

Alpha-Ketoglutarate Stimulates Procollagen Production in Cultured Human Dermal Fibroblasts, and Decreases UVB-Induced Wrinkle Formation Following Topical Application on the Dorsal Skin of Hairless Mice

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Alpha-ketoglutarate is a key intermediate in the Krebs cycle, and a rate-limiting cofactor of prolyl-4-hydroxylase. It also has a potent effect on increasing the proline pool during collagen production, but the details underlying the boosting effect on collagen production by α -ketoglutarate remain as yet unreported. To investigate the effects of α -ketoglutarate on procollagen production and wrinkle formation, we conducted experiments in cultured human dermal fibroblasts and UVB-irradiated hairless mice. Based on ELISA measurements, α -ketoglutarate (10 μ M) stimulated procollagen production in fibroblasts by $25.6 \pm 4.6\%$ compared to vehicle (dH₂O)-treated control cells. Also, we demonstrated that α -ketoglutarate increased activities of prolylase, which is known to play an important role in collagen metabolism, in fibroblasts and *N*-benzyloxycarbonyl-L-proline (Cbz-Pro), prolylase inhibitor, inhibited procollagen synthesis by α -ketoglutarate in fibroblasts. To determine the effect of topically applied α -ketoglutarate on wrinkle formation, α -ketoglutarate (1%) and vehicle (70% propylene glycol, 30% ethanol) were applied on the dorsal skin of UVB-induced hairless mice for twelve weeks. We found that α -ketoglutarate decreased wrinkle formation upon long-term topical application. These results suggest that α -ketoglutarate diminishes UVB-induced wrinkle formation by increasing collagen production, through a pathway that involves prolylase activation. Therefore, application of α -ketoglutarate may represent an effective anti-wrinkle agent for the cosmetic field.

Key words α -ketoglutarate; procollagen; prolylase; prolylase inhibitor; prolyl-4-hydroxylase; anti-wrinkle

Skin aging generally results in a reduction in the amount of connective tissue, and concomitant disorganization of its structure. The most abundant connective proteins are members of the collagen family, with more than 20 different collagen types identified to date. Collagen makes up 70–80% of the dry weight of the skin, and contributes to the stability and structural integrity of tissues. A number of additional functional roles are carried out by additional protein domains.^{1–4} The deposition of collagen is finely controlled and is dependent on the physiological status of the body. Thus, the control of collagen metabolism could potentially be very useful in a variety of therapeutic and cosmetic applications.

Alpha-ketoglutarate (α -KG), a rate-determining intermediate in the Krebs cycle, plays a crucial role in cellular energy metabolism. It also functions as a source of glutamate and glutamine, and as an ammonium ion scavenger in hepatocytes, *via* its transformation into glutamate.^{5–7} Moreover, enteric feeding of α -KG was found to increase circulating plasma levels of such hormones as insulin, growth hormone, and insulin like growth factor-1 (IGF-1).^{8,9}

It has been reported that α -KG is involved in collagen metabolism through a variety of mechanisms. First, α -KG is a cofactor of prolyl-4-hydroxylase (P4H). P4H is located within the endoplasmic reticulum (ER), and catalyzes the formation of 4-hydroxyproline, which is essential for formation of the collagen triple helix. Incomplete hydroxylation of proline residues within the repeated amino acid motif: any amino acid-proline-glycine (X-Pro-Gly), results in incomplete formation of the collagen triple helix. Incorrectly folded triple helices are not secreted into cytoplasm, and are

subsequently degraded in the ER.^{10–12} Second, α -KG contributes to collagen synthesis by increasing the pool of proline residues *via* glutamate.¹³ Proline is a primary substrate for collagen synthesis, and plays a significant role in collagen metabolism. As seen in Fig. 1, proline is generated through the conversion of pyrroline 5-carboxylate (P5C), an intermediate in the interconversion of proline, ornithine and glutamate. Recently, it was shown that in addition to being a source of proline residues through the P5C-pathway, P5C activated collagen production through the activation of prolylase, a key enzyme in proline recycling.¹⁴ This is significant,

