.88	0.000
Beta-defensin	DEFB103A	16.98	0.000
Psoriasin/S100A7	S100A7	8.29	0.000

The results of various gene expression analyses are presented as ratios, with the values of untreated cells (control) normalized to 1. Means (n=3), Student's *t*-test.

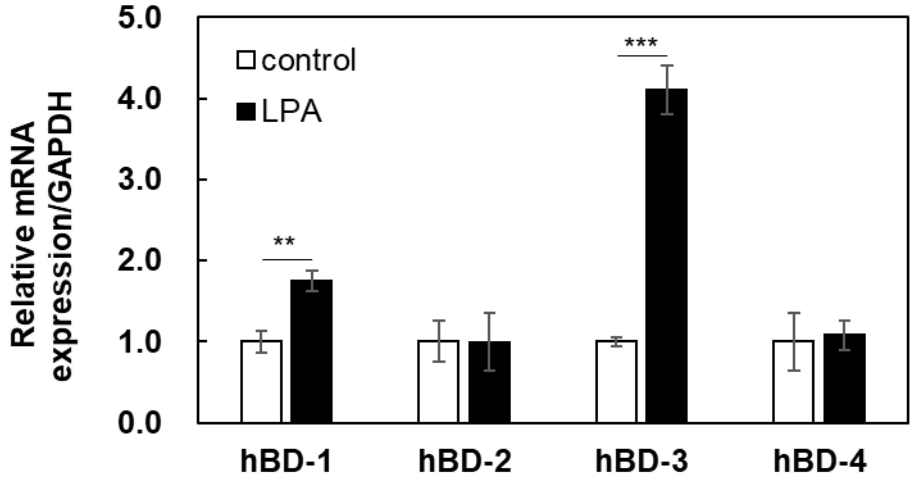
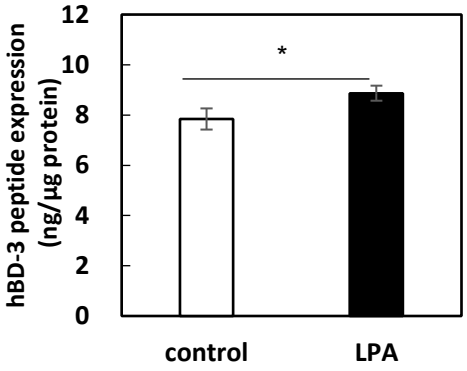


Figure 1. mRNA expression levels of hBDs in NHEKs treated with LPA

NHEKs were treated with LPA for 4 h. Expression of *hBD-1*, *hBD-2*, *hBD-3*, and *hBD-4* mRNAs in NHEKs treated with LPA. Means ± SD (n=3), Significant: **p*<0.05, ***p*<0.01, ****p*<0.001 vs control, Student's *t*-test.

3.2. LPA promoted hBD-3 peptide in RHE models

Because hBD-3 was significantly increased in NHEKs, we investigated whether similar effects could be observed in RHE models, which closely mimic the structure of the human skin, by considering the penetration process of LPA. In RHE models treated with LPA, expression of the hBD-3 peptide was examined using immunofluorescence staining and ELISA. Consequently, we confirmed that the expression of the hBD-3 peptide increased following LPA treatment (Figure 2).



(**a**) (**b**)

Figure 2. Expression of the hBD-3 peptide in RHE models treated with LPA

RHE models were treated with LPA for 48 h. (a) Expression of the hBD-3 peptide in RHE models treated with LPA was observed using immunofluorescence staining (red: hBD-3, blue: nuclei). Scale bar: 100 μm. (b) Expression of the hBD-3 peptide in RHE models treated with LPA was quantified using ELISA. Means ± SD (n=3), Significant: **p*<0.05, vs control, Student's *t*-test.

3.3. The supernatant from NHEKs treated with LPA inhibited proliferation of *C.acnes*

We evaluated whether the LPA-induced increase in hBD-3 affected the proliferation of *C.acnes*. The conditioned media from NHEKs treated with LPA inhibited the proliferation of *C.acnes* in a time-dependent and effective manner (Figure 3).

