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Carnosine and Retinol Synergistically Inhibit UVB-Induced PGE_2 Synthesis in Human Keratinocytes through the Up-Regulation of Hyaluronan Synthase 2

In Guk Park[1], Sun Hee Jin[1], Seungchan An[1], Min Won Ki[1], Won Seok Park[2], Hyoung-June Kim[2], Yongjoo Na[2,*] and Minsoo Noh[1,*]

[1]Natural Products Research Institute, College of Pharmacy, Seoul National University, Seoul 08826,
[2]Basic Research & Innovation Division, AmorePacific Corporation R&I Center, Yongin 17074, Republic of Korea

Abstract

Skin aging results from complex interactions of intrinsic and extrinsic factors, leading to structural and biochemical changes such as wrinkles and dryness. Ultraviolet (UV) irradiation leads to the degradation of hyaluronic acid (HA) in the skin, and the fragmented HA contributes to inflammation. This study revealed that the synergistic combination of carnosine and retinol (ROL) increases HA production in normal human epidermal keratinocytes (NHEKs) by upregulating hyaluronan synthase 2 (HAS2) gene transcription. Simultaneously, the combined treatment of carnosine and ROL significantly attenuates UVB-induced prostaglandin E_2 (PGE_2) synthesis in NHEKs. A significant correlation exists between the increase of HA synthesis and the inhibition of PGE_2 production. This study suggests that combined treatment of carnosine and ROL can improve skin aging phenotypes associated with UVB irradiation.

Key Words: Skin aging, Inflammation, PGE_2, Hyaluronic acid, Carnosine, Retinol

INTRODUCTION

Skin aging is promoted by diverse extrinsic factors such as UV irradiation, pollutants, smoking, and air humidity. These extrinsic factors trigger cutaneous inflammation and maintain a low level of chronic inflammatory status, leading to wrinkles, irregular pigmentation, and skin dryness (Koohgoli *et al*., 2016). Especially, UV irradiation is the most significant extrinsic factor that causes DNA damage, cellular oxidative stress, and upregulation of pro-inflammatory lipid mediators like prostaglandin E2 (PGE_2) (Camillo *et al*., 2022; Lee *et al*., 2022). UVB irradiation also promotes the degradation of hyaluronic acid (HA) by upregulation of hyaluronidase and downregulation of HA synthases (HAS) (Šínová *et al*., 2022).

HA is an essential component of the extracellular matrix composed of repeating polymeric disaccharides of D-glucuronic acid and N-acetyl-D-glucosamine, which is important in its hydrophilic nature. Low molecular weight (LMW) HA fragments have been shown to increase inflammation, whereas high molecular weight (HMW) HA (>1,000 kDa) fragments have been shown to inhibit inflammation (Muto *et al*., 2019). In humans, three distinct isoforms of hyaluronan synthase have been characterized, and each displays varying temporal expression profiles throughout development, cellular specificity, and differential synthesis of HA polymers in terms of size. HAS1 and HAS2 are associated with the synthesis of HMW HA, whereas HAS3 is involved in the synthesis of LMW HA (Itano *et al*., 1999).

Retinol (ROL), a derivative of vitamin A, is extensively prescribed in anti-aging cosmetic formulations. ROL is well-known for maintaining a positive collagen mass balance in the human dermis, not only by increasing collagen biosynthesis but also by inhibiting matrix metalloproteinase (MMP) expression (Romana-Souza *et al*., 2019). In addition, recent studies have shown that ROL increases HA levels in reconstructed human tissues and in human clinical studies (McCook *et al*., 2016; Li *et al*., 2017). In the epidermis, ROL may be effective in restoring epidermal thickness. However, ROL can induce cutaneous inflammation in some populations (Griffiths *et al*., 2023). Consequently, there are many studies that have in-

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***Corresponding Authors**
E-mail: minsoonoh@snu.ac.kr (Noh M), nay@amorepacific.com (Na Y)
Tel: +82-2-880-2481 (Noh M), +82-31-280-5966 (Na Y)
Fax: +82-2-762-8322 (Noh M), +82-31-284-8478 (Na Y)

vestigated the compounds that enhance the pharmacological benefits of ROL and reduce its adverse outcomes.

