NEW BENEFITS OF A NOVEL ASCORBIC ACID DERIVATIVE ON SKIN HOMEOSTASIS - 3-O-LAURYLGLYCERYL ASCORBATE -

Yushi Katsuyama\(^1\), Tatsuya Tsuboi\(^1\), Sayaka Nakamura\(^1\), Norihisa Taira\(^1\), Hitoshi Masaki\(^2\), Masato Yoshioka\(^1\)

\(^1\)SEIWA KASEI CO., LTD., 1-2-14, Nunoichicho, Higashiosaka, Osaka, 579-8004, Japan.
\(^2\)Tokyo University of Technology, 1404-1, Katakuracho, Hachiouji, Tokyo 192-0982, Japan

Summary
To improve the disadvantages of existing water-soluble ascorbic acid (AsA) derivatives on their skin moisturizing effects, we added a laurylglyceryl group to AsA to produce 3-O-laurylglyceryl ascorbate (3LGA). In studies to assess the efficacy of 3LGA, we found that it had new functions not seen in AsA. Although 3LGA had less ability to scavenge reactive oxygen species (ROS), it elicited a superior reduction of intracellular ROS with or without extracellular stimuli such as exposure to \(\text{H}_2\text{O}_2\) or UVB. 3LGA up-regulated the expression of mRNAs encoding peroxisome proliferator activated receptor-\(\gamma\) (PPAR-\(\gamma\)) and nuclear factor E2-related factor 2 (Nrf2), which resulted in increases of glutathione (GSH) and catalase due to the up-regulation of mRNAs encoding \(\gamma\)-glutamyl cysteine synthetase (\(\gamma\)-GCS) and catalase. In addition, 3LGA increased ceramide synthesis associated with the stimulation of serine palmitoyl transferase (SPT) mRNA expression. Thus, we report novel functions of a novel AsA derivative on the homeostasis of the skin in this study.

Introduction
In skin care, “moisturizing” is the most basic and important function needed to maintain homeostasis in the skin. Directly, an enhancement of skin moisture prevents skin roughness and fine wrinkles. Skin moisture is regulated by two factors, one of which is its barrier function and the other is its water holding function. The skin barrier function depends on intercellular lipid lamellae constructed of ceramide, cholesterol and free fatty acids\(^1\). However, the skin barrier function is reduced with aging and is impaired after UV exposure. These facts suggest that oxidative stress caused by the aging process and by UV exposure might be an important contributor to the skin barrier dysfunction.

Ascorbic acid (AsA) is a well-known vitamin that provides a scavenging effect against various types of reactive oxygen species (ROS). However, AsA not only has poor stability in aqueous solutions and in cosmetic formulations, but it also influences the stabilities of those formulations. Thus far, to improve the stability of AsA in cosmetic formulations, several stabilized water soluble derivatives of AsA have been developed and launched on the market\(^2\), \(^3\). Among those water soluble AsA derivatives, magnesium ascorbyl phosphate (MAP) is widely used in cosmetic formulations, and has inhibitory effects against oxidative stress after conversion to AsA via dephosphorylation by some phosphatases in the skin\(^4\). However, it is known that MAP causes skin dryness during long-term use by consumers. Thus, the cosmetic industry is trying to develop new

\[\text{HO} \quad \text{HO} \quad \text{H}^+ \quad \text{O} \quad \text{1} \quad \text{2} \quad \text{3} \quad \text{O} \quad \text{OH} \quad \text{n-C}_{12}\text{H}_{25} \quad \text{O} \]

Figure 1. The structure of 3-O-laurylglyceryl ascorbate (3LGA)
AsA derivatives which have improved water solubilities and moisturizing effects.

