Phosphatidylserine prevents UV-induced decrease of type I procollagen and increase of MMP-1 in dermal fibroblasts and human skin in vivo

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Abstract **In an effort to find topical agents that prevent or retard cutaneous aging, seven functional lipids were screened for their procollagen-upregulating and matrix metalloproteinase (MMP)-1-downregulating activities in human dermal fibroblasts by Western blotting. The preventive effect on ultraviolet (UV)-induced decrease of procollagen was demonstrated in phosphatidylserine (PS), lysophosphatidylserine (LPS), lysophosphatidic acid (LPA), *N*-acetyl phytosphingosine (NAPS), and tetraacetyl phytosphingosine (TAPS). Furthermore, PS, LPS, and LPA upregulated procollagen expression in unirradiated basal conditions. The inhibitory effect on UV-induced MMP-1 expression was seen in NAPS, TAPS, LPA, PS, lysophosphatidylglycerol, and LPS. PS was chosen as the most suitable candidate anti-aging chemical for the subsequent in vivo studies. We investigated the effects of PS on acute UV response and chronologic skin aging by topically applying it to young skin before UV irradiation and to aged human skin, respectively. Real-time PCR and Western blot revealed that in the young skin, PS treatment prevented UV-induced reduction in procollagen expression and inhibited UV-induced MMP-1 expression. PS also blocked UV-induced IL-6 and COX-2 gene expression in cultured fibroblasts dose-dependently. In the aged skin, PS caused increased procollagen transcription and procollagen immunostaining in the upper dermis, and a significant decrease in MMP-1 expression at both mRNA and protein levels. These results indicate that topical PS has anti-skin-aging properties and point to the potential use of PS as a therapeutic agent in the prevention and treatment of cutaneous aging.**—Cho, S., H. H. Kim, M. J. Lee, S. Lee, C-S. Park, S-J. Nam, J-J. Han, J-W. Kim, and J. H. Chung. **Phosphatidylserine prevents UV-induced decrease of type I procollagen and increase of MMP-1 in dermal fibroblasts and human skin in vivo.** ***J. Lipid Res.*** **2008.** 49: **1235–1245.**

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Skin aging is the sum of intrinsic aging and photoaging caused by repeated exposure to ultraviolet (UV) light. Whereas naturally aged skin is smooth, pale, and finely wrinkled, photoaged skin shows coarse, deep wrinkles and dyspigmentation and telangiectasia (1). In both chronologic aging and photoaging, decreased procollagen expression and increased matrix metalloproteinases (MMPs), a group of matrix-degrading enzymes secreted by epidermal keratinocytes and dermal fibroblasts, are characteristic. The alterations in collagen, the major structural component of the dermis, are thought to be responsible for wrinkle formation (2).

MMP expression is increased by various stimuli, including UV light, oxidative stress, and cytokines. UV irradiation induces MMPs, including MMP-1 (collagenase), MMP-3 (stromelysin), and MMP-9 (gelatinase) (3). UV-induced MMP-1 initiates collagen breakdown by cleaving the fibrillar collagen (types I and III) at a single cleavage site. Once collagen is cleaved by MMP-1, it is further degraded by MMP-3 and MMP-9, whose expression levels are also increased by UV irradiation (3).

The search for topical agents that prevent or retard cutaneous aging has become the quest of many basic researchers as well as the pharmaceutical and cosmeceutical industries. As part of the endeavor, in this study, seven functional lipids, including two sphingolipids and five phospholipids, were screened for their effects on UV-induced MMP-1 expression in human dermal fibroblasts.

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Among sphingolipids, sphingosine is known for its anti-inflammatory and antiproliferative activities in mouse models of irritant contact dermatitis (4). Phytosphingosine, which is structurally similar to sphingosine, is abundant in fungi and plants but is also found in mammalian epidermis. Recently, phytosphingosine was shown to stimulate differentiation of human keratinocytes and inhibit inflammatory epidermal hyperplasia in hairless mouse skin (5). In this study, phytosphingosine derivatives *N*-acetyl phytosphingosine (NAPS) and tetraacetyl phytosphingosine (TAPS) were examined for their anti-aging properties. Five phospholipids, lysophosphatidic acid (LPA), lysophosphatidylserine (LPS), lysophosphatidylglycerol (LPG), lysophosphatidylinositol (LPI), and phosphatidylserine (PS), were examined for their anti-aging activities. LPA has been shown to regulate cell proliferation, enhance cell motility, increase the production of MMPs in vitro (6), and promote wound healing in vivo (7). Phospholipid metabolites lysophospholipids, including LPS, LPG, and LPI, may affect cellular signaling, as evidenced by their inhibition of phospholipase D, which is important in signal transduction in mammalian cells (8).

PS, a precursor of LPS, is a membrane phospholipid that is ubiquitously present in membranes of prokaryotic and eukaryotic cells (9). It plays the structural role in membranes and is required for diverse cellular functions, serving as a cofactor of signaling enzymes, such as protein kinase C and cRaf1 protein kinase (9). Some researchers have suggested that externalized PS reduces inflammation, serving as a signal recognized by phagocytic macrophages in normal human keratinocytes during oxidative stress (10). The externalization of PS to the cell surface is a hallmark of apoptosis (11). The recognition of surface-exposed PS by the PS receptor on macrophages is necessary for the removal of apoptotic cells (12). Additionally, PS has the potential to act as an effective antioxidant, especially in response to iron-mediated oxidation (13). However, the physiological roles of PS in the skin have not been understood well. It has been shown that PS interacts with the peroxisome proliferator-activated receptor (PPAR) α protein as strongly as a known PPARα ligand, linoleic acid, in an in vitro binding assay, suggesting that PS could be a novel ligand of PPARα (14). PPARs are members of the nuclear hormone receptor super-family that have been reported to play an important role in maintaining epidermal homeostasis (15). Because activators of PPARα decrease UVB-induced cutaneous inflammation (16), PS may be expected to decrease UV-induced inflammation and retard photoaging. However, no study has been undertaken to investigate the anti-skin-aging effects of topical PS treatment on human skin in vivo.

In this study, we initially screened the seven functional lipids for anti-skin-aging properties, and subsequently investigated whether topical application of PS prevents UV-induced acute skin response and improves intrinsic skin aging in vivo, by applying it to young skin prior to UV irradiation and to aged skin, respectively.

MATERIALS AND METHODS

Materials

NAPS, TAPS, LPS, LPA, LPG, LPI, and PS were provided by Doosan Biotech Division (Gyeonggido, Korea). The chemical structure of each functional lipid is shown in **Fig. 1**. Fatty acid compositions of lysophospholipids are summarized in **Table 1**. The fatty acid composition of the PS used in the experiments was 16:0 (20%), 18:0 (6%), 18:1 (13%), 18:2 (57%), and 18:3 (4%).