In this study, we found out that carnosine, an endogenous dipeptide consisting of alanine and histidine, synergistically stimulates ROL-induced HA production by inhibiting PGE_2 and upregulating HAS2 gene transcription in normal human epidermal keratinocytes (NHEKs).

MATERIALS AND METHODS

Cell culture and viability assay

NHEKs were purchased from Lonza (Walkersville, MD, USA) and were cultured in KBM supplemented with human epidermal growth factor, insulin, bovine pituitary extract, epinephrine, hydrocortisone, transferrin, and gentamicin/amphotericin B at 36°C with 10% CO_2. NHEKs were passaged at 80-90% confluence. Carnosine and ROL were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell viability was evaluated using a CCK-8. NHEKs were cultured in 48-well plates up to 100% confluence. To measure cell viability, carnosine and/or ROL were treated to NHEKs for 24 h. NHEKs were washed 3 times with phosphate-buffered saline (PBS) and treated with 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) solution. At 2 h After WST-8 treatment, the absorbance at 450 nm was measured with a microplate reader (BioTek, Winooski, VT, USA).

Enzyme linked immunosorbent assay (ELISA)

NHEKs were subcultured in 48-well plates and were grown to 100% confluence. After UVB (20 mJ/cm^2) was irradiated to NHEKs by using a UV irradiation system (Bio-Sun, Vilber Lourmat, Collégien, France), carnosine or ROL were treated in NHEKs. At 2 h after UVB irradiation, culture supernatants were harvested. The PGE_2 levels were measured with ELISA kits (Cayman Chemical, Ann Arbor, MI, USA). To quantify HA, a hyaluronan ELISA kit was used (R&D Systems, Minneapolis, MN, USA).

Western blot

Cell lysates were prepared by harvesting cells and lysing in RIPA buffer containing protease and phosphatase inhibitors (Sigma-Aldrich). Western blot was performed as previously described (Pyo *et al*., 2019) with anti-HAS2 antibody (SC-66916, Santa Cruz, CA, USA) and anti-GAPDH (SC-25778, Santa Cruz). Protein bands were visualized using an enhanced chemiluminescence detection system and ImageQuant LAS 4000 (Fujifilm, Tokyo, Japan).

Quantitative real-time reverse transcription polymerase chain reaction

Total RNA was extracted using TRIzol Reagent (Invitrogen, MA, USA). cDNA was synthesized by reverse transcription using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, MA, USA). The expression levels of target mRNAs were quantified by quantitative real-time reverse transcription-polymerase chain reaction (Q-RT-PCR) using the TIi RNaseH Plus kit and an AB7500 Real Time PCR system (Applied Biosystems, MA, USA). PCR primers used included for human HAS1 are as follows: 5'-TCA AGG CGC TCG GAG ATT C-'3 (forward) and 5'-CTA CCC AGT ATC GCA GGC T-'3 (reverse); HAS2: 5'-CTC TTT TGG ACT GTA TGG TGC C-'3 (forward) and 5'-AGG GTA GGT TAG CCT TTT CAC A-'3 (reverse); HAS3: 5'-CGC AGC AAC TTC CAT GAG G-'3 (forward) and 5'-AGT CGC ACA CCT GGA TGT AGT-'3 (reverse); GAPDH: 5'-ACA ACT TTG GTA TCG TGG AAG G-'3 (forward) and 5'-GCC ATC ACG CCA CAG TTT C-'3 (reverse). GAPDH was used as an internal control.

Statistical analysis

The experiments were independently replicated at least three times. The results were expressed as the mean value ± standard deviation (SD). Statistical analyses were conducted using Student's t-test or ANOVA.

