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포스파티딜세린의 각질세포 분화 유도를 통한 피부장벽 기능 강화

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Phosphatidylserine Enhances Skin Barrier Function Through Keratinocyte Differentiation

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요 약: 포스파티딜세린(Phosphatidylserine; PS)은 생체막에서 구조적인 역할을 담당하는 인지질로서, 생체 내 다양한 세포작용에 필수적인 신호전달 효소의 보조인자로서 작용하는 것으로 알려져 있다. 하지만, PS의 생리활성에 대한 연구는 거의 이루어지지 않았고, 특히 피부에서의 생리활성에 대한 연구는 전무한 실정이다. 본 연구에서는 무모생쥐의 피부에 tape-stripping으로 경표피수분손실(TEWL)의 증가를 유도한 후, PS를 도포함으로써 그 손실을 현저히 감소시켰다. 또한, PS 도포군의 피부에서 세라마이드 함량이 증가된 사실을 확인한 바 있다. PS 도포군에서 non-hydroxyl 세라마이드와 glucosyl 세라마이드의 함량이 비처리군과 비교하여 각각 1.4배와 1.6배로 증가하였다. PS는 또한 피부각질세포의 분화를 촉진하였다. 피부각질세포에 PS를 처리함으로써 세포 형태가 분화상을 띄고 있음을 현미경 상에서 확인하였고, 표피분화의 특이적 표지 단백질인 Involucrin (INV)과 Transglutaminase 1 (TG'ase 1)의 발현이 각각 3.5배와 3배로 현저히 증가하였음을 웨스턴 블랏을 통하여 확인하였다. 또한 무모생쥐 피부에 PS를 도포한 결과 INV와 loricrin단백질 발현이 증가하였다. 본 연구는 PS가 피부에서 생리활성을 나타낸다는 최초의 증거를 제시하며, 구체적으로는 각질세포 분화를 촉진함으로써 피부 세라마이드 함량을 증가시키고 경표피수분손실을 감소시켜 궁극적으로 피부장벽을 강화하는 작용을 한다는 것을 보여준다.

Abstract: Phosphatidylserine (PS) is a phospholipid which plays the structural role in membranes and serves as a cofactor of signaling enzymes for diverse cellular functions. In this study, we observed that topical treatment with PS significantly decreased trans-epidermal water loss (TEWL) induced by tape-stripping in hairless mice. Also, ceramides in epidermis were increased in PS-treated group compared to vehicle-treated one *in vivo*. the amounts of non-hydroxyl ceramide (NHCER) (1.4 fold) and glucosylceramide (glucosylCER) (1.6 fold), in the skin of hairless mice, were increased by topical treament with PS. Also, we demonstrated that PS stimulated keratinocyte differentiation. We observed that PS treatment morphologically altered normal human keratinocyte (NHK) from the proliferating phase to the differentiating one, suggesting that PS stimulated epidermal differentiation in NHK. We also showed that the expressions of the specific markers for epidermal differentiation, involucrin (INV) (3.5 fold up) and transglutaminase 1 (TG'ase 1) (3 fold up), were significantly increased by PS treatment, compared to untreated control *in vitro*. In addition, topical treatment with PS resulted in a progressive increase in INV and loricrin protein levels *in vivo*. In conclusion, we provide the first evidence for the physiological activities of PS in skin, and we suggest that PS strengthen the epidermal permeability barrier by stimulation of keratinocyte differentiation.