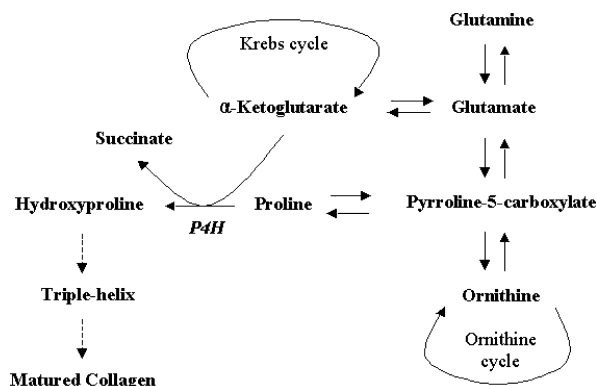


Fig. 1. Schematic Representation of the Roles of α -KG in Collagen Production

P4H, prolyl-4-hydroxylase. α -KG influences collagen production by increasing the proline pool, and also by functioning as a cofactor for prolyl-4-hydroxylase, which catalyzes the formation of 4-hydroxyproline, an essential event in the formation of correctly folded collagen triple helices in the endoplasmic reticulum.

because the P5C-pathway is a minor contributor to the proline pool during collagen synthesis; the major source of proline is through recycling of proline from collagen degradation products.^{15–17} In this regard, α -KG, which is a precursor of P5C, also has a close relationship to proline metabolism. In a study performed in growing pigs, it was shown that enteral α -KG administration increased the level of proline in the portal and arterial blood by 45% and 20%, respectively, when compared to animals that were not given α -KG. Through improved proline and hydroxyproline formation, enteral α -KG is believed to enhance bone tissue formation.^{18,19}

Although we can infer the importance of α -KG in collagen metabolism based on these studies, the direct effects of α -KG on collagen production have yet to be reported. The purpose of this study was to investigate the effect of α -KG on collagen production and UVB-induced wrinkle formation.

MATERIALS AND METHODS

Fibroblast Cell Culture Primary cultures of dermal fibroblasts were established from human neonatal foreskins, and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.48 mg/ml glutamine, 100 IU/ml penicillin, 50 mg/ml streptomycin, and 10% fetal bovine serum in a 37 °C humidified, 5% CO₂ incubator. The fibroblasts were cultured until they were 90% confluent before being passaged. Fibroblasts that had been cultured for <seven passages were used for the experiments. Compounds (α -KG) under investigation were dissolved in vehicle (dH₂O) and diluted with culture media at the indicated concentration (0.1, 1, 10, 100 μ M).

MTT Assay Cytotoxicity of cell was determined by MTT assay,²⁰ which is based on the reduction of the soluble yellow MTT tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to its blue insoluble MTT formazan product by mitochondrial succinic dehydrogenase. For treatment, primary human dermal fibroblasts (1 × 10⁴ cells/well) were maintained on culture media without FBS for 24 h. After α -KG treatment, the cells were cultured for 24 h. The cells were washed with PBS, 200 μ l of MTT (0.05 mg/ml) was added to each well, and the cells were incubated for 4 h at 37 °C. The supernatant was then removed, and 200 μ l of DMSO was added to each well to dissolve the formazan product. Wells without cells were used as blanks. Absorbance was determined at 570 nm, spectrophotometrically, using an ELISA reader.

ELISA for Type I Procollagen Protein The levels of type I procollagen protein in cell-free supernatants were determined by ELISA (Takara, Japan). The supernatants of cultured fibroblast treated with various concentrations of α -KG were harvested, and stored at -70 °C. The ELISA was performed according to the manufacturer's instructions accompanying the product.

Western Blotting Proteins were separated using SDS-PAGE, then transferred to 0.2 μ m pore-sized nitrocellulose membranes at 160 mA for 3 h. Nitrocellulose membranes were blocked with 5% non-fat dried milk in PBS-T for 1 h, then incubated with anti-type I collagen monoclonal antibody (Calbiochem, 1/100), followed by anti-mouse polyclonal secondary antibody conjugated to horseradish peroxidase (Amersham, 1/1000), sequentially. Secondary antibody

was detected using an ECL western blotting detection system (Amersham, U.S.A.) or Opit-4CN substrate kit (Biorad, U.S.A.).