RESULTS

Effects of carnosine and ROL on PGE_2 production in NHEKs

To investigate the synergistic anti-inflammatory effect of carnosine and ROL, the UVB irradiation-induced upregulation of PGE_2 in NHEKs was evaluated (Fig. 1A). Up to a concentration of 2 mM, carnosine did not affect the UVB-induced PGE_2 synthesis in NHEKs (Fig. 1B). Although ROL at 10 μM reduced PGE_2 synthesis in UVB-irradiated NHEKs by 13%, its effect was not significant (Fig. 1B). However, carnosine and ROL exhibited a synergistic inhibition of UVB-induced PGE_2 synthesis in NHEKs (Fig. 1C). For example, the co-treatment of 2 mM carnosine and 10 μM ROL decreased PGE_2 production in NHEKs by 35% compared to that of the vehicle control (Fig. 1C). Notably, this synergistic inhibition exhibited a concentration dependency (Fig. 1C).

Effects of carnosine and ROL on HAS2 gene transcription in NHKEs

HA was reported to inhibit PGE_2 synthesis in synovial fibroblasts (Mitsui *et al*., 2008). To investigate whether carnosine and ROL directly affect HA synthesis in NHEKs, their effects on the gene transcription of HAS were evaluated (Fig. 2). Similar to the effect on PGE_2 synthesis, carnosine had no effect on the gene transcription of any HAS subtype (Fig. 2). ROL significantly increased the gene transcription of HAS2 and HAS3 in NHEKs whereas it had no effect on mRNA levels of HAS1 (Fig. 2). Compared to the vehicle control, 10 μM ROL increased HAS2 and HAS3 gene transcription by 1.52-fold and 1.72-fold in NHEKs, respectively. Notably, the co-treatment of 2 mM carnosine and 10 μM ROL resulted in the synergistic upregulation of HAS2 gene transcription by 2.66-fold compared to the vehicle control in NHEKs (Fig. 2B). However, there was no significant synergistic effect on HAS3 in the carnosine and ROL co-treated NHEKs (Fig. 2C). Therefore, these results suggest that carnosine synergistically affects the ROL-induced upregulation of HAS2 gene transcription in NHEKs.

Effects of carnosine and ROL co-treatment on the protein synthesis of HAS2 in NHEKs

Next, Western blot analysis was performed to determine whether protein levels of HAS2 were changed in carnosine and ROL co-treated NHEKs (Fig. 3). The results showed that 10 μM ROL increased the HAS2 protein expression by 1.29-fold at 24 h and by 1.67-fold at 48 h, while carnosine did not affect the protein expression as consistent with its effect on gene transcription. When co-treated with carnosine and ROL in NHEKs, HAS protein synthesis was increased by 1.82 fold