Keywords: *phosphatidylserine, keratinocyte differentiation, ceramide, TEWL*

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1. Introduction

Phosphatidylserine (PS) is an essential phospholipid which is ubiquitously present in membranes of eukaryotic and prokaryotic cells[1]. PS plays the structural role in membranes, and is involved in biological processes as a cofactor of enzymes such as protein kinase C (PKC) and c-Raf1 protein kinase. It is also reported that PS is particularly concentrated in brain and enhances brain function. Although numerous studies on PS have been carried out, the physiological functions of PS, except for those in brain, have not been understood well, and the activities of PS in skin have never been reported. The epidermis, the outer layer of skin, has multiple barrier functions to protect skin from environmental stress[2]. It is comprised of morphologically distinct cell layers formed by a progressively more advanced stage of differentiation. Each step of differentiation is characterized by the expression of specific marker genes[3]. The transition from the spinous to the granular layer is accompanied by upregulation of genes encoding structural proteins of the cornified envelope such as involucrin and transgluta minase[4]. Competent skin barrier requires the intercellular lipids that form the only continuous domain in the stratum corneum (SC). The intercellular SC lipids consist mainly of free fatty acids, ceramides and cholesterol and particularly, ceramides is the major components of these multilayered membranes[5]. In this study, we observed that PS significantly decreased trans-epidermal water loss (TEWL) increased by tape-stripping in hairless mice. Also, we showed that NHCER and glucosyCER were increased in PS-treated group in comparison with vehicle-only group *in vivo*. Here, we demonstrated that PS strengthened the epidermal barrier function in *in vitro* and *in vivo* model, increasing the expressions of involucrin (INV), TG'ase 1 (Transglutaminase 1), and loricrin. This work provides the first evidence that PS has the physiological activities in skin, thus we suggest that PS might be a cosmeceutically or pharmaceutically great material in the treatment of skin diseases.

2. Materials and Methods

2.1. Cell Culture

Normal human keratinocyte was grown in keratinocyte serum-free medium (Gibco BRL/Life Technologies) supplemented with 50 μg/mL bovine pituitary extract at 37℃ in a 5% CO_2 humidified atmosphere.

2.2. Chemicals and Treatment

Clofibrate (Clo) was purchased from Sigma Chemical Company. Phosphatidylserine (PS) was provided by Dr. Jeong Jun Han (Doosan, Korea). Clo was dissolved in DMSO and PS was dissolved in water. Treatments were added directly to cell culture media and topically applied to hairless mice. Appropriate vehicle controls were used in all cases.

2.3. Epidermal Permeability

Transepidermal water loss (TEWL), a measurement of cutaneous permeability barrier function, was measured by a Tewameter (TM810, Courage and Khazaka, Germany). Barrier recovery was determined following repeated applications of cellophane tape (Scotch type, 3 M) until TEWL reached 68 mg/cm^2/h. Barrier recovery was determined by measuring TEWL at 6 and 12 h following barrier disruption. SC integrity was defined as the number of tape strippings required to produce defined elevations in TEWL and cohesion of SC as the amount of protein removed with each stripping. The protein content per stripping was measured with the protein assay kit (Bio-rad, USA), using bovine plasma γ-globulin as the standard.

2.4. Analysis of Ceramide Production

SC was collected by trypsinization of the skin with trypsin (0.25% in PBS) from a skin area of about 3 mm diameter on the dorsal region of mouse. The collected SCs were subjected to chloroform : methanol [2 : 1]. Lipid extracts were applied under a flow of nitrogen on the HPTLC plate (Merck) using Linomat IV (CAMAG, Muttenz, Switzerland) and separated by using the following development condition (chloroform : methanol : acetic acid [190 : 9 : 1]) twice. The quantification was performed after staining (7.5% Cu-acetate and 2.5% $CuSO_4$ in 8% H_3PO_4 (w/w)) and charring at 150℃. Analysis of lipid fractions was based on the

known quantities of the co-migrated standards.