Cell culture and functional lipid treatment

Human foreskin fibroblasts (Hs27) were purchased from American Type Culture Collection (Manassas, VA) and cultured in DMEM media containing 10% FBS. The phytosphingosines (NAPS, TAPS) were dissolved in DMSO (2 μM), diluted with culture media at the indicated concentrations (final DMSO concentration, 0.1%), and all phospholipids (LPS, LPA, LPG, LPI, PS) were dissolved in water (20 μM). The cells were pretreated with the functional lipids for 24 h before UV irradiation. After pretreatment, cells were washed with PBS and then irradiated with UV in PBS. Hs27 cells were also used for time course and dose-response studies of PS on UV-irradiated cells.

Reporter assay

NIH3T3 fibroblasts, which contain the pMMP1 *luci*/pProcollagen *luci*, were grown in DMEM containing 10% FBS until 80–90% confluent. The cells were then washed with PBS, and fresh DMEM containing 20 μM PS was added. After incubation for 24 h, cells were washed with PBS and then irradiated with UV. After 24 h, cells were harvested using reporter lysis buffer (Promega; Madison, WI). The luciferase activities in aliquots were normalized by protein concentration and described as relative to untreated cells.

Topical application of PS in vivo

PS was dissolved in ethanol-polyethyleneglycol (50:50) containing 1% (w/v) tocopherol for topical application on human skin in vivo. Young Korean adults (20–30 years of age, male, average age = 24, $n = 6$), volunteers without current or prior skin disease, were studied for the effect of PS on acute UV response. The subjects were treated twice, 24 h apart, with 2% PS or its vehicle, which was left on the skin for 24 h after each treatment under occlusion, and irradiated by UV [2 minimal erythema dose (MED)]. Forty-eight hours postirradiation, buttock skin was biopsied for real-time PCR, Western blot, and immunohistochemical analyses. Prior to the experiments, we confirmed that PS does not absorb UV light (200–400 nm in wavelength) at all and will not act as a sunscreen (data not shown).

Aged Korean adults (>70 years of age, male, average age = 75, $n = 6$), volunteers without current or prior skin disease, were treated with 2% PS and its vehicle for 2 weeks (three times per week, total six times) under occlusion. Buttock skin was biopsied for real-time PCR, Western blot, and immunohistochemical analyses.

This study was conducted according to the Declaration of Helsinki Principles. All procedures received prior approval from the Institutional Review Board at Seoul National University Hospital, and all subjects gave written informed consent.

UV irradiation

For all in vitro studies, Philips TL (Einthoven, Netherlands) 20W/12RS fluorescent sun lamps with an emission spectrum between 275 and 380 nm (peak, 310–315 nm) were used as the

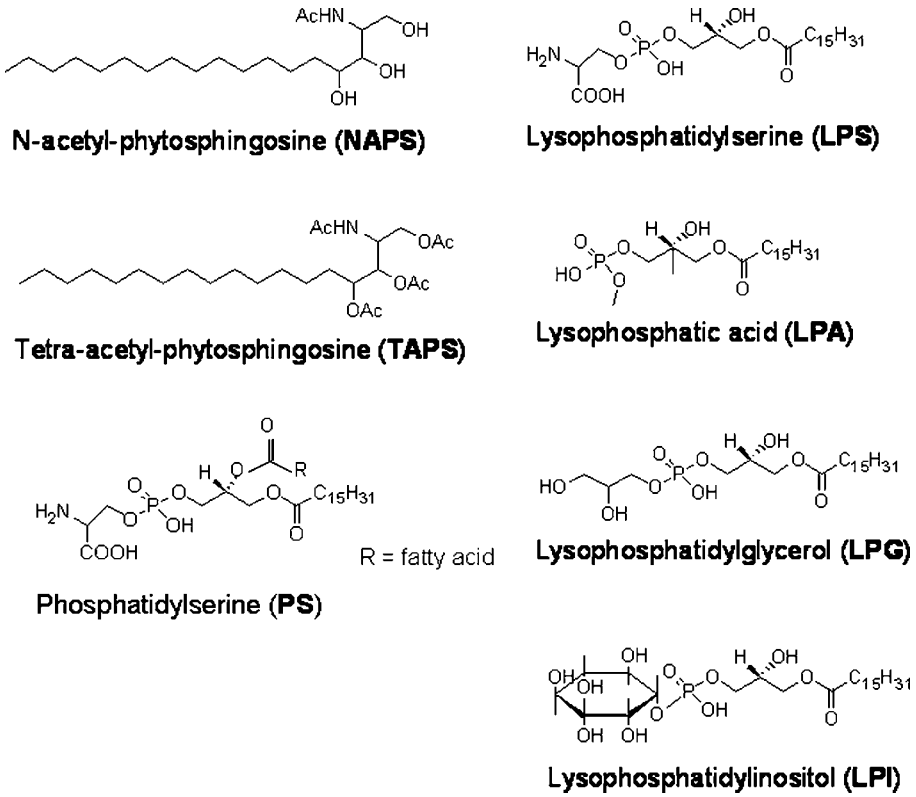

Fig. 1. Chemical structures of the seven functional lipids used in the experiments. R denotes the fatty acid group in phosphatidylserine (PS), which consists of 16:0 (20%), 18:0 (6%), 18:1 (13%), 18:2 (57%), and 18:3 (4%).

UV source (17), and a Kodacel filter (TA401/407; Kodak, Rochester, NY) was used to block UVC, which has wavelengths of <290 nm. UV strength was measured using a UV meter (Model 585100; Waldmann, Villingen-Schwenningen, Germany).

For in vivo studies, a Waldmann UV-800 phototherapy device was used as the UV source. UV light source was an F75/85W/UV21 fluorescent sun lamp, with an emission spectrum between 285 and 350 nm (peak at 310–315 nm), as described previously (18). Strength of UV irradiation at the skin surface was measured with a Waldmann UV meter (Model 585100). The buttock skin was irradiated with filtered UV, and the MED was determined at 24 h after irradiation. MED ranged between 70 mJ/cm^2 and 90 mJ/cm^2 for the skin of Koreans.