Measurement of Prolidase Activity We measured prolidase activity according to a modified protocol based on the method of Palka *et al.*¹⁷ Cultured fibroblasts were washed with 0.15 M NaCl and harvested. The cells were centrifuged at 1000 rpm and the supernatant was discarded. The cell pellet was re-suspended in 0.05 M Tris-HCl (pH 7.8), sonicated, and centrifuged at 14000 rpm. Supernatants were recovered, and used for the prolidase activity assay and protein determination. Prolidase was first activated by incubating the supernatant in 0.05 M Tris-HCl (pH 7.8) containing 2 mM MnCl₂ for 24 h at 37 °C. Prolidase reactions were initiated by adding 94 mM Glycine-Proline to the pre-activated mixture, followed by incubation for 1 h at 37 °C. The reaction was terminated using 0.45 M trichloroacetic acid (TCA), then the mixture was centrifuged at 12000 rpm for 10 min. Proline was measured by mixing 0.5 ml of the supernatant with a 1 : 1 mixture of glacial acetic acid: Chinard's reagent (25 g of ninhydrin dissolved at 70 °C in 600 ml of glacial acetic acid and 400 ml of 6 M orthophosphoric acid). The mixture was incubated for 10 min at 90 °C, and the UV-absorbance of the mixture was measured at 515 nm. The level of proline was quantified based on a calibration curve derived using a set of proline standards. Protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, Illinois, U.S.A.). Prolidase activity is expressed as nanomoles of released proline per minute per milligram of protein.

To test the direct effect of prolidase inhibitor in α -KG-induced procollagen synthesis, cells were co-cultured with 10 μ M α -KG and 5 mM Cbz-Pro,²¹ a potent inhibitor of prolidase, for 48 h. Type I procollagen proteins in supernatant were determined by ELISA.

Application Studies in Mouse The application study was performed in long-term topical application of α -KG on the dorsal skin of UVB-irradiated mice three times a week for 12 weeks. This study was conducted in conformity with the policies and procedures of the Institutional Animal Care and Use Committee of the AmorePacific R&D center. Female albino hairless mice (Skh:hr-1) purchased from Charles River Laboratories (Wilmington, Mass, U.S.A.) were used for the application of α -ketoglutarate (1%) and the vehicle solution (polyethyleneglycol: ethanol = 7 : 3). UVB irradiation protocols were as follows. During the first week, a UVB dose of 1 MED (minimal erythema dose) was applied. The intensity was then increased by 1 MED per week for up to 4 weeks, after which the mice were exposed to 4 MED for the duration of the experiment.

Generatio of Replicas, and Image Analysis Replicas of mouse dorsal skin were obtained using SILFLO resin (Cuderm, U.S.A.). A photograph of the dorsal skin of each mouse was taken just before the animals were sacrificed. Image analysis of the replicas was performed by IEC Korea (Suwon, Korea). The impression replicas were set on a horizontal sample stand, and wrinkle shadows were produced by illumination with light of a fixed intensity at an angle of 20° using an optical light source (Minebea, China). The shadow images were photographed with a CCD camera (Samsung, Korea) and analyzed by Skin-Visiometer SV 600 software (CK electronic GmbH, Germany). Arbitrary units were as-