2.5. Involucrin and Transglutaminase 1 Protein Levels

Whole cell lysate was prepared by lysis in 2% sodium dodecyl sulfate. The following proteins were quantified by Bradford assay (Bio-rad, Hercules, CA) and equal amounts of protein were electrophoresed on 7.5% polyacrylamide gels and transfered to PVDF membrane (Bio-rad). INV protein was detected by a mouse monoclonal anti-human INV antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (1 : 500 in Tween 20, 0.5%, and nonfat milk) and anti-mouse IgG-HRP (Sigma). TG'ase 1 was detected by a goat polyclonal anti-human TG'ase 1 antibody (Santa Cruz Biotechnology) and donkey anti-goat IgG-HRP (Santa Cruz Biotechnology) (1 : 1000 in Tween 20, 0.5%, and nonfat milk). Blotting proteins were visualized by enhanced chemiluminescence (Amersham, Buchinghamshire, England).

2.6. Differentiation Markers Detection by Lmmunohistochemistry

Immunohistochemistry was performed as described previously[10]. Tissue was fixed in 3.7% paraformaldehyde and embedded in paraffin, and 4 to 6 μM-thick sections were prepared. INV was detected with a rabbit antipeptide antibody, specific to mouse INV, and a rabbit antibody specific to mouse loricrin was used to localize loricrin antibody (BabCo, Berkeley, CA, USA). Slides were subsequently incubated with a biotinylated goat anti-rabbit or anti-mouse antibody and visualized using a standard streptavidin biotin (LSAB) horseradish peroxidase technique. The slides were counterstained in hematoxylin and mounted. The staining area was analyzed by image quantitative analysis software at immunolocalization.

3. Result

3.1. PS Accelerates the Recovery Rate of TEWL in Hairless Mice

The vital function of epidermis is to provide a barrier to transepidermal water movement. Here, we investigated the effect of topical treatment with PS on epidermal permeability barrier function. As shown in

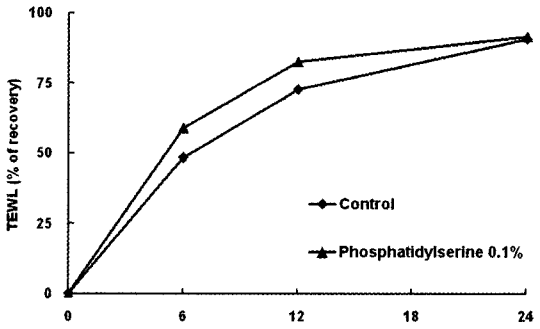

Figure 1. PS accelerates the recovery rate of TEWL. The skin barrier was disrupted by repeated applications of tape-stripping. Topical treatment with 0.1% of PS recovered the epidermal barrier function.

Figure 1, the barrier of hairless mice was disrupted by tape-stripping and then, topical treatment with PS accelerated the recovery rate of TEWL.

3.2. PS Increases Ceramide Production

Intercellular lipids, especially ceramides in SC are responsible for the barrier function of skin. Here, we examined the effect of PS on ceramide production in epidermis of hairless mice. Figure 2 shows that topical treatment with PS increases the amounts of NHCER and glucosyl CER compared to untreated group.

3.3. Morphological Change of Keratinocyte by PS

The high concentration of extracellular calcium stimulates keratinocyte differentiation and morphologically changed keratinocyte from small round shape to long differentiated shape[11]. We determined whether PS changed morphology of the cells. As shown in Figure 3, untreated keratinocytes incubated in low calcium (0.09 mM) were small and round. However, cells treated for 24 h with 20 μM of PS were morphologically changed as similar as those treated with high calcium (1.2 mM). This result indicates that PS stimulates keratinocyte differentiation.

