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Original Article

3,6-Anhydro-L-galactose increases hyaluronic acid production via the EGFR and AMPKα signaling pathway in HaCaT keratinocytes

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ABSTRACT

Background: Hyaluronic acid (HA) is an important factor in skin hydration maintenance. In mammalian keratinocytes, hyaluronan synthase 2 (HAS2) is a critical enzyme in HA production. Therefore, the promotion of HAS2 expression in keratinocytes may be a strategy for maintaining skin moisture.
Objective: The aim was to determine the skin hydration effect and regulatory mechanisms of 3,6-anhydro-L-galactose (L-AHG), a main component of red macroalgal carbohydrates in human keratinocytes.
Methods: L-AHG was applied to an immortalized human epidermal keratinocyte cell line (HaCaT cells). HA production, HAS2 protein and mRNA levels, and the activation of the signaling pathways involved in HAS2 expression were measured. HA levels were also evaluated for three dimensional (3D) reconstructed human skin.
Results: Our results suggest that L-AHG upregulates HA production and may enhance HAS2 expression by activating EGFR-mediated ERK, PI3K/Akt, and STAT3 signaling pathways. We confirmed that L-AHG activated the AMPKα signaling pathway which in turn could regulate HAS2 expression in HaCaT cells. The effects of L-AHG on HA production were observed in the 3D reconstructed human skin model.
Conclusion: Our results suggest that L-AHG may enhance skin moisture retention by increasing HA synthesis in human epidermal keratinocytes.

1. Introduction

Hyaluronic acid (HA) is a major extracellular matrix molecule. It is a nonsulfated glycosaminoglycan consisting of repeating *N*-acetyl-D-glucosamine and D-glucuronic acid disaccharide units. Despite its simple structure, HA has a variety of physiological functions including attachment, apoptosis, proliferation, differentiation, and migration [1–3]. It is also a component of all tissues and fluids and is ubiquitous in human body [4]. HA is most abundant in skin including the epidermis and dermis [5]. In the skin, HA participates in wound healing [6,7]. Especially, it retains large amounts of water and is therefore very important in maintaining moisture and regulating osmotic pressure in the skin [8].

HA is synthesized in the extracellular space by six transmembrane glycosyltransferases known as hyaluronan synthases (HASs). In mammalian cells, the HASs have three isoforms (HAS1, HAS2, and HAS3) which differ in catalytic activity or cell type. The order of their relative catalytic activity is HAS1 < HAS2 < HAS3 [9]. HAS2 is expressed mainly in normal human cells, but HAS3 is predominantly expressed in tumor cells [10]. The most highly expressed isoform in keratinocytes is HAS2 [11] which is downregulated in aged skin [12]. Therefore, the control of HAS2 expression in keratinocytes could maintain skin moisture and homeostasis by regulating HA synthesis.

Li *et al.* reported that platelet-derived growth factor (PDGF)-BB upregulated HAS2 by activating the extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)/Akt and signal transducers and activators of transcriptions (STATs) signaling pathway in human dermal fibroblasts [13]. Epidermal growth

Abbreviations: AMPK, 5′ adenosine monophosphate-activated protein kinase; CREB, cyclic adenosine monophosphate-responsive element-binding protein; DMEM, Dulbecco's modified Eagle's media; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FBS, Fetal bovine serum; GPCR, G protein-coupled receptors; H&E, hematoxylin-eosin; HA, hyaluronic acid; HaCaT, immortalized human epidermal keratinocytes; HAS, hyaluronan synthase; IKK, inhibitor kappa-B kinase; IκB, inhibitor kappa-B; L-AHG, 3,6-anhydro-L-galactose; MEK, mitogen-activated protein kinase/ERK kinase; NF-κB, nuclear factor immunoglobulin kappa chain enhancer-B; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3-kinase; PPAR, peroxisome proliferator-activated receptor; RSK, ribosomal S6 kinase; S.D., standard deviation; STAT, signal transducers and activators of transcription.

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factor (EGF) is one of the most powerful factors influencing keratinocytes. It induces the expression of HAS2 mRNA in epidermal keratinocytes and increases HA production [14,15]. EGF receptor (EGFR), a tyrosine kinase receptor, may activate various signal pathways including ERK and PI3K/Akt in HaCaT cells [16,17]. EGFR activation may also induce STATs via tyrosine kinase Src [18]. These signaling pathways may play an important role in regulating epidermal HAS2 expression [11,19,20]. However, the role of another pathway in upstream HAS2 regulation mechanisms has also been investigated [21–23].