Real-time quantitative PCR and RT-PCR

Total RNA was isolated from human foreskin fibroblasts (Hs27) and buttock skin using TRI_{ZOL} reagent (Invitrogen Life Technologies; Carlsbad, CA). Total RNA (1–2 μg) was reverse transcribed into cDNA using a first-strand cDNA synthesis kit (MBI Fermentas; Vilnius, Lithuania). Quantitation of procollagen α1(I), MMP-1, and endogeneous reference 36B4 cDNA was performed using a fluorescence detection method, the 7500 Real-time PCR System (Applied Biosystems; Foster City, CA). Sequence-specific PCR primer sets and the TaqMan MGB probe (FAM^{TM} dye-labeled) were purchased from Applied Biosystems. For real-time PCR, cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Data were analyzed using the $2^{-\Delta\Delta CT}$ methods (19); the data were presented as the fold change in gene expression normalized to an endogenous reference gene and relative to the untreated control sample. For the untreated control sample, $^{\Delta\Delta}C_T$ equals 0 and 2° equals 1, so that the fold change in gene expression relative to the untreated control equals 1, by definition. For the treated samples, evaluation of $2^{-\Delta\Delta CT}$ indicates the fold change in expression relative to the untreated control.

For semi-quantitative RT-PCR, oligonucleotide primers were as follows: human MMP-1 sense primer, 5′-ATT CTA CTG ATA

TABLE 1. Fatty acid composition of lysophospholipids

	Fatty Acid Composition							
Lysophospholipid	14:0	16:0	16:1	18:0	18:1	18:2	18:3	others
LPS	0.2	25.2	1.4	5.6	8.9	52.1	6.3	0.3
LPA	0.2	25.2	1.4	5.6	8.9	52.1	6.3	0.3
LPI	nd	65.0	nd	11.0	7.0	15.0	2.0	nd
LPG	nd	67.0	2.0	23.0	6.0	1.0	nd	1.0

LPS, lysophosphatidylserine; LPA, lysophosphatidic acid; LPI, lysophosphatidylinositol; LPG, lysophosphatidylglycerol; nd, not detected.

TCG GGG CTT TGA-3′; human MMP-1 antisense primer, 5′-ATG TCC TTG GGG TAT CCG TGT AG-3′; human procollagen α1(I) sense primer, 5′-CTC GAG GTG GAC ACC ACC CT-3′; human procollagen α1(I) antisense primer, 5′-CAG CTG GAT GGC CAC ATC GG-3′; human IL-6 sense primer, 5′-CTC CTT CTC CAC AAG CGC C-3′; human IL-6 antisense primer, 5′-GCC GAA GAG CCC TCA GGC-3′; human COX-2 sense primer, 5′-TTC AAA TGA GAT TGT GGG AAA ATT GCT-3′; human COX-2 antisense primer, 5′-AGA TCA TCT CTG CCT GAG TAT CTT-3′; human 36B4 sense primer, 5′-TGG GCT CCA AGC AGA TGC-3′; human 36B4 antisense primer, 5′-GGC TTC GCT GGC TCC CAC-3′. After predenaturation at 94°C for 3 min, each cycle of PCR amplification consisted of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s; 21 cycles were performed for 36B4 and procollagen α1(I), 24 cycles for MMP-1 and IL-6, and 30 cycles for COX-2, after a final extension at 72°C for 5 min. PCR products were resolved on 2.0% agarose gels, followed by quantification with a densitometry analysis (TINA; Raytest Isotopenme gerate, Straubenhardt, Germany) of bands (relative density normalized to 36B4).

Western blot

Western blot analysis was performed as described previously (18). Briefly, human dermal fibroblasts and punch-biopsied skin samples were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 5 mM PMSF, and 1 mM DTT) containing 1% (w/v) Triton X-100. Lysates were centrifuged at 12,000*g* for 15 min, and supernatants were collected to perform Western blot. Protein concentration of samples was determined by Bradford assay. Equal amounts of proteins were loaded onto Tris-glycine gels and then electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were subsequently blocked with 5% skim milk in TBS/T (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.05% Tween-20) and incubated with the indicated antibodies. For detection of type I procollagen, monoclonal anti-procollagen type I N-terminal extension peptide (SP1.D8) antibody was obtained from culture media of hybridoma (Developmental Studies Hybridoma Bank; Iowa City, IA). Monoclonal anti-MMP-1 antibody was purchased from Oncogene (Boston, MA). Polyclonal anti-β-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Blotting proteins were visualized by enhanced chemiluminescence (Amersham; Buckinghamshire, England).

Immunohistochemical analysis

Immunohistochemical analyses were performed as described in a previous report (20). Human skin samples were fixed in 10% formalin for 24 h, and embedded in paraffin. Serial sections (4 μm) were mounted onto silane-coated slides (Dako; Glostrup, Denmark) and allowed to dry at 58°C for 1 h. Sections were stained with hematoxylin and eosin. Acetone-fixed frozen sections were stained with monoclonal anti-human procollagen type I-C peptide (PIP) antibody (Takara; Shiga, Japan), polyclonal anti-TNF-α antibody (Santa Cruz Biotechnology), and polyclonal anti-IL-1α antibody (Santa Cruz Biotechnology) in a humidified chamber at 4°C for 18 h. After washing in PBS, sections were visualized using a Histostatin-plus kit (Zymed; San Francisco, CA) with a biotinylated secondary antibody and a horseradish-streptavidin conjugate. 3-Amino-9-ethylcarbazole (Dako) was used as a chromogenic substrate. Sections were counterstained with Mayer's hematoxylin (Dako). Negative control staining was performed with normal rabbit immunoglobulin, which demonstrated no immunoreactivity.

Statistical analysis

Statistical analyses were performed using the Mann-Whitney U-test, Wilcoxon's signed rank test, and ANOVA. For all analyses, a P value of less than 0.05 was considered statistically significant.

RESULTS

LPS and LPA inhibited UV-induced decrease of type I procollagen and increase of MMP-1 in human dermal fibroblasts

The effects of the seven functional lipids on UV-induced decrease of procollagen and increase of MMP-1 are summarized in **Fig. 2**. By Western blotting, the preventive effect on UV-induced decrease of collagen was seen in PS, LPS, LPA, NAPS, and TAPS in decreasing order. Furthermore, PS, LPS, and LPA upregulated procollagen expression under unirradiated basal conditions. The inhibitory effect on UV-induced MMP-1 expression was seen in NAPS, TAPS, LPA, PS, LPG, and LPS in decreasing order. In unirradiated HDFs, NAPS, PS, LPS, and LPG downregulated MMP-1 expression. LPI did not demonstrate any upregulating activity on procollagen or downregulating activity on MMP-1 expression with or without UV irradiation, followed by LPG. Although NAPS and TAPS were shown to inhibit UV-induced decrease of collagen and UV-induced MMP-1 expression, the two phytosphingosines were excluded from further experiments because they were reported to cause apoptosis in cultured keratinocytes (21). Because PS demonstrated the most promising biochemical profiles regarding procollagen induction and MMP-1 inhibition, PS was chosen for the subsequent in vivo studies.