3.4. Protein Levels of INV and TG'ase 1 Are Increased by PS

Keratinocytes maintained in low calcium remain in a proliferating state, and express relatively low levels of differentiation-specific proteins such as INV and TG'-ase 1[9], whereas keratinocytes incubated in high

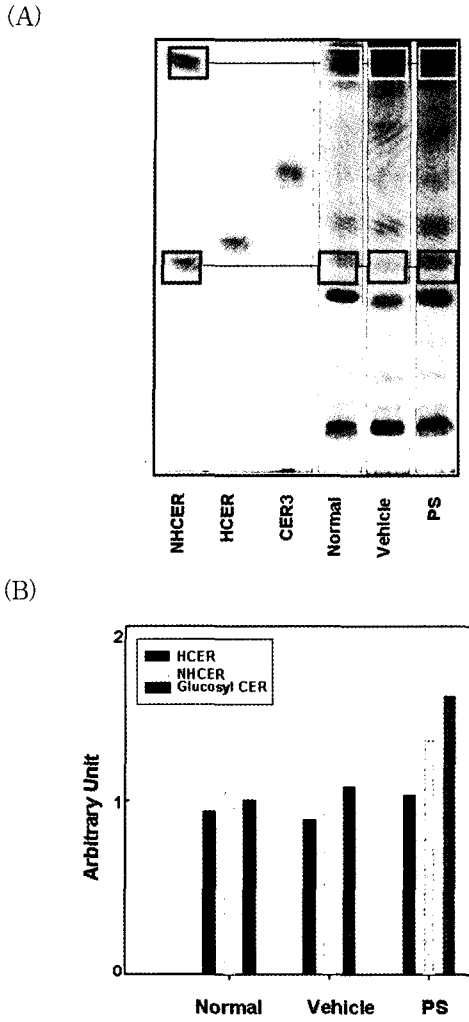

Figure 2. Topical treatment with PS increases ceramide production. The skin of hairless mice was topically applied with PS for 3 days. Ceramide (NHCER and glucosyl CER) production were measured by HPTLC method.

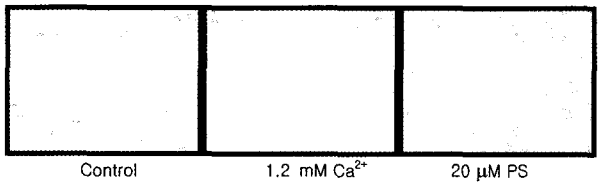

Figure 3. PS alters keratinocyte morphology. NHK were incubated for 48 h in media with or without PS. 1.2 mM Ca^{2+} was used as a positive control of keratinocyte differentiation. The cells were then photographed using a Nikon inverted brightfield microscope.

calcium express increased levels of INV and TG'ase 1 [3,12,13]. Here, we examined protein levels of INV and

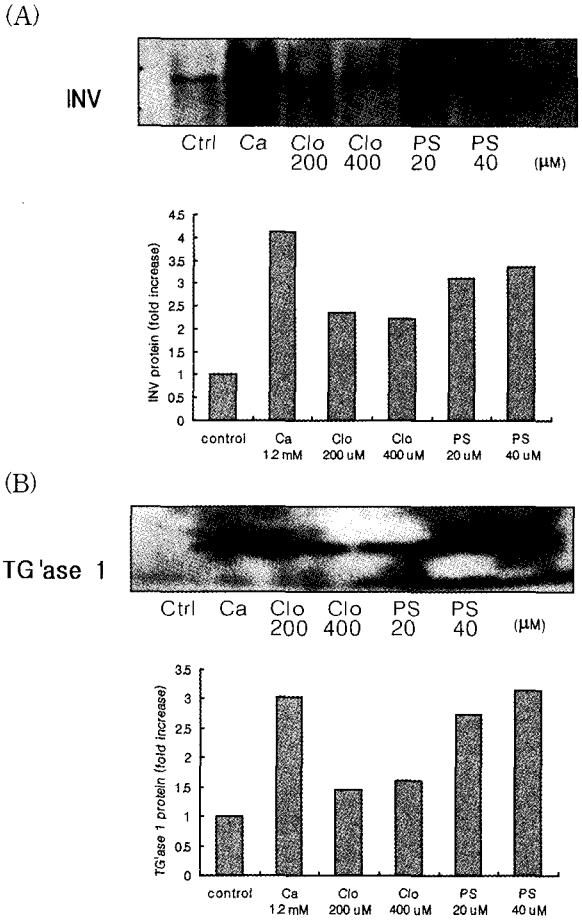

Figure 4. PS increases protein levels of INV and TG'ase 1. NHK were incubated for 24 h in KSFM containing 1.2 mM of Ca^{2+} or 200, 400 μM of Clo or 20, 40 μM PS. Western analysis was performed and autoradiograms were quantified as described in Materials and Methods. Representative autoradiograms are shown here.