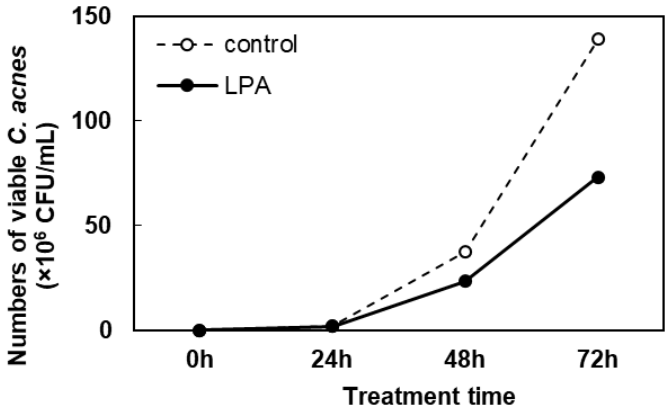
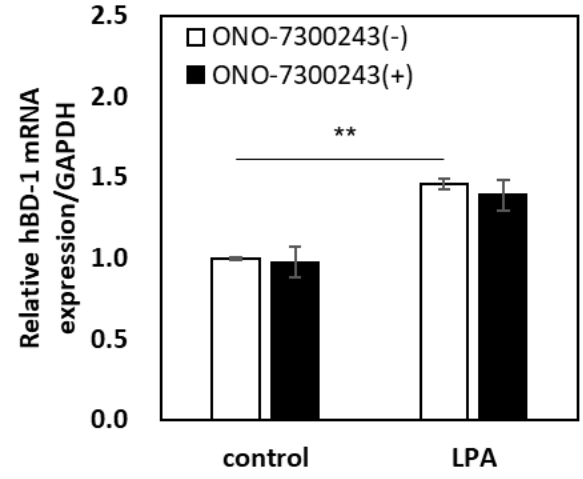


Figure 3. Time-dependent changes in the effect of supernatant from NHEKs treated with LPA on *C.acnes*

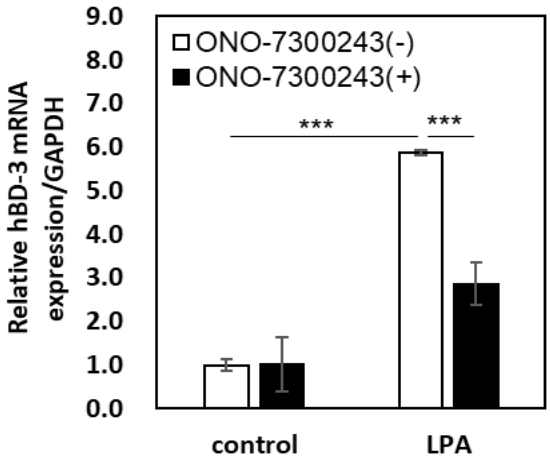
Supernatants from NHEKs treated with LPA for 48 h were applied to *C.acnes*, and the number of viable *C.acnes* was measured using the colony counting method.

3.4. LPA promoted hBD-3 expression via LPA1 receptor

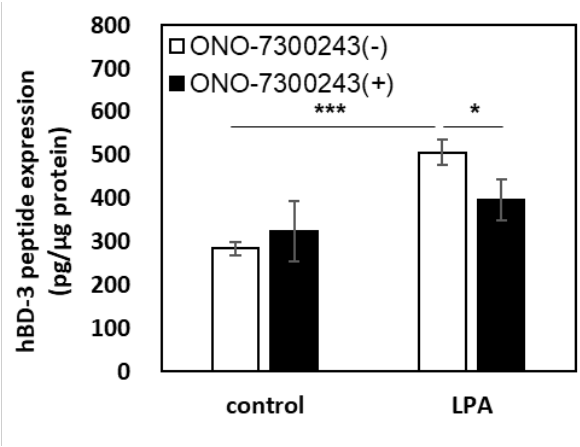
Since LPA promotes the mRNA expression of *hBD-1* and *hBD-3*, we examined the relationship between LPA receptors and the expression of hBD-1 or hBD-3. The increase in *hBD-1* mRNA levels was not affected by ONO-7400234 treatment (Figure 4a). However, the LPA-induced increase in *hBD-3* mRNA levels was partially inhibited by ONO-7400234 (Figure 4b). This effect was similar to that observed during the quantification of peptide expression using ELISA (Figure 4c).



(**a**)



(**b**)



(**c**)

Figure 4. hBD-1 and hBD-3 expression in NHEKs treated with LPA and LPA1 receptor inhibitor

NHEKs were pretreated with ONO-74000234 for 1 h and treated with LPA and ONO-74000234 for 4 h or 48 h. (a), (b) Expression of the *hBD-1* or *hBD-3* mRNA in NHEKs treated with LPA and ONO-74000234 for 4 h. (c) Expression of the hBD-3 peptide in supernatants form NHEKs treated with LPA and ONO-74000234 for 48 h. Means ± SD, Significant: **p*<0.05, ***p*<0.01, ****p*<0.001 vs control, Tukey-Kramer's test.

3.5. LPA promoted PGD_2 expression via LPA1 receptor

To further analyze the mechanism of action of LPA and hBD-3, we investigated the effect of LPA and the LPA1 receptor on PGD_2, which promotes hBD-3 expression. PGD_2 expression was promoted by LPA and significantly inhibited by an LPA1 receptor antagonist (Figure 5).