To meet this demand, we developed an amphipathic derivative of AsA, 3-O-laurylglyceryl ascorbate (3LGA), by introducing a laurylglyceryl group into AsA (Figure 1). We found that 3LGA up-regulates the intracellular anti-oxidation system and restores ceramide synthesis that is down-regulated by H$_2$O$_2$. In this study, we report the novel functions and benefits of the AsA derivative, 3LGA, on maintaining skin homeostasis.

**Methods**

**Radical scavenging ability:** Scavenging against 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma, USA) and H$_2$O$_2$ (Nacalai Tesque, Japan) were measured. In the DPPH scavenging assay, 250 μM DPPH were mixed with the samples and changes in absorbance at 524 nm due to the scavenging of DPPH radicals were measured using a microplate reader (Enspire™ 2300 multilabel plate reader, Perkin Elmer, USA). In the H$_2$O$_2$ scavenging assay, 5 mM H$_2$O$_2$ was incubated with the samples for 30 min at 37°C. The resulting solution was then mixed with 4-aminoantipyrine (Tokyo Chemical Industry, Japan), phenol (Kanto Chemical, Japan) and horseradish peroxidase (MP Biomedicals, USA). After 10 min of incubation at 37°C, the absorbance of the solution at 490 nm was measured.

**Intracellular ROS in normal human epidermal keratinocytes (NHEKs):** NHEKs (2×10$^4$ cells/well) were seeded into 96-well plates in HuMedia-KG2 (Kurabo, Japan). After pre-culture with samples for 24 h, cells were loaded with 2',7'-dichlorofluorescein diacetate (DCFHDA, Invitrogen, USA) as the probe for intracellular ROS. After exposing the cells to H$_2$O$_2$ (0.2 mM for 2 h) or UVB (40 mJ/cm$^2$), intracellular ROS levels were evaluated by measuring the fluorescence intensity (Ex; 485 nm, Em; 530 nm). The UVB source used was a TL20W/12RS UVB broadband lamp (Philips, USA) and irradiation energies were measured with a UVX radiometer (UVP, Canada). In addition, protein concentrations were determined using a BCA protein assay kit (Thermo, USA).

**Protective effect of cell damage induced by oxidative stress:** NHEKs (2×10$^4$ cells/well) were seeded into 96-well plates in HuMedia-KG2. After pre-culture with samples for 24 h, cells were exposed to an oxidative stress such as H$_2$O$_2$ (0.2 mM for 2 h) or UVB (40 mJ/cm$^2$). Survival rates of cells were measured using the Alamar blue assay (Invitrogen).

**Stimulating effect of the intracellular antioxidant system:** NHEKs (5×10$^4$ cells/well) were seeded into 48-well plates in HuMedia-KG2. After pre-culture with samples for 6 or 24 h, total RNAs were extracted from the cells and cDNAs were synthesized using the Power SYBR Green Cells-to-CT kit (Life Technologies, USA). Real-time PCR was performed with SYBR Green Master Mix (Applied Biosystems, USA) using the StepOnePlus™ Real-Time PCR system (Applied Biosystems).

**Quantification of glutathione (GSH):** NHEKs (5×10$^4$ cells/well) were seeded into 48-well plates in HuMedia-KG2. After pre-culture with samples for 24 h, cell homogenates were used to measure total GSH by the GSH reductase recycling assay$^5$. 
Immunofluorescence for Nrf2: NHEKs (3 × 10^5 cells) were seeded into type I collagen-coated cover slips (IWAKI, Japan). After pre-culture with samples for 6 h, cells were fixed with 4% formaldehyde (Wako, Japan), and then were permeabilized with 0.2% Triton X-100. After blocking nonspecific binding with 1% IgG-free BSA (Sigma), 2 μg/mL rabbit anti-Nrf2 polyclonal antibody (Abcam, UK) was used for immunofluorescent staining. 2 μg/mL donkey anti-rabbit IgG H&L (Alexa Fluor 647, Abcam) was used to label antibody bound to Nrf2. Cell nuclei were stained with 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI, Dojindo Laboratories, Japan). Confocal images were obtained using a laser scanning confocal microscope (LSM510, Carl Zeiss, Germany).