Agarose consists of repeating disaccharide units composed of 3,6-anhydro-L-galactose (L-AHG) and D-galactose linked with α-1,3 and β-1,4 glycosidic linkages. It is the major carbohydrate component of red macroalgae. Agarooligosaccharides from agarose have beneficial effects on skin health including antioxidant, anti-inflammatory, whitening, and moisturizing properties [24–27]. Recently, the chemical and enzymatic production and purification of the agarose monosaccharide L-AHG has been investigated [28,29]. Currently, the only known effect of L-AHG on skin health is whitening. However, its other possible physiological effects have not been fully elucidated.

In the present study, we demonstrated the roles of L-AHG in HA and HAS2 production and its molecular mechanisms in HaCaT cells. We also evaluated its induction of HA biosynthesis in 3D-reconstructed human skin.

2. Materials and methods

2.1. Cell culture

Immortalized human epidermal keratinocyte (HaCaT) cells were cultured at 37 °C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin.

2.2. Hyaluronan enzyme-linked immunosorbent assay

HaCaT cells were added to a 24-well plate at a density of 10^4 cells/well. After 24 h, the cells were washed twice with serum-free DMEM to remove all HA accumulated during cell growth. The cells were starved for more than 12 h in serum-free DMEM to eliminate

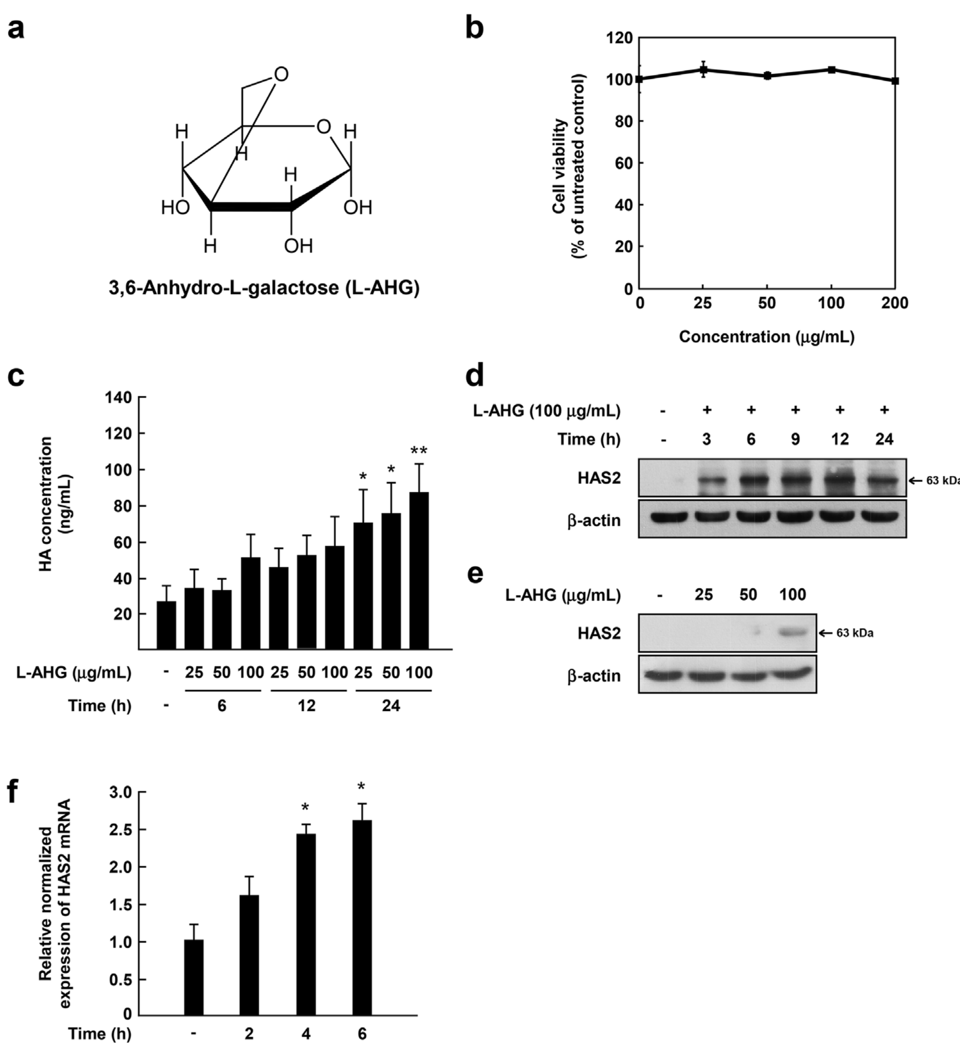

Fig. 1. Effects of L-AHG on HA production and HAS2 expression in HaCaT cells. (a) Chemical structure of L-AHG. (b) Cytotoxicity of indicated L-AHG concentrations on HaCaT cells determined by MTT assay as described in the Supplementary Data S1. (c) Effect of L-AHG on HA production in HaCaT cells. Cells were starved in serum-free DMEM for more than 12 h then treated with L-AHG under the indicated conditions. HA production