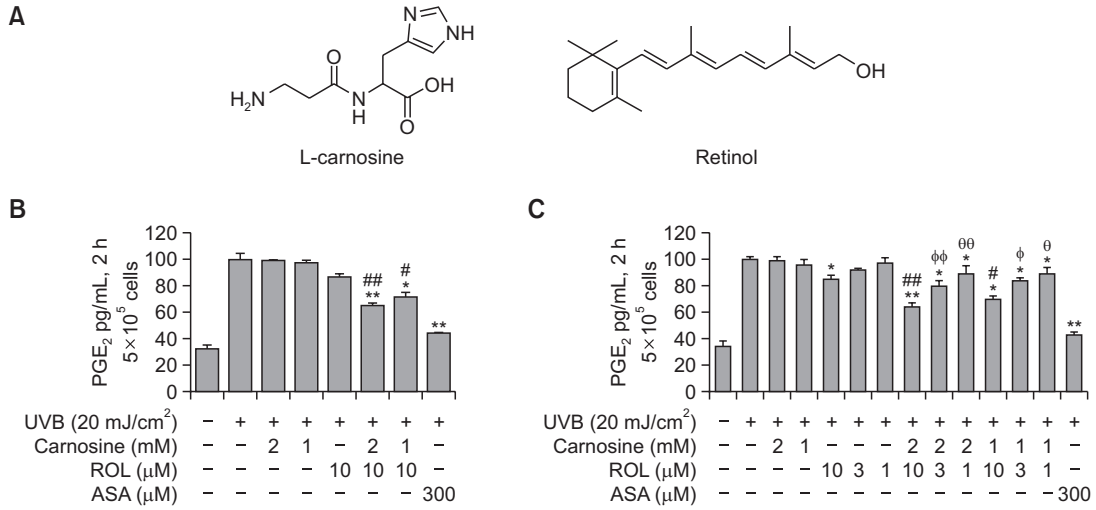

Fig. 1. Effects of carnosine and ROL on PGE_2 production in NHEKs. (A) Structures of L-carnosine and ROL. NHEKs were cultured in 48-well plates. When NHEKs reached 100% confluence, UVB irradiation was applied at a dose of 20 mJ/cm^2, and varying concentrations of carnosine and ROL were treated. (B) The quantification of PGE_2 levels in cell culture supernatants was performed using ELISA. Aspirin (ASA, 300 μM) was used as a positive control drug. (C) The concentration dependency of carnosine and ROL co-treatment on PGE_2 production was determined. Values represent the mean expression ± standard deviation (SD) (n=3). *p≤0.05 vs. UVB (+) vehicle control; **p≤0.01 vs. UVB (+) vehicle control; $^{\#}p$≤0.05 vs. ROL 10 μM; $^{\#\#}p$≤0.01 vs. ROL 10 μM; $^{\phi}p$≤0.05 vs. ROL 3 μM; $^{\phi\phi}p$≤0.01 vs. ROL 3 μM; $^{\theta}p$≤0.05 vs. ROL 1 μM and $^{\theta\theta}p$≤0.01 vs. ROL 1 μM.

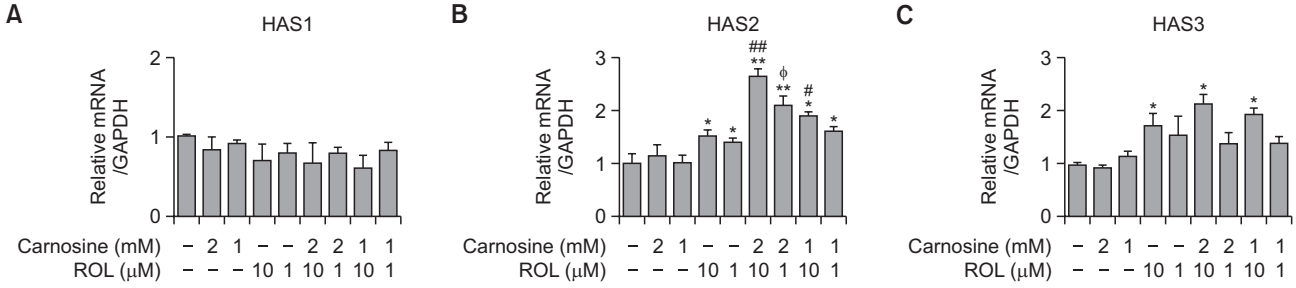

Fig. 2. Effects of carnosine and ROL on HAS mRNA levels in NHEKs. NHEKs were treated with various concentrations of carnosine and ROL for 48 h. The mRNA expression levels of HAS1 (A), HAS2 (B), and HAS3 (C) were measured by Q-RT-PCR. Data are expressed as fold change vs. vehicle control. Values represent the mean expression ± standard deviation (SD) (n=3). *p≤0.05 vs. vehicle control; **p≤0.01 vs. vehicle control; $^{\#}p$≤0.05 vs. ROL 10 μM; $^{\#\#}p$≤0.01 vs. ROL 10 μM and $^{\phi}p$≤0.05 vs. ROL 1 μM.