Restoration of SPT (serine palmitoyl transferase) mRNA decreased by H_2O_2: NHEKs (5×10^4 cells/well) were seeded into 48-well plates in HuMedia-KG2. After pre-culture with samples for 24 h, cells were exposed to H_2O_2 for 1 h. Cells were then incubated for 6 h in fresh medium and SPT mRNA was quantified by Real-time PCR.

Skin barrier function in reconstructed human epidermis equivalents (RHEEs): RHEEs (Labcyte, J-TEC, Japan) were topically treated with samples on their stratum corneum and were incubated for 10 days. During that period, the medium and samples were replaced every other day. After the incubation, skin barrier function was evaluated by measuring transepidermal water loss (TEWL) with a handy TEWL measuring instrument (VAPO SCAN AS-VT100RS, Asahi Techno Lab, Japan). In addition, ceramide amounts of RHEEs were measured using high performance thin layer chromatography (HPTLC, Merck, USA).

Results and Discussion

Anti-oxidative potential

We compared the anti-oxidative ability of 3LGA with AsA. Although 3LGA had scavenging abilities against DPPH radicals (Figure 2a) and H_2O_2 (Figure 2b), its abilities were lower than those of AsA. However, 3LGA showed an excellent suppression of the elevation of intracellular ROS levels in NHEKs after treatment with H_2O_2 (Figure 3) or UVB (Figure 4). MAP, which is easily converted to AsA in cells and causes reduced intracellular ROS at high concentrations, didn’t show any effect in the concentration range tested. Further, 3LGA protected cells from damage induced by H_2O_2 treatment and by UVB irradiation (Figure 5). Considering those results, the anti-oxidative effect of 3LGA in cells was hypothesized to be due to the following reason(s): 1) 3LGA is converted to AsA in cells, and/or 2) 3LGA stimulates the intracellular anti-oxidation system. If #1 was the main reason, 3LGA had a higher cell permeability compared with MAP.
Figure 2. Radical scavenging abilities of 3LGA
Scavenging abilities against (a) DPPH radicals and (b) H₂O₂ were measured using colorimetric analyses. Bars indicate means ± S.D. (n = 3). **p<0.01 indicates a significant difference against 0 μM (Student’s T-test).

Figure 3. Intracellular ROS suppression of NHEKs treated with or without 3LGA after H₂O₂ treatment
Bars express means ± S.D. (n = 4). **p<0.01 indicates a significant difference between groups (Student’s T-test).

Figure 4. Intracellular ROS suppression of NHEKs treated with or without 3LGA after UVB irradiation
Bars express means ± S.D. (n = 4). **p<0.01 indicates a significant difference between groups (Student’s T-test).
Stimulating effects of the intracellular anti-oxidation system

It is well known that the intracellular anti-oxidation system is regulated by the Nrf2 (nuclear factor E2-related factor 2) - ARE (antioxidant response element) signaling pathway\(^6\). Under stress conditions, Nrf2, which is activated through the modification of Keap1 (Kelch-like ECH-associated protein 1), binds to the ARE and promotes the expression of anti-oxidant genes, such as superoxide dismutase (SOD), catalase and \(\gamma\)-glutamyl cysteine synthetase (\(\gamma\)-GCS), which is the rate-limiting enzyme of \textit{de novo} glutathione (GSH) synthesis\(^7\). Therefore, we examined mRNA expression levels of intracellular antioxidants of cells treated with 3LGA. 3LGA significantly up-regulated the mRNA expression levels of catalase and \(\gamma\)-GCS at 24 h after treatment, although MAP failed to up-regulate those mRNAs (Figure 6). In addition, we found that 3LGA increased the level of intracellular GSH (Figure 7). Further, in order to assess the effect of 3LGA on catalase, we investigated changes of intracellular ROS levels using buthionine sulfoximine (BSO), which is an inhibitor of \(\gamma\)-GCS\(^8\). Intracellular ROS levels were increased by treatment with BSO in a dose-dependent manner, and MAP did not suppress the increased intracellular ROS levels elicited by BSO. In contrast, 3LGA suppressed the increased intracellular ROS levels elicited by BSO (Figure 8). In this system, intracellular GSH defected due to BSO treatment, however 3LGA suppressed intracellular ROS. These results suggest that increased catalase activity induced by 3LGA is responsible for the suppression of intracellular ROS and indicate that 3LGA increases levels of GSH and catalase by up-regulating levels of mRNAs encoding \(\gamma\)-GCS and catalase.