PS prevented UV-induced changes of promoter activities and mRNA expression of procollagen and MMP-1

To confirm whether PS has a stimulatory effect on procollagen and an inhibitory effect on MMP-1 expression, a promoter assay on procollagen and MMP-1 was performed. PS stimulated procollagen promoter activity and suppressed MMP-1 promoter activity significantly under basal conditions ($P < 0.05$). In UV-irradiated samples, PS pretreatment resulted in significant restoration of UV-induced reduction in procollagen promoter activity and inhibition of UV-induced activation in MMP-1 promoter, and these effects were statistically significant ($P < 0.05$) (**Fig. 3A**).

Next, the effects of PS on mRNA expression of procollagen and MMP-1 were investigated. We demonstrated that the inhibitory effect of PS on UV-induced changes of procollagen and MMP-1 mRNA expression in cultured fibroblasts peaked at 24 h after UV irradiation (data not shown). Therefore, the dose-response effects of PS were observed at 24 h after UV irradiation. PS prevented UV-induced decrease of procollagen mRNA expression and UV-induced increase of MMP-1 expression dose-dependently, up to 20 μM, in cultured fibroblasts (Fig. 3B). Western blot revealed similar patterns in procollagen and MMP-1 expression, depending on PS

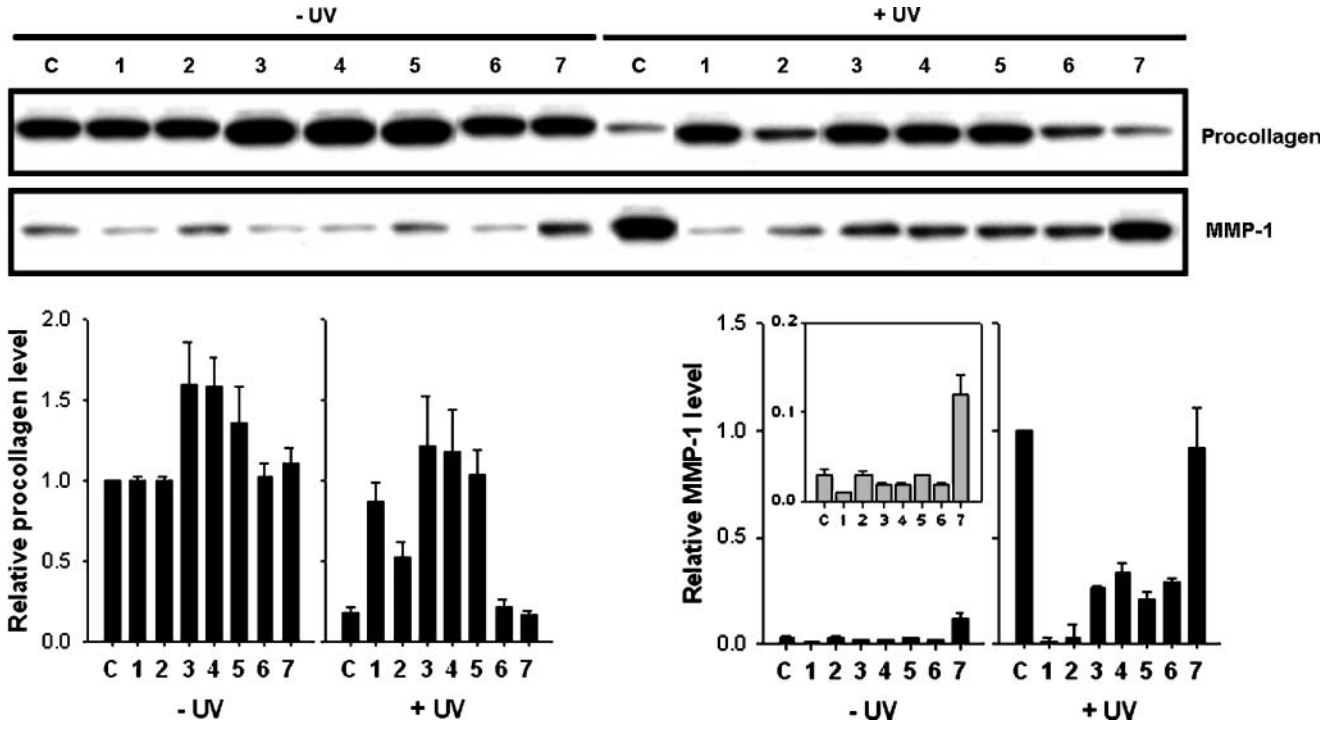

Fig. 2. The effects of the seven functional lipids on ultraviolet (UV)-induced decrease of procollagen and increase of matrix metalloproteinase (MMP)-1. Human foreskin fibroblasts (Hs27) were pretreated with seven functional lipids for 24 h before UV irradiation for Western blot analysis. Relative procollagen-upregulating and MMP-1-downregulating activities of two phytosphingosines, *N*-acetyl phytosphingosine (NAPS) and tetraacetyl phytosphingosine (TAPS), at 2 μM and five phospholipids, PS, lysophosphatidylserine (LPS), lysophosphatidic acid (LPA), lysophosphatidylglycerol (LPG), and lysophosphatidylinositol (LPI) at 20 μM are shown (C, control; lane 1, NAPS; lane 2, TAPS; lane 3, PS; lane 4, LPS; lane 5, LPA; lane 6, LPG; lane 7, LPI). The relative MMP-1 levels in unirradiated samples are shown in the insert. Error bars indicate ± SD.

concentration. Thus, these results suggest that PS pretreatment prevents UV-induced changes of procollagen and MMP-1 expression at the transcriptional level.

PS blocked UV-induced increase of IL-6 and COX-2 mRNA expression

We also investigated the effects of PS on UV-induced mRNA expression of IL-6 and COX-2 in cultured fibroblasts. By UV irradiation, IL-6 and COX-2 mRNA expression was increased more than two-fold and five-fold, respectively, and PS prevented UV-induced IL-6 and COX-2 mRNA expression in a dose-dependent manner up to 20 μM (Fig. 3C).

PS prevented UV-induced decrease in type I procollagen and induction of MMP-1 in young human skin in vivo

Decrease in procollagen expression caused by repeated UV irradiation has been considered to be a cause of photoaging. We investigated the effect of PS on UV-induced decrease of procollagen expression by real-time PCR, Western blot, and immunohistochemical analysis in human skin in vivo. Young human buttock skin was treated with PS and then was irradiated by UV (2 MED). Forty-eight hours post-irradiation, skin was biopsied to assess the effect of PS on type I procollagen and MMP-1 expression level.