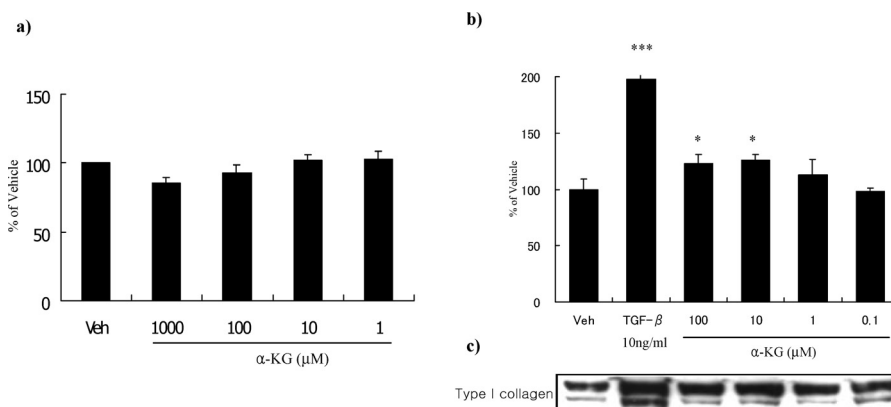


Fig. 2. Concentration-Dependent Effect of α -KG on Collagen Production in Cultured Human Dermal Fibroblast

(a) Cytotoxicity of α -KG was tested by an MTT-based cytotoxicity assay. The results shown are from 3 independent experiments. The bars indicate the standard. (b) Type I procollagen production by confluent monolayers was measured by ELISA (Takara, Japan) according to the manufacturer's instructions. Results are expressed as percentage of vehicle-treated cultures. Values are the means \pm S.D. from three experiments. *** p <0.001, * p <0.05 compared with vehicle-treated group. (c) Western blot analysis of type I collagen in culture supernatants was performed using antibodies specific for type I collagen (Calbiochem, U.S.A.). TGF- β (10 ng/ml) was used as a positive control for procollagen production.

signed to each sample, R1—R5, based on the principle of measuring the depth of furrows according to shadow size and brightness due to inflection under illumination. Skin roughness, referred to as R1, is defined as the difference between the highest crest and lowest furrow. To exclude the possibility of artifact, the program cut the line into five equal parts. R3 represents an average of the maximum distance (R1) derived from each of the five parts of the line; R2 represents the largest value of these five distances. R4 represents the mean area surrounded by a horizontal line drawn at the highest crest and the furrows profile. R5 stands for the mean deviation of the furrow's profile to the middle line. Skin specimens obtained from dorsal skin sacrificed at the end of the study. Dorsal skin were fixed in 10% buffered formalin, embedded in paraffin and sectioned (3—4 μ m) for light microscopy. Sections were stained with Masson-Trichrome for collagen fiber evaluation.

Statistical Analysis Statistical analyses were performed using the Student's *t*-test or Mann Whitney test. A *p*-value of less than 0.05 was considered statistically significant. All analyses were performed using Minitab software (Minitab, U.S.A.).

RESULTS

α -Ketoglutarate (α -KG) Stimulated Procollagen Production in Cultured Human Dermal Fibroblasts An MTT-based cell viability assay revealed that α -KG was slightly cytotoxic at 1000 μ M (Fig. 2a). Thus, fibroblasts were cultured with 0.1, 1, 10 and 100 μ M α -KG. Also, the morphology of fibroblast was not affected by α -KG. In this studies, the effect of α -KG on procollagen production in cultured human dermal fibroblasts was investigated. Cells were treated for 48 h with various concentrations of α -KG, and cell-free supernatants were examined for type I procollagen using a commercially available ELISA kit (Takara, Japan). Treatment with α -KG resulted in an increase in the synthesis of type I procollagen protein in a concentration-dependent manner (Fig. 2b). The levels were: $98.4 \pm 2.4\%$, $112.6 \pm 13.8\%$, $125.6 \pm 4.6\%$, and $122.7 \pm 8.0\%$ of vehicle-treated control at concentrations of 0.1, 1, 10, and 100 μ M of α -KG, respectively. Also western blot assay for type I collagen