**Figure 5. Protective effects against oxidative stress induced cell damage by 3LGA in NHEKs**

Bars express means ± S.D. (n = 4). **\(p<0.01\) indicates a significant difference between groups (Student’s T-test).**
Figure 6. Stimulating effects of intracellular antioxidant mRNAs by 3LGA in NHEKs
Bars express means ± S.D. (n = 4). **p<0.01 indicates a significant difference against 0 µM (Student’s T-test).

Figure 7. Stimulating effects of intracellular GSH by 3LGA in NHEKs
Bars express means ± S.D. (n = 3). **p<0.01 indicates a significant difference against 0 µM (Student’s T-test).

Figure 8. Influence of BSO on the intracellular ROS of NHEKs treated with 3LGA
Bars express means ± S.D. (n = 4). **p<0.01 indicates a significant difference between groups (Student’s T-test).
Stimulating ability of catalase and GSH via activation of PPAR-γ

PPAR-γ (peroxisome proliferator activated receptor-γ), which is a key regulator involved in differentiation, proliferation and inflammation, is one of the most important regulators of the Nrf2 pathway. It has been reported that PPAR-γ regulates the expression of the catalase and γ-GCS genes through binding to the PPRE (peroxisome proliferator response element) in their promoter regions. Thus, we examined the signaling pathways of the 3LGA stimulation of γ-GCS and catalase focusing on PPAR-γ and Nrf2. In cells treated with 3LGA for 6 h, PPAR-γ and Nrf2 mRNA levels were up-regulated significantly (Figures 9, 10). Further, in 3LGA-treated cells, Nrf2 protein was increased compared with untreated control cells (Figure 11). In order to address the contribution of PPAR-γ, we examined intracellular catalase and γ-GCS mRNA levels using an agonist and an antagonist of PPAR-γ. GW1929, which is an agonist of PPAR-γ, up-regulated the mRNA expression levels of both catalase and γ-GCS. In contrast, BADGE, which is an antagonist of PPAR-γ, abolished the up-regulated mRNA expression levels of both catalase and γ-GCS stimulated by 3LGA (Figure 12). These results support a pathway where 3LGA promotes intracellular catalase and GSH through the pathway of PPAR-γ and Nrf2, and suggest that 3LGA might be a ligand of PPAR-γ.

![Figure 9. Effect of 3LGA on the expression of PPAR-γ mRNA](image1)
Bars express means ± S.D. (n = 4). **p<0.01 indicates a significant difference against 0 μM groups (Student’s T-test).

![Figure 10. Effect of 3LGA on the expression of Nrf2 mRNA](image2)
Bars express means ± S.D. (n = 4). *p<0.05 indicates a significant difference against 0 μM (Student’s T-test).
Skin barrier function

The results reported above suggest that 3LGA might be a ligand for PPAR-γ and result in the up-regulation of anti-oxidative substances. PPAR-γ is well known as a key regulator of differentiation and also of lipid synthesis. Thus, we attempted to clarify the potential contribution of 3LGA on ceramide synthesis and the relationship between oxidative stress and ceramide synthesis. First, we found that H₂O₂-exposed NHEKs showed a down-regulation of SPT mRNA expression. That result indicated the possibility that oxidative stress initiated by the accumulation of ROS in cells leads to an impaired barrier function through the down-regulation of SPT mRNA. SPT is a rate-limiting enzyme in the synthesis of sphingosine during the de novo synthesis of ceramide. The effects of 3LGA on mRNA expression of SPT under physiological conditions and oxidative stress loading