By real-time PCR, UV irradiation caused an insignificant decrease in procollagen mRNA level to 82 ± 23% of the control ($P > 0.05$), and PS showed a tendency to prevent this, keeping the procollagen mRNA level at 120 ± 30% of control; however, this effect was not statistically significant. PS pretreatment without UV irradiation increased the procollagen mRNA level to 139 ± 61% of the control level ($P > 0.05$, n = 5). In UV-irradiated young skin, MMP-1 mRNA level was significantly increased to 482 ± 140% ($P < 0.02$, n = 6), and PS-pretreated skin had a lower MMP-1 mRNA level (381 ± 120%), compared with vehicle-pretreated skin, although this difference was not statistically significant (**Fig. 4A**).

Western blot demonstrated that UV irradiation reduced the level of procollagen expression significantly ($P < 0.05$ compared with UV-untreated control group, n = 4) (Fig. 4B). In PS-treated skin, UV-induced decrease of procollagen was prevented ($P < 0.05$ versus UV-only-treated group, n = 4). From these results, we found that topical application of PS partially prevents the UV-induced decrease of procollagen in human skin in vivo. Moreover, PS was shown to reduce the upregulated MMP-1 expression in UV-irradiated skin ($P < 0.05$ versus UV-only-treated group, n = 5), in a pattern similar to real-time PCR results.

Immunohistochemistry revealed that UV decreased procollagen expression in the fibroblasts and also throughout the dermis (**Fig. 5A**). The type I procollagen antibody we used, PIP antibody, detects procollagen I C-terminal peptides and thus stains dermal fibroblasts both intracellularly and extracellularly. PS-pretreated skin demonstrated greater intracellular procollagen staining in dermal fibro-

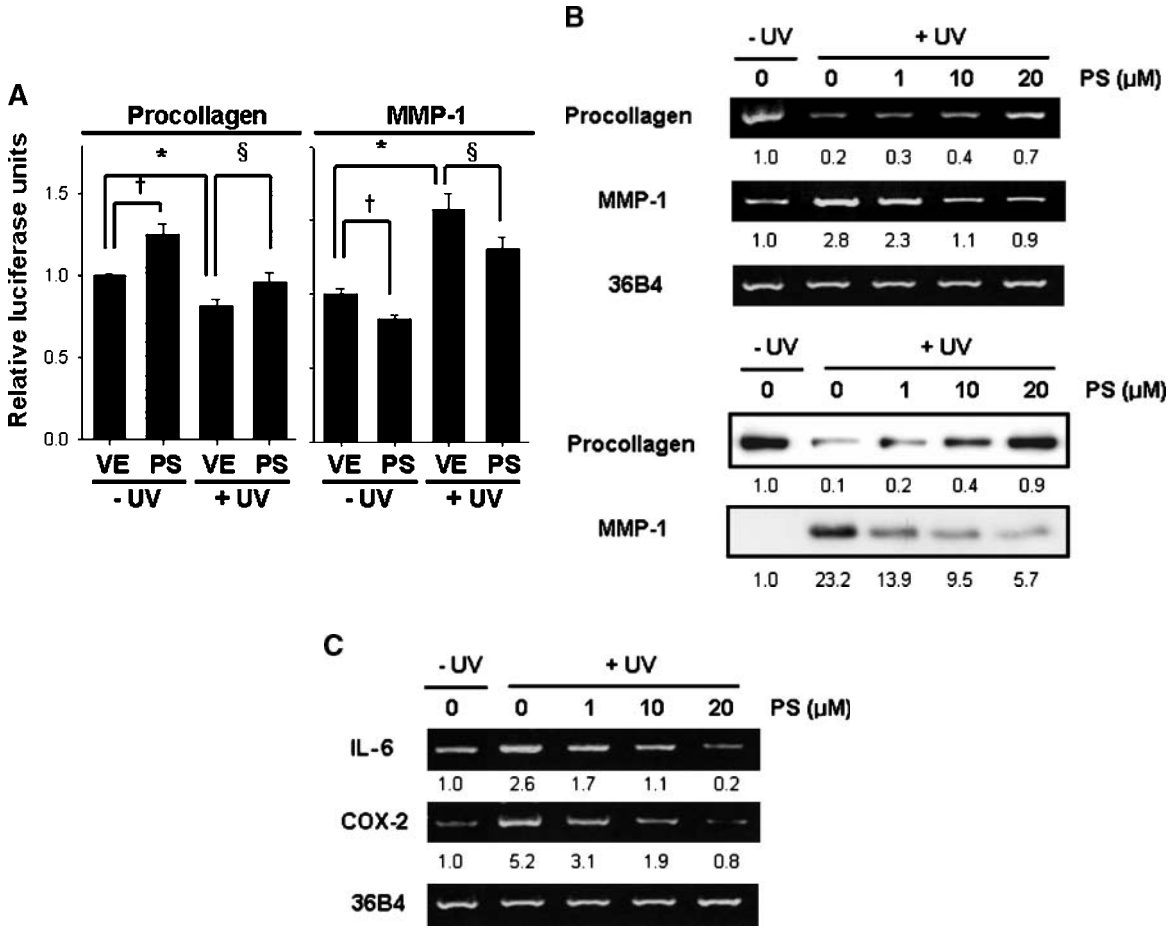

Fig. 3. PS prevented UV-induced changes of promoter activities and mRNA expression of procollagen and MMP-1, and blocked UV-induced increase in IL-6 and COX-2 mRNA in cultured fibroblasts. A: NIH3T3 fibroblasts, which contain the pMMP1 *luci*/pProcollagen *luci*, were treated with 20 μM PS for 24 h, irradiated with UV (75 mJ/cm^2), and harvested for luciferase activity. Luciferase activities were normalized by protein concentration and described as relative to untreated cells. The data presented are representative of three independent experiments. VE, unirradiated vehicle-treated group; †, statistically significant differences in procollagen promoter and MMP-1 promoter activities between vehicle-treated skin and PS-treated skin under basal conditions; *, statistically significant differences in procollagen promoter and MMP-1 promoter activities between unirradiated control and irradiated skin; §, significant differences in UV-induced procollagen and MMP-1 promoter activities between vehicle-treated control skin and PS-treated skin. Error bars indicate ± SD. B: Human dermal fibroblasts were incubated with PS for 24 h and irradiated with UV (75 mJ/cm^2) for RT-PCR and Western blot analyses. The bands are representative of results from three independent experiments. 36B4, housekeeping gene; numbers underneath each band, densitometer value of each band. C: Human dermal fibroblasts were incubated with PS for 24 h and irradiated with UV (75 mJ/cm^2). RT-PCR results demonstrate dose-response relationship of PS concentration with IL-6 and COX-2 mRNA expression. The bands are representative of results from three independent experiments. 36B4, housekeeping gene; numbers underneath each band, densitometer value of each band.

blasts, compared with vehicle-pretreated skin, after UV irradiation, indicating that the PS pretreatment counteracted the downregulating effects of UV on procollagen and stimulated protein synthesis (Fig. 5A). In addition, even in unirradiated skin, PS-treated skin demonstrated increased procollagen expression compared with the vehicle-treated control.