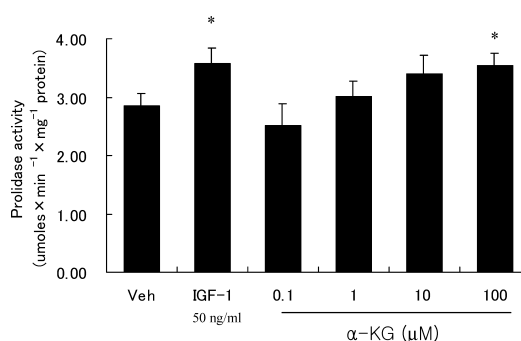


Fig. 3. The Effect of α -KG on Prolidase Activity in Cultured Human Dermal Fibroblasts

Individual cultures were treated with 0.1, 1, 10, 100 μ M α -KG for 24 h, then prolidase activity was measured. IGF-1 (50 ng/ml) was used as a positive control for prolidase activation. Values are the means \pm S.D. from three experiments. * p <0.05 compared with vehicle treated group. IGF-1 (50 ng/ml) was used as a positive control to elevate prolidase activity.

showed that α -KG stimulated the collagen production concentration-dependently up to 1 μ M (Fig. 2c). As a positive control, we also treated the NHFs with 10 ng/ml transforming growth factor- β (TGF- β), which is known to elevate collagen expression.²²⁾

α -Ketoglutarate (α -KG) Enhanced Prolidase Activity in Cultured Human Dermal Fibroblasts On the basis of reports that prolidase plays a crucial role in collagen metabolism, we investigated prolidase activity in human dermal fibroblasts incubated with various concentrations of α -KG. In these experiments, cells were grown to confluency, in order to provide the same experimental conditions as those for examining procollagen biosynthesis. Following α -KG treatment at various concentrations for 24 h, we observed maximal stimulation of prolidase activity at a concentration of 100 μ M. This phenomenon paralleled the pattern of procollagen production. Based on the profiles of procollagen production and prolidase activity, we hypothesize that prolidase activation may be a cause of procollagen production by α -KG. As a positive control, we also treated the NHFs with 50 ng/ml insulin like growth-1 (IGF-1), which is known to elevate prolidase activity.²³⁾ Also, IGF-1 is stimulator of collagen biosynthesis. It is important metabolic and mitogenic

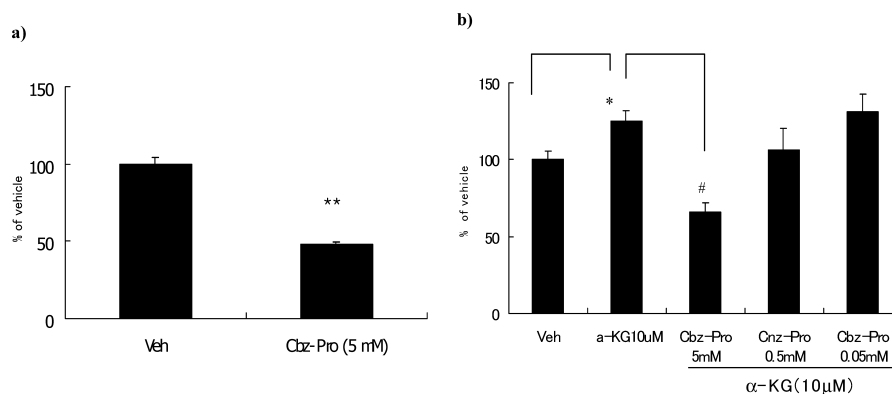


Fig. 4. Cbz-Pro, Prolidase Inhibitor, Inhibited α -KG-Induced Procollagen Synthesis by Inhibiting Prolidase Activity

(a) Cells were cultured with 5 mM Cbz-Pro, prolidase inhibitor, for 24 h, and prolidase activity was measured as described in Materials and Methods. (b) 0.05, 0.5, 5 mM Cbz-Pro was added to culture medium of dermal fibroblast with 10 μ M α -KG and culture media were harvested 48 h later. Type 1 procollagen levels measured in cell free supernatant by Elisa. Values are the means \pm S.D. from three experiments. ** $p < 0.01$, * $p < 0.05$ compared with vehicle treated group. # $p < 0.05$ compared with α -KG treated group.