in the cell culture supernatants were measured by ELISA as described in the Materials and Methods. Data shown in (b) and (c) are means ± S.D. (n ≥ 3). *, $P < 0.05$ and **, $P < 0.01$ vs. control. (d, e) Effect of L-AHG on HAS2 expression in HaCaT cells. Cells were starved in serum-free DMEM then treated with 100 μg/mL L-AHG for 6, 12, or 24 h (d), or with 25, 50, or 100 μg/mL L-AHG for 6 h (e). HAS2 and β-actin expression levels in cell lysates were determined by western blot. The data are representative of more than two experiments with similar results. (f) Effect of L-AHG on HAS2 mRNA level in HaCaT cells. Cells were starved in serum-free DMEM then treated with 100 μg/mL L-AHG for 2, 4, or 6 h. Data represents means ± SED of relative expression of HAS2 mRNA normalized to GAPDH (n=3). *, $P < 0.05$ vs. control.

any effect of FBS. They were then either left untreated or treated with L-AHG in 0.5 mL serum-free DMEM at different time points. At the end of the incubation, aliquots were collected and centrifuged at 11,400 × *g* for 5 min at 4 °C. The supernatants were analyzed for HA with an enzyme-linked immunosorbent assay (ELISA) kit (Echelon Bioscience, Salt Lake City, UT, USA).

2.3. Western blot analysis

The cells were cultured at a density of 10^5/mL in a 60-mm dish for 24 h then starved in serum-free DMEM to eliminate any influence of FBS on kinase activation. After another 24 h, the cells were treated with L-AHG at the indicated times and concentrations. The cells were harvested with lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and a protease inhibitor cocktail tablet]. The protein concentration was determined with a dye-binding protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Lysate protein (20–60 μg) was subjected to 8% or 10% sodium dodecyl sulfate–polyacrylamide gel