PS did not prevent UV-induced TNF-α and IL-1α expression in young human skin in vivo

To investigate whether topical PS inhibits UV-induced TNF-α and IL-1α expression in human skin, immunohistochemistry was performed using antibodies against TNF-α and IL-1α. The expression of the two cytokines was increased significantly by UV irradiation in human skin. We found that topical treatment of PS did not prevent UV-induced expression of TNF-α and IL-1α significantly, although there were some individual variations: two subjects showed slightly reduced expression of TNF-α and IL-1α by PS treatment, and three subjects did not demonstrate any difference by PS treatment (Fig. 5B).

PS increased type I procollagen expression and decreased MMP-1 expression in intrinsically aged human skin in vivo

To investigate the effects of PS on intrinsic aging, aged human buttock skin was treated with 2% PS for 2 weeks (three times per week, total six times) under occlusion. By real-time PCR, the procollagen mRNA levels were increased (217 ± 65% of control, n = 6) and MMP-1 mRNA levels were significantly decreased (50 ± 14% of

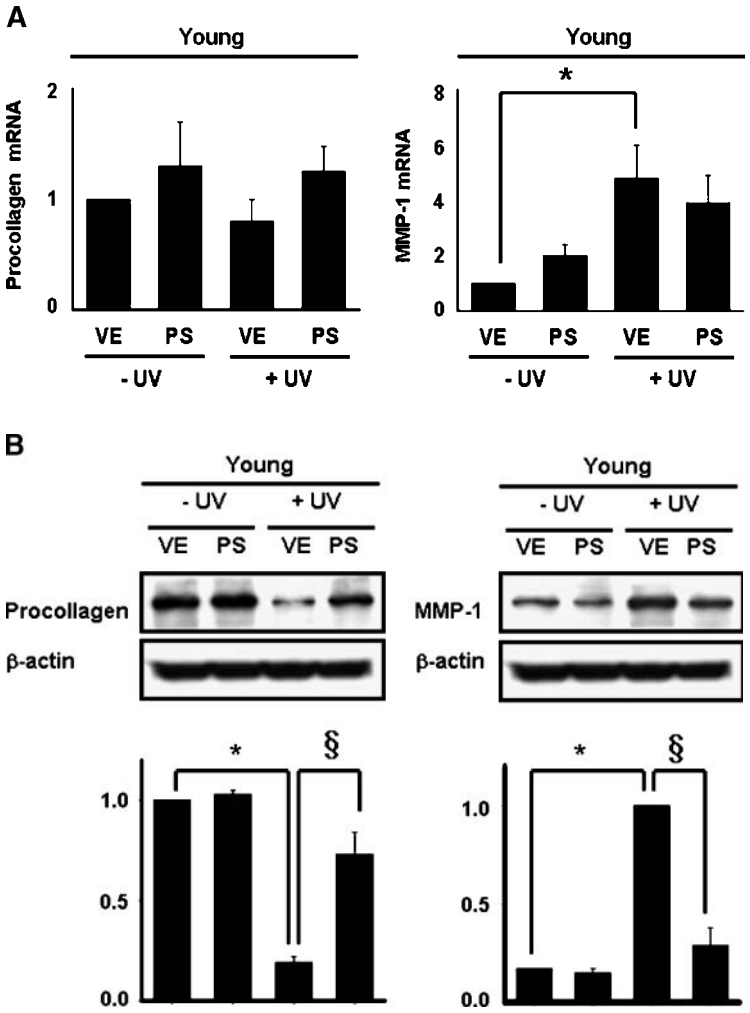

Fig. 4. PS prevented UV-induced decrease in type I procollagen and induction of MMP-1 in young human skin in vivo. A: Buttock skin of young male subjects was treated twice, 24 h apart, with 2% PS or its vehicle under occlusion, and irradiated by UV [2 minimal erythema dose (MED)]. Skin samples were taken 48 h postirradiation for real-time PCR of type I procollagen (n = 5) and MMP-1 (n = 6) genes. B: Western blot demonstrates that PS restores UV-induced reduction in procollagen expression (n = 4) and inhibits UV-induced MMP-1 expression (n = 5) in young human skin in vivo. Error bars indicate ± SD. VE, vehicle-treated skin; PS, PS-treated skin; *, statistically significant differences between unirradiated control and irradiated skin; §, significant differences between vehicle-treated and PS-treated skin, after UV irradiation; β-actin, loading control for Western blot.

control, n = 6, $P < 0.02$) in PS-pretreated aged human skin in vivo (**Fig. 6A**). Western blot demonstrated that PS increased procollagen expression by 15 ± 7% (versus vehicle-treated control, n = 5) and significantly decreased the MMP-1 expression to 75 ± 9% ($P < 0.05$ versus vehicle-treated control, n = 5) (Fig. 6B). Compared with vehicle-treated skin, PS-treated skin demonstrated increased procollagen immunostaining of fibroblasts throughout the dermis as well as denser extracellular staining in the dermo-epidermal junction (**Fig. 7**).

DISCUSSION

This study demonstrates for the first time that PS, LPS, LPA, and NAPS increase basal procollagen expression and prevent UV-induced decrease of procollagen in cultured dermal fibroblasts. In addition, NAPS, TAPS, LPA, PS, LPG, and LPS were found to downregulate UV-induced MMP-1 expression in dermal fibroblasts. These results suggest that these chemicals may have the potential to be anti-photoaging agents. As shown in Fig. 2, the fact that PS, LPS, and LPA increase collagen synthesis in unirradiated skin implies that these three phospholipids may be of benefit in the treatment or prevention of intrinsic aging as well as photoaging. Of the two phytosphingosines, NAPS demonstrated stronger anti-aging activity with respect to procollagen I and MMP-1. However, because NAPS and TAPS were reported to cause apoptosis in cultured keratinocytes (21), they were excluded from further studies. Among the five phospholipids, PS revealed the most procollagen-stimulating activity, followed by LPS and LPA. In MMP-1-inhibitory activity, PS was second only to LPA. Because PS demonstrated the most promising biochemical profiles regarding procollagen induction and MMP-1 inhibition, it was chosen as the first phospholipid to be tested in human skin in vivo.