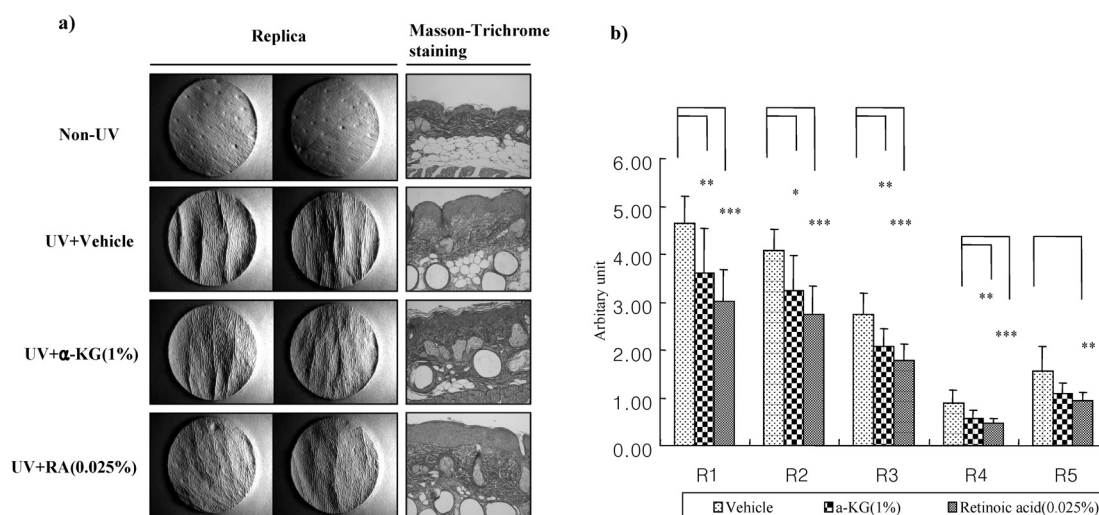


Fig. 5. Wrinkle Formation Was Decreased after Long-Term Topical Treatment of α -KG (1%) Three Times Weekly for 12 Weeks on the Dorsal Skin of Hairless Mice Exposed to UVB Irradiation

The dorsal skin of ten SKH-1 hairless mice was topically treated with 1% α -KG, or vehicle solution (70% propylene glycol, 30% ethanol). Replica and image analysis was performed after 12 weeks. 0.025% retinoic acid (RA) was used as a positive control in order to compare the level of wrinkle formation. (a) Replica photographs showed the inhibition of wrinkle formation upon treatment with α -KG and RA. In Masson-Trichrome staining, RA group and α -KG treated groups increased collagen fibers in dermal-epidermal junction. Original magnification $\times 80$. (b) Graphic representation of the Replica image analysis. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared with vehicle treated group.

factors involved in cell growth and metabolism.

α -KG-Induced Procollagen Expression Decreased by *N*-Benzylloxycarbonyl-L-proline (Cbz-Pro), Prolidase Inhibitor, in Human Dermal Fibroblasts To determine whether Cbz-Pro, prolidase inhibitor, inhibits α -KG-induced procollagen expression, we were tested inhibition of prolidase activity after Cbz-Pro treatment for 24 h, and procollagen expression in co-cultured medium with α -KG and Cbz-Pro in human dermal fibroblasts for 48 h. Cbz-Pro (5 mM), prolidase inhibitor, was found to decrease the activity of prolidase to $48.1 \pm 1.8\%$, compared with vehicle treated human dermal fibroblasts (Fig. 4a). Moreover, Cbz-Pro inhibited α -KG-induced procollagen synthesis in dose-dependent manner to $66 \pm 5.83\%$ at 5 mM and $106 \pm 14.1\%$ at 0.5 mM, versus the α -KG treated group ($125.6 \pm 4.6\%$) (Fig. 4b). These results suggest that α -KG-induced procollagen synthesis may mediate, at least in part, through a pathway that involves prolidase activation in human dermal fibroblasts.