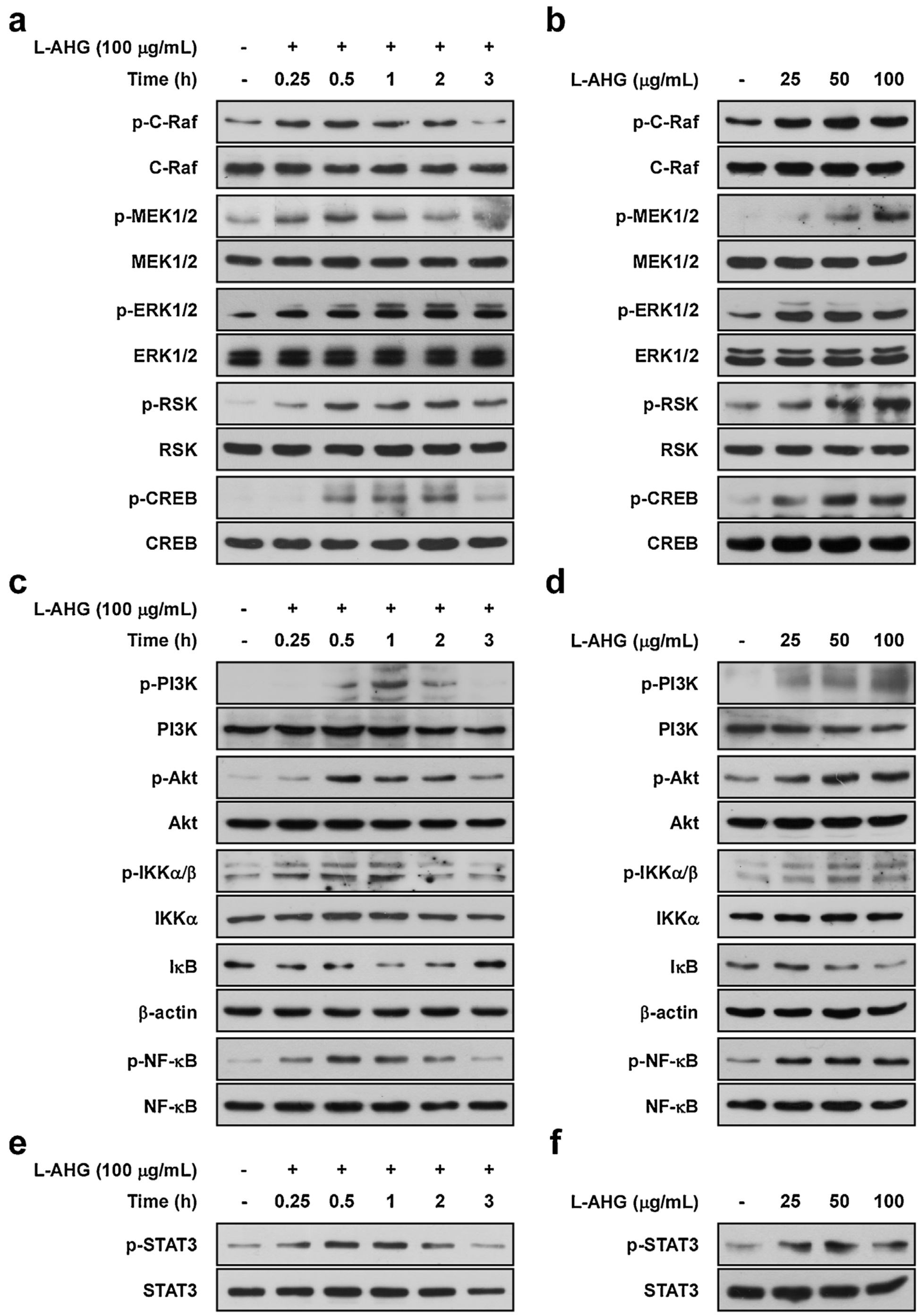

Fig. 2. Effects of L-AHG on ERK, PI3K/Akt, and STAT3 signaling pathways activation in HaCaT cells. (a, c, e) Cells were starved in serum-free DMEM then subjected to 100 μg/mL L-AHG for 0.25, 0.5, 1, 2, or 3 h. (b, d, f) Cells were starved in serum-free DMEM then subjected to 25, 50, or 100 μg/mL L-AHG for 1 h. Proteins levels in the phosphorylated and total forms determined by western blot. The data are representative of more than two experiments with similar results.

electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). After blotting, the membranes were blocked in 5% skim milk for 2 h and incubated with primary antibodies overnight at 4 °C. The membranes were then incubated with secondary antibodies for 2 h and antibody-bound protein was identified with a chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.4. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA of HaCaT cells cultured according to the experimental conditions was extracted using RNeasy kit from Qiagen (Valencia, CA, USA). cDNA was synthesized from 1 μg of purified RNA via reverse transcription with oligo-dT primers using a PrimeScript First Strand cDNA Synthesis Kit (TaKaRa Bio Inc., Kusatsu, Japan). qRT-PCR was performed using TB Green Premix Ex Taq (TaKaRa Bio Inc., Kusatsu, Japan), and determined using CFX96 Real-Time PCR detection system (Bio-Rad, Hercules, California, USA). The expression level of HAS2 mRNA was analyzed by Bio-Rad CFX Maestro 1.1 and presented relative level normalized to GAPDH. The primer sequences for qRT-PCR are presented in Supplementary Table S1.