TG'ase 1 in NHK incubated in the presence or absence of PS. Figure 4(A) shows that PS stimulates a further increase in INV. TG'ase 1 protein level was also increased by PS treatment. (Figure 4(B)). These results indicate that PS dramatically increases the differentiation protein levels of both INV and TG'ase 1, suggesting that PS stimulates keratinocyte differentiation.

3.5. PS Stimulates Keratinocyte Differentiation *In Vivo*

We next examined the changes in the expression of INV and loricrin following topical treatment with PS *in vivo*. These protein markers at the late keratinocyte

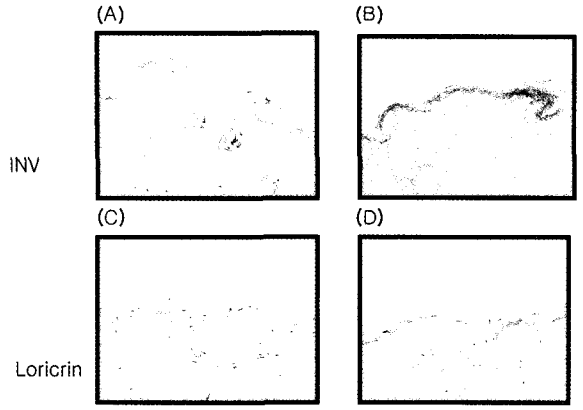

Figure 5. Topical PS treatment increases the expression of INV and loricrin. PS was topically applied to hairless mice twice a day for 2 weeks. Mouse INV (A, B) and loricrin (C, D) were detected using specific antibodies.

differentiation, are localized to the spinous/granular layers in the epidermis. INV (Figure 5(B)) and loricrin (Figure 5(D)) were markedly increased in the epidermis of PS-treated group compared to vehicle-only group. Therefore, PS stimulates keratinocyte differentiation *in vivo*.

4. Disucussion

This study first demonstrated that PS, which has been known to exist abundantly in brain and enhance brain function such as memory, also possessed the physiological activity in skin. In this study, we observed that topical treatment with PS accelerated permeability barrier recovery following acute barrier disruption by tape-stripping in hairless mice. At 6 h and 12 h following skin barrier disruption, barrier recovery of PS-treated group was accelerated (Figure 1). We also showed that ceramides (NHCER and glucosylCER) were increased in PS-treated group compared to vehicle-only group (Figure 2). These results mean that PS enhances skin barrier function by induction of increase in ceramide production and acceleration of barrier recovery. We also revealed that PS stimulated epidermal differentiation. Keratinocyte morphology is changed from the proliferating phase to differentiating phase by treatment with PS, and the expression of specific markers for epidermal differentiation, INV, TG'ase 1, and loricrin, were markedly increased *in vitro* and *in vivo* (Figure 3, 4). PS treatment dramatically increased INV and TG'ase 1 protein in NHK. And, topical treatment with PS also increased the expression of IVN and loricrin presented in epidermis of hairless mice. The differentiation process of keratinocyte is highly essential for the development of epidermal permeability barrier and enhancement of skin barrer function. These data suggest that PS might develop epidermal permeability barrier effectively and enhance skin barrer function through keratinocyte differentiation. In this work, we provide the first evidence that PS has the physiological activities in skin and in detail, PS strengthens skin barrier through epidermal differentiation. Therefore, we carefully suggest that PS might be a cosmeceutically and pharmaceutically great material.

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