Decreased UVB-Induced Wrinkle Formation Following Long-Term Topical Application of α -KG on the Dorsal

Skin of Hairless Mice To determine whether long-term topical treatment of α -KG effected wrinkle formation in UVB-irradiated hairless mice, the dorsal skins of hairless mice were treated with α -KG (1%) or vehicle solution (70% polyethyleneglycol, 30% ethanol), three times weekly for 12 weeks, following UVB irradiation. Briefly, mice were divided into three groups of 10 mice each, and all the groups were exposed to the same level of UVB irradiation according to the protocol referred to in 'Materials and Methods'. Immediately after each session of UVB irradiation, samples (100 μ l) were applied to the dorsal skin of the mice at the same time each day, three times a week for 12 weeks.

After 12 weeks, a visual assessment and replica assay were performed on each mouse. According to our visual assessment of close-up photos, there was an inhibition UVB-induced wrinkle formation on the skin of mice that received α -KG, as compared to the vehicle-treated control mice (Fig. 5a). To compare of collagen fibers in the α -KG or vehicle-treated skin ($n=3$), we performed the Masson-Trichrome staining. The collagen fibers stained strongly in dermal areas.

After α -KG and retinoic acid (RA) application, the collagen fibers (blue color) of RA or α -KG treated skin were less damaged than vehicle-treated skin (Fig. 5a). Also, replica image analysis revealed inhibition of UVB-induced wrinkle formation by α -KG and retinoic acid (RA) application (Fig. 5b). We treated with 0.025% retinoic acid (RA) as a positive control. It is known to increase collagen synthesis *in vivo*.²⁴⁾

DISCUSSION

In this study, we investigated the effect of α -KG on procollagen production and wrinkle formation, using both *in vitro* and *in vivo* approaches. *In vitro* studies in human dermal fibroblasts revealed that α -KG treatment stimulated production of type I procollagen protein and prolydase activity. Our results also showed that the increase in procollagen production by α -KG in these cells might be due to enhanced prolydase activity, in addition to the mechanisms described by others, including increased proline pools and prolyl-4-hydroxylase cofactor activity.^{10–14)}

Prolydase is a cytosolic enzyme that cleaves imidodipeptides with C-terminal prolines or hydroxyprolines, catalyzing the final step of their degradation into free amino acids in the cytoplasm.¹⁶⁾ The main substrate for prolydase is Gly-Pro, which comes from endogenous and exogenous proline-containing proteins, mainly collagen. Collagen contains the highest amount of imido-bonds of any other protein. The Gly-Pro sequence pair occurs 25 times in the $\alpha 1$ chains of type I collagen. Thus, through its role in recycling proline, prolydase plays an important role in collagen synthesis. The efficiency of proline recycling by prolydase is about 90%; the remainder of the cellular pool of proline seems to be provided by P5C-derived proline donation.¹⁵⁾ Although α -KG was shown to upregulate prolydase activity in this study, we don't yet know exactly what the mechanism of α -KG-stimulated prolydase activity is. According to a recent report, intermediates in the interconversion of glutamine (Gln) to proline, including glutamate (Glu) and pyrroline-5-carboxylate (P5C), as well as Gln itself, induced an increase in collagen biosynthesis in cultured human skin fibroblasts, and the increase correlated with an increase in prolydase activity.¹⁴⁾ It's possible that prolydase activation by α -KG may be related to the induction potential of P5C, since α -KG is a precursor of glutamate. In order to accurately understand the underlying mechanism(s), however, additional studies are required.

In order to investigate the effects of α -KG on skin aging *in vivo*, we used a long-term topical application on the dorsal skin of hairless mice. Following *in vivo* application, we found that α -KG (1%) diminished UVB-induced wrinkle

formation. From these results, we conclude that α -KG may be an efficient anti-wrinkle agent.

In summary, our study provided detailed evidence that α -KG increases procollagen production by stimulating prolydase activity and inhibits UVB-induced wrinkle formation. Also, our results provided another clue to the underlying mechanism of collagen boosting by α -KG, in addition to those previously reported. In conclusion, we suggest that α -KG may be used as an efficient anti-wrinkle agent in the cosmetic field.

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