In this study, interestingly, PS prevents UV-induced changes of promoter activities, and mRNA and protein expression of procollagen and MMP-1 significantly in the cultured fibroblasts. In young human skin in vivo, topical PS was found to significantly restore UV-induced decrease in procollagen protein expression, and inhibit UV-induced MMP-1 protein expression. However, we could not observe these UV-protective effects of PS at the mRNA levels of procollagen and MMP-1 in young skin. The reasons for this discrepancy between protein and mRNA in young skin may be explained by the fact that the skin samples were taken only at one time point (48 h) after UV irradiation. The time course of changes at the mRNA and

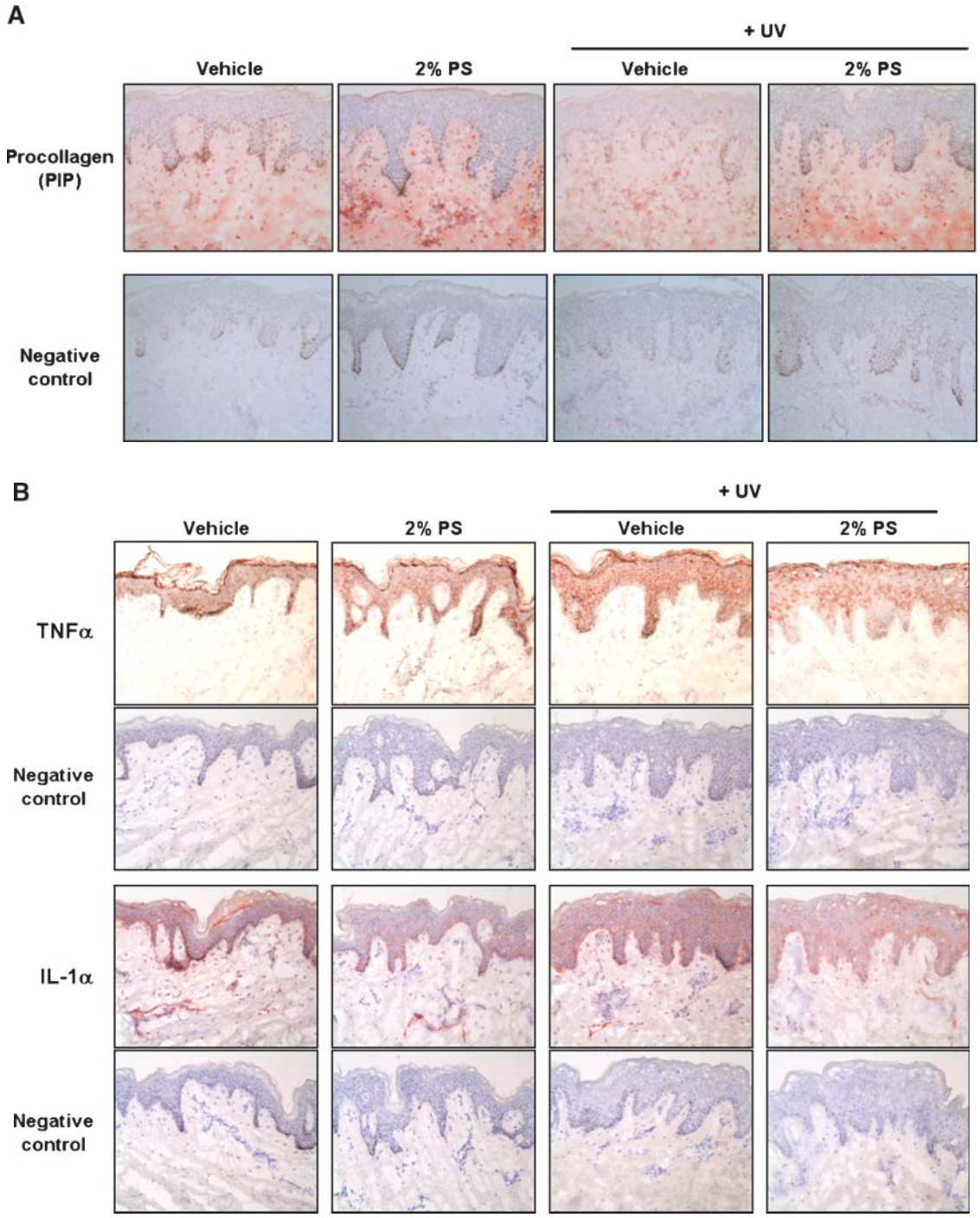

Fig. 5. Immunohistochemical staining of type I procollagen, TNF-α, and IL-1α expression in the young human skin in vivo. Young human skin (n = 5) was topically treated with 2% PS or its vehicle under occlusion, twice, 24 h apart, and irradiated with UV (2 MED). Buttock skin was biopsied 48 h postirradiation. Four serial sections (4 μM) per subject were mounted onto silane-coated slides. The sections were stained with antibodies against (A) type I procollagen (PIP), and against (B) TNF-α and IL-1α. Negative control staining was performed with normal rabbit immunoglobulin without primary antibody (magnification × 200).

protein levels is different in each. Usually mRNA levels change earlier, followed by the changes in protein level. In addition, PS was shown to increase procollagen expression and decrease MMP-1 expression in intrinsically aged human skin in vivo. No previous research has been reported on the collagen-stimulating and MMP-inhibiting effects of topically applied PS in aged human skin in vivo.

Although the exact mechanism of cutaneous aging is unclear, it is known that intrinsic biological cutaneous aging results in a loss of collagen and an increase in MMP-1 expression (22). Photoaging is thought to occur through the generation of reactive oxygen species, subsequent activation of activator protein 1 (AP-1) and nuclear factor κB (NF-κB), and resultant induction of MMPs,