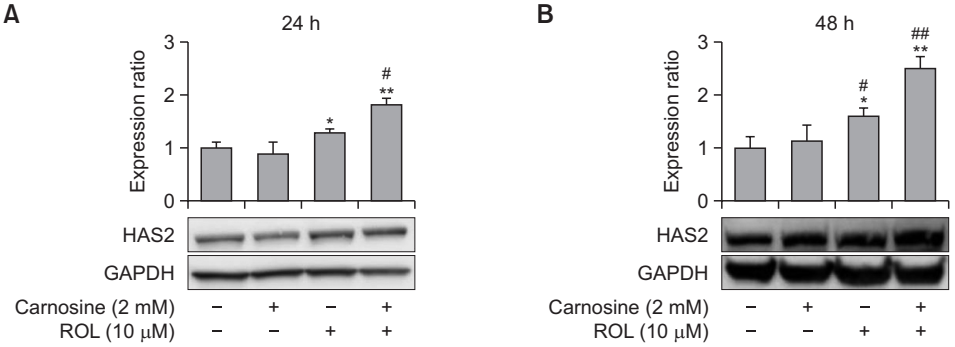

Fig. 3. Effects of carnosine and ROL on HAS protein expression in NHEKs. Western blot analysis was performed to evaluate HAS expression after 24 h and 48 h treatment with carnosine and ROL individually or in combination. NHEKs were cultured in 6-well plates, and protein extraction was conducted. Values represent the mean expression ± standard deviation (SD) (n=3). *p≤0.05 vs. vehicle control; **p≤0.01 vs. vehicle control; $^{\#}p$≤0.05 vs. ROL 10 μM and $^{\#\#}p$≤0.01 vs. ROL 10 μM.

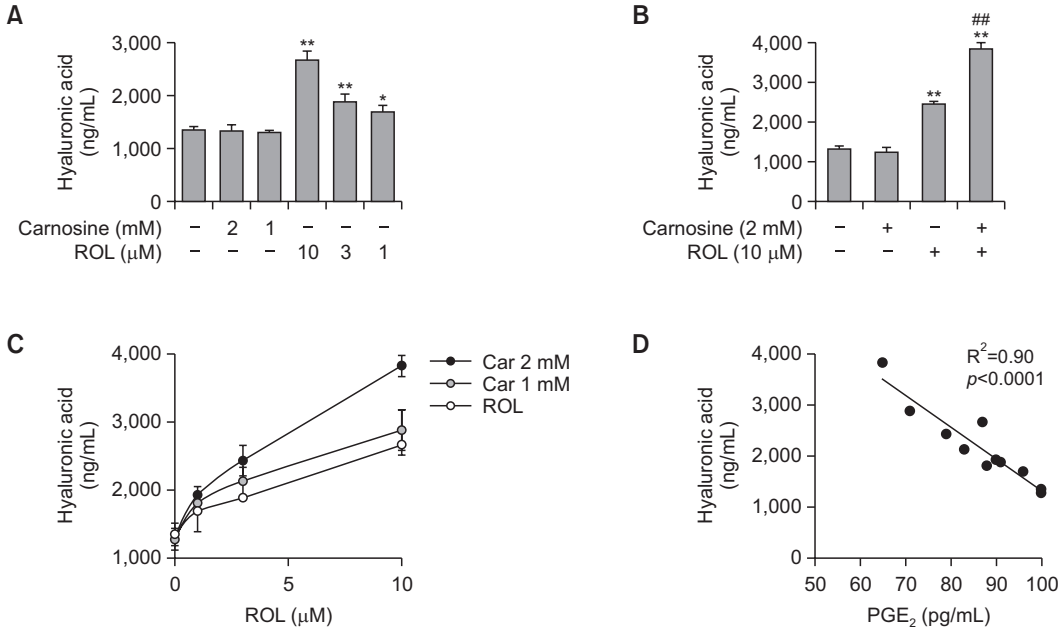

Fig. 4. Effects of carnosine and ROL on HA secretion in NHEKs. NHEKs were cultured in 6-well plates. Upon reaching confluence, carnosine and ROL were treated. Cell culture supernatants were analyzed for HA secreted content by ELISA. The concentrations of HA induced by carnosine and ROL individually (A) and in combination (B) were quantified 48 h after the treatment. (C) The concentration dependent effectsof carnosine and ROL co-treatment on HA production were determined. (D) Pearson's correlation coefficient (R^2) between the HA levels and PGE_2 levels were calculated. Values represent the mean expression ± standard deviation (SD) (n=3). *p≤0.05 vs. vehicle control; **p≤0.01 vs vehicle control and $^{\#\#}p$≤0.01 vs. ROL 10 μM.

at 24 h and 2.5 folds at 48 h (Fig. 3). These results support that carnosine synergistically promoted the ROL-induced HAS2 protein synthesis in NHEKs.