Figure 11. Nrf2 expression in 3LGA-treated NHEKs
Detected by immunofluorescent staining and confocal microscopy. (Magnification: 100 × )

Figure 12. Effect of a PPAR-γ agonist or antagonist on the expression of catalase and γ-GCS mRNAs
Bars express means ± S.D. (n = 4). *p<0.05, **p<0.01 indicates a significant difference between groups (Student's T-test).
BADGE: PPAR-γ antagonist, GW1929: PPAR-γ agonist
conditions were then examined. 3LGA up-regulated the mRNA expression of SPT in physiological conditions, and further, restored the down-regulation of SPT mRNA following H₂O₂ exposure (Figure 13). In addition, 3LGA-treated RHEEs exhibited significantly lower TEWL values associated with quantitative increases of various ceramides (Figures 14, 15). Those results indicate that 3LGA reinforces skin barrier function by stimulating ceramide synthesis.

**Figure 13. Influence of H₂O₂ on SPT mRNA expression of NHEKs treated with 3LGA**

Bars express means ± S.D. (n = 4). *p<0.05, **p<0.01 indicates a significant difference between groups (Student’s T-test).

**Figure 14. Effect of 3LGA on the skin barrier function in RHEEs by TEWL analysis**

Bars express means ± S.D. (n = 3). *p<0.05 indicates a significant difference against 0 μM (Student’s T-test).

**Figure 15. Effect of 3LGA on the skin barrier function in RHEEs by HPTLC analysis**

Ceramide levels in RHEEs was observed by HPTLC. S: standard, 1: non-treated cells, 2-4: 3LGA-treated cells (2: 10 μg/mL, 3: 30 μg/mL, 4: 100 μg/mL)

**Conclusion**

AsA is the most popular active ingredient in the cosmetic field, although it has many defects regarding its own stability and disturbing the stability of products containing it. To overcome these defects, several derivatives of AsA have been developed and launched on the market. However, existing water-soluble derivatives are not effective to improve skin moisture levels. To achieve that, we developed 3LGA by introducing a laurylglyceryl group into AsA to improve its hygroscopic property due to the effect of glycerin.
3LGA reduced intracellular ROS levels with or without extracellular stimulations such as H$_2$O$_2$ and UVB, although it did not have the anti-oxidative properties of AsA. Those effects were elicited since 3LGA stimulated anti-oxidative substances such as catalase and GSH through the PPAR-$\gamma$ and Nrf2 signaling pathway. In addition, 3LGA exhibited a lower TEWL associated with ceramide synthesis.

Taken together, the results show that 3LGA interacts with PPAR-$\gamma$ like a kind of ligand, and initiates the signal to activate Nrf2 and finally results in maintaining cellular redox levels through the induction of GSH and catalase. Further, the PPAR-$\gamma$ signal may stimulate ceramide synthesis through the up-regulation of SPT mRNA expression, resulting in the reinforcement of the skin barrier function (Figure 16).

Oxidative stress initiated by ROS accumulation plays a critical factor in skin homeostasis. A bias towards oxidation in the cellular redox balance causes most of the problems in skin beauty such as a decreased moisture function, as well as increased pigmentation and wrinkling. In this study, we demonstrate the novel function of a newly developed AsA derivative, 3LGA, on ROS scavenging and reinforcement of the skin barrier function. We expect that 3LGA will be an effective novel derivative of AsA in the future.

![Figure 16. Proposed mechanism of 3LGA on its skin moisturizing effect via the activation of PPAR-$\gamma$](image)

References