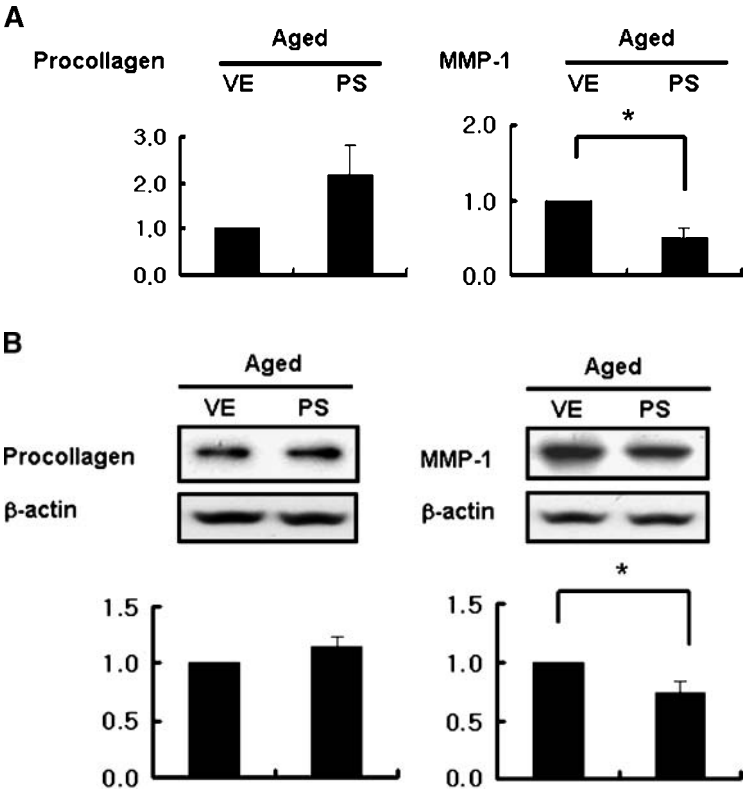

Fig. 6. PS increased type I procollagen expression and decreased MMP-1 expression in intrinsically aged human skin in vivo. A: Buttock skin of aged (>70 years) male subjects was treated with 2% PS and its vehicle for 2 weeks (three times per week, total of six times) under occlusion and biopsied for real-time PCR of procollagen and MMP-1 genes (n = 6). B: Results of Western blot analysis for procollagen and MMP-1 (n = 5). Error bars indicate ± SD. VE, vehicle-treated skin; PS, PS-treated skin; *, statistically significant difference between vehicle-and PS-treated skin; β-actin, loading control for Western blot.

decreased synthesis of collagen, and inflammation (2). AP-1-induced MMPs are secreted from keratinocytes and fibroblasts and break down collagen and other proteins that comprise the dermal extracellular matrix. Transcription factor NF-κB amplifies expression of 92-kd gelatinase and stimulates proinflammatory cytokine genes, including TNF-α, IL-1β, IL-6 and IL-8, and adhesion molecules. NF-κB recruits neutrophils, thereby introducing preformed neutrophil collagenase (MMP-8) into UV-irradiated skin. Despite the repair process that follows, including tissue inhibitors of MMPs, invisible solar scars result. A lifetime of acute exposures with accumulation of invisible solar scars will eventually lead to visible solar scars, causing the characteristic wrinkling of photodamaged skin

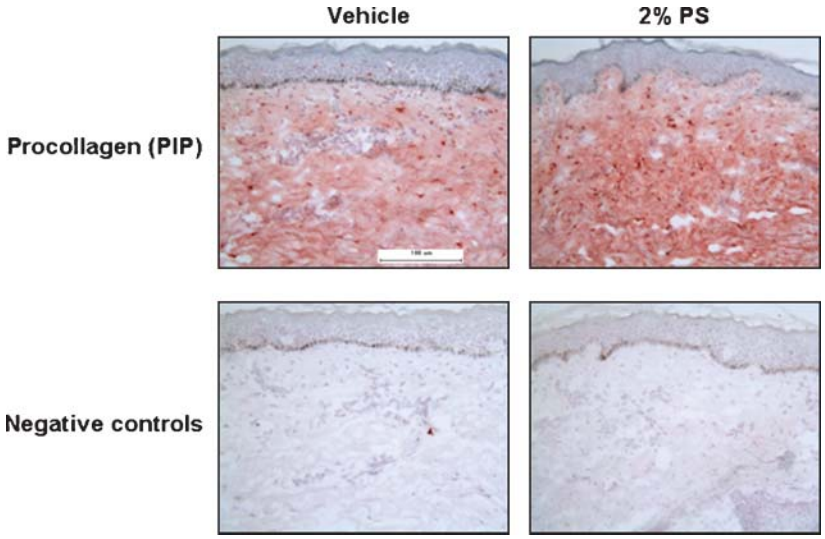

Fig. 7. Immunohistochemical staining of type I procollagen expression in the aged human skin in vivo. Buttock skin of aged (>70 years) male subjects was treated with 2% PS and its vehicle for 2 weeks (three times per week, total of six times) under occlusion and biopsied. Four serial sections (4 μM) per subject were mounted onto silane-coated slides and stained with an antibody against type I procollagen (PIP). Negative control staining was performed with normal rabbit immunoglobulin without primary antibody (n = 6, magnification × 200).

(23). In this research, PS demonstrates anti-skin-aging activities in both intrinsically aged skin and UV-irradiated skin. In this study, because we used whole-skin samples that contain epidermis and dermis, the differential effects of PS on the epidermal keratinocytes and dermal fibroblasts were not clear and remain to be investigated.

PS is a ubiquitous phospholipid species that is normally located within the inner leaflet of the cell membrane. During apoptosis, PS is exposed on the outer leaflet of the cytoplasmic membrane and serves as a target for the PS receptor of phagocytes. The latter is responsible for anti-inflammatory signaling and the induction of TGF-β (24). It is possible that topically applied PS may coat the cytoplasmic membrane in a manner similar to the exposed membrane PS and serve as a signal for TGF-β induction, thereby stimulating collagen synthesis. In photoaging, UVB irradiation triggers cutaneous inflammatory responses by directly inducing epidermal keratinocytes to produce specific proinflammatory cytokines such as IL-1α (25), IL-6 (25, 26), and TNF-α (25, 27). PS might also exert its anti-aging effects by binding to PPARα and thus decreasing UV-induced inflammation. Activators of PPARα protect human skin from UVB-induced cell damage by reducing UVB-induced secretion of proinflammatory cytokines such as IL-6 and IL-8 (16). Topical PPARα activators are known to reduce inflammation in irritant and allergic contact dermatitis animal models (28), and activators of PPARα such as clofibrate, WY-14643, and fatty acids have been shown to repress inflammation-related gene expression by interfering with other signaling pathways, such as the AP-1 and NF-κB, by protein-protein interactions and cofactor competition. As a result, they prevent the activities of these transcription factors (29), and then may reduce the production of the proinflammatory cytokines IL-6 and IL-8 (16). The present study demonstrates that PS inhibits the UV-induced increase of the IL-6 and COX-2 genes in cultured fibroblasts (Fig. 3C), which suggests anti-inflammatory properties of PS. However, topical PS treatment to human skin did not prevent UV-induced IL-1α and TNF-α significantly (Fig. 5B). Further study is necessary to investigate the anti-inflammatory effects of PS in human skin in vivo.

In conclusion, this study demonstrates for the first time that topical application of PS increases procollagen synthesis and inhibits MMP-1 induction in both UV-irradiated skin and intrinsically aged skin in vivo. These results suggest that topical PS application could be of therapeutic benefit in the treatment of skin aging. Further studies on the mechanism of the beneficial effects of PS on skin aging, including signaling pathways, are necessary in order to fully elucidate the function of topically applied PS in human skin.

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