Synergistic effects of carnosine on the ROL-induced HA synthesis in NHEKs

Next, HA levels of NHEKs in response to carnosine and ROL were determined (Fig. 4). ROL is well known to increase HA synthesis in mammalian cells (Takahashi and Takasu, 2011). In accordance with the mRNA and protein levels of HAS; expectedly, upregulated HA secretion in NHEKs in a concentration-dependent manner, while carnosine had no effect (Fig. 4A). Importantly, the co-treatment of 2 mM carnosine and 10 μM ROL enhanced HA production in NHEKS by 2.89-fold compared to that of vehicle control and by 1.56-fold compared to that of the ROL mono-treatment (Fig. 4B). The synergic effects of carnosine on the ROL-induced HA upregulation in NHEKs showed a concentration dependency (Fig. 4C). Notably, a significant correlation existed between the promotion of HA synthesis and the inhibition of PGE_2 synthesis (R^2=0.90, p<0.0001).

DISCUSSION

Skin aging is promoted by extrinsic factors such as UVB irradiation and exposure to pollutants (Krutmann *et al*., 2021). Among diverse external stimuli, UVB irradiation-cutaneous inflammation stimulates the skin aging process. PGE_2 biosynthesis is increased in response to UVB irradiation in skin cells and plays a role in the regulation of cutaneous inflammation (Rahman *et al*., 2023). In this study, it was shown that carnosine and ROL synergistically inhibit the UVB-induced PGE_2 synthesis in NHEKs, suggesting their synergistic skin antiaging effect. Although anti-inflammatory properties of carnosine have been reported (Caruso *et al*., 2019), carnosine itself did not affect the synthesis of PGE_2. The synergistic effect of carnosine and ROL was also observed in HA synthesis in NHEKs, which may be associated with the upregulation of HAS2 gene transcription. Especially, with co-treatment of the carnosine and ROL, the upregulation of HA synthesis exhibited a significant negative correlation with the PGE_2 synthesis in NHEKs.

HA levels in human skin decrease as skin ages (Lee *et al*., 2016). The anti-inflammatory activity of HA is significantly associated with antiaging mechanisms in the skin because inflammation promotes skin aging processes (Lee *et al*., 2021). In U937 human myeloid leukemia cells, HA attenuated the lipopolysaccharide (LPS)-induced upregulation of cyclooxygenase-2 (COX-2); consequently, reducing PGE_2 levels (Yasuda, 2007). In addition, HA was known to decrease PGE_2 production in cytokine-stimulated synovial fibroblasts, although its mechanism is not fully elucidated (Bollyky *et al*., 2009). ROL increases the synthesis of HA in NHEKs and dermal fibroblasts (Bjerke *et al*., 2021). In this study, ROL inhibited PGE_2 synthesis at high concentrations. In contrast, the effect of ROL on HA synthesis was significantly potent compared to that on PGE_2 synthesis because ROL increased the cellular production of HA at low concentrations. It is possible that ROL-induced downregulation of PGE_2 synthesis may be mediated by the indirect effect of ROL-induced upregulation of HA synthesis in NHEKs. Three distinct HAS subtypes, HAS1, HAS2, and HAS3, are present in human skin (Itano *et al*., 1999). In

NHEKs, ROL increased mRNA levels of HAS2 and HAS3. Carnosine exhibited a synergistic effect on the ROL-induced HAS2 gene transcription, while there was no observed alteration in the ROL-induced HAS3 transcription. HAS2 is associated with the biosynthesis of HMW HA (Itano *et al*., 1999). The role of HMW HA in cutaneous inflammation is controversial. Future studies should elucidate the direct or indirect effects of the ROL-induced HAS2 upregulation on PGE_2 synthesis and its synergistic role with carnosine in cutaneous inflammation.

In conclusion, this study demonstrated that carnosine synergistically upregulated ROL-induced HA production via the modulation of HAS2 gene transcription. In addition, the carnosine and ROL co-treatment inhibited the UVB-induced PGE_2 synthesis in NHEKs. Considering the age-related decline in HA levels and the progressive increase in cutaneous inflammation, this study supports that the co-treatment of carnosine and ROL has therapeutic potentials in modulating skin aging processes.

CONFLICT OF INTEREST

Authors Won Seok Park, Hyoung-June Kim and Yongjoo Na are employees of AmorePacific Corporation. The other authors have no conflict of interest to declare.

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