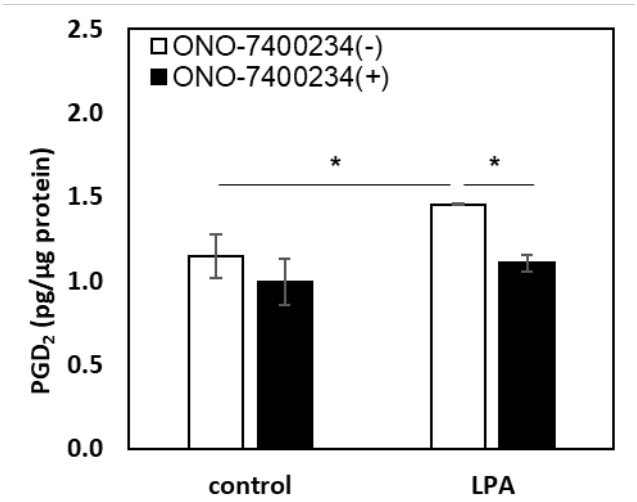


Figure 5. PGD_2 expression in NHEKs treated with LPA and LPA1 receptor inhibitor

NHEKs were pretreated with ONO-74000234 for 1 h and then treated with LPA and ONO-74000234 for 1 h. PGD_2 expression in supernatants from NHEKs treated with LPA and ONO-74000234 for 4 h. Means ± SD (n=3), Significant: *p<0.05, vs control, Tukey-Kramer's test.

4. Discussion

Our results show that LPA may promote several antimicrobial peptides in NHEKs. Specifically, the expression levels of hBD-1 and hBD-3 increased. In particular, the gene expression of hBD-3 was significantly increased, and the promotion of the hBD-3 peptide by LPA was also observed in RHE models that consider the penetration process. Additionally, the supernatant of NHEKs treated with LPA significantly suppressed the proliferation of *C.acnes*. Since LPA itself does not have antimicrobial effects against *C.acnes* (data not shown) and hBD-3 exhibits antimicrobial activity against *C.acnes* [10], hBD-3 promotion by LPA may influence this effect. Therefore, cosmetics containing LPA are expected to effectively treat acne by inhibiting the proliferation of *C.acnes*. Furthermore, a decrease in skin barrier function is associated with the worsening of acne [8]. As LPA improves skin barrier function [5,6], LPA can approach acne care from multiple perspectives.

In the present study, we aimed to elucidate the mechanisms by which LPA promotes hBDs. Given that LPA receptors are deeply involved in various physiological activities of LPA, we examined their roles. Although the promotion of hBD-1 expression by LPA was not inhibited by ONO-7400234, the promotion of hBD-3 expression was partially suppressed by ONO-7400234. This result suggests that the promotion of hBD-3 expression by LPA is partly mediated by the LPA1 receptor. Further examination of the downstream signals revealed that PGD_2, which has been reported to promote hBD-3 expression, was increased by LPA and suppressed by ONO-7400234. This finding indicates that PGD_2 facilitates LPA-mediated hBD-3 through the LPA1 receptor. In summary, as shown in Figure 6, LPA induces PGD_2 and hBD-3 expression through the LPA1 receptor, thereby inhibiting the proliferation of *C.acnes*.

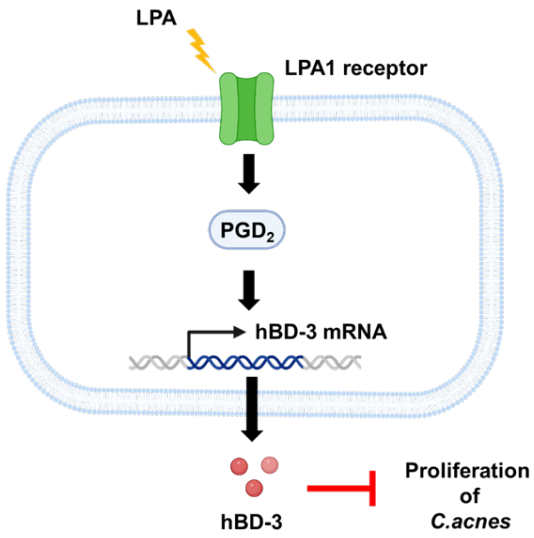


Figure 6. Mechanism of hBD-3 expression promotion and *C.acnes* suppression by LPA

In this study, we focused on the hBD-3-promoting effect of LPA. However, LPA exhibits high biological activity while potentially inducing sensory stimulation via the activation of Transient Receptor Potential Vanilloid 1 (TRPV1) [12]. We found that combining LPA with aluminum hydroxide suppresses the influx of calcium ions into cells induced by TRPV1 activation [5]. This combination is expected to alleviate skin stimulation and make it safer for use.

5. Conclusion

In conclusion, LPA may promote hBD-3 expression through PGD_2 induction via the LPA1 receptor, thereby inhibiting the proliferation of *C.acnes*. Therefore, LPA is expected to be a novel compound that can contribute to acne care.

6. References

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