2.5. 3D reconstructed human skin model and histological staining

A 3D reconstructed human skin model (EpiDermFT) was purchased from MatTek (Ashland, MA, USA) and used as a full-thickness skin model. EpiDermFT was stabilized by incubation overnight at 37 °C in 5% CO_2 in a six-well plate containing 2.5 mL dedicated medium provided by MatTek. After stabilization, the media with L-AHG at concentrations of 0, 12.5 or 25 μg/mL were replaced every day for 7 days. After the incubation, the sections for histological staining were prepared as described in the Supplementary Data S1. For histochemical staining of HA, the sections were deparaffinated, hydrated, and treated with 3% v/v H_2O_2 for 10 min. Thereafter, the sections were incubated for 20 min with normal goat serum (Vector Laboratories, CA, USA), followed by incubation with 5 μg/mL of biotinylated HA binding protein (bHABP; Sigma-Aldrich Corp., MO, USA) at 4 °C for overnight. The sections were then washed with phosphate-buffered saline (PBS) and incubated with avidin-biotin peroxidase (Vector Laboratories, CA, USA) for 1 h. Then 3,3-diaminobenzidine tetrahydrochloride (DAB) reagent was added and the sections were incubated, washed with water, and counterstained with hematoxylin for 1 min. After dehydration and mounting, the stained sections were observed under a Nikon phase-contrast microscope (Nikon, Tokyo, Japan) at 400× magnification. To verify the specificity of HA staining by bHAPB, HA removed by preincubating the section with hyaluronidase from *Streptomyces hyalurolyticus* (Sigma-Aldrich Corp., MO, USA).

2.6. Statistics

Quantitative data were expressed as means ± standard deviation (S.D.) for at least triplicate experiments. Student's *t*-test was used to compare treatment means, and statistical significance was established at $P < 0.05$.

3. Results

3.1. L-AHG upregulates HA production in HaCaT cells

L-AHG (Fig. 1a) treatments up to 200 μg/mL did not significantly affect cell viability for 24 h ($P > 0.05$; Fig. 1b). Therefore, subsequent experiments were conducted such that HaCaT cell viability was not affected by L-AHG. As a result of confirming the effect of L-AHG on HA production, L-AHG significantly induced HA production in a dose-dependent manner after 24 h (Fig. 1c).

Since the control of HAS2 expression is considered important in mediating HA production in human keratinocytes, we determined whether L-AHG regulates HAS2 expression in HaCaT cells. L-AHG enhanced HAS2 expression at all measured time intervals, but maximum induction was observed after 6 h (Fig. 1d). L-AHG upregulated HAS2 in a dose-dependent manner after 6 h (Fig. 1e). In addition, the level of mRNA of HAS2 increased in a time-dependent manner, reaching more than two-fold after 4 h of treatment (Fig. 1f). These results suggest that L-AHG upregulates HA production by increasing HAS2 expression.

3.2. L-AHG activates the ERK, PI3K/Akt, and STAT3 signaling pathways in HaCaT cells

It was reported that the ERK signaling pathway is associated with the regulation of HAS2 expression [13]. Therefore, we explored whether L-AHG increases ERK phosphorylation in HaCaT cells. Here, 100 μg/mL L-AHG activated the ERK signaling pathway in HaCaT cells via the serial phosphorylation of C-Raf, mitogen-activated protein kinase/ERK kinase1/2 (MEK1/2), ERK1/2, ribosomal S6 kinase (RSK), and cyclic adenosine monophosphate-responsive element-binding protein (CREB) (Fig. 2a). In HaCaT cells, C-Raf, MEK1/2, ERK1/2, and RSK were phosphorylated at 0.25–2 h, whereas CREB phosphorylation was observed at 0.5–2 h. L-AHG induced maximum ERK signaling pathway activation at 1 h. Therefore, the dose-dependent effects of 25, 50, and 100 μg/mL L-AHG on ERK signaling pathway activation were evaluated at 1 h. L-AHG increased ERK signaling pathway phosphorylation in a dose-dependent manner (Fig. 2b).

The PI3K/Akt signaling pathway is also a critical HAS2 expression mechanism in human skin cells [13]. PI3K/Akt activation leads to phosphorylate and activate inhibitor kappa-B kinase alpha/beta (IKKα/β) and inhibitor kappa-B alpha (IκB), sequentially. Phosphorylated IκB is degraded by ubiquitination, resulting nuclear factor immunoglobulin kappa chain enhancer-B (NF-κB) is liberated from the complex with IκB and activated, which enhances expression of target protein as a transcription factor. NF-κB has known to act as a transcription factor for human HAS2 mRNA [30]. Therefore, we determined whether L-AHG-mediated HAS2 expression was involved in the PI3K/Akt signaling pathway leading to NF-κB activation. Maximum the signaling pathway activation was observed with 100 μg/mL L-AHG after 1 h (Fig. 2c). Moreover, PI3K/Akt/NF-κB activation by L-AHG was increased in a dose-dependent manner at 1 h (Fig. 2d).

STAT3 is another signaling pathway in HAS2 expression [11,13]. Here, we confirmed that he temporal pattern of L-AHG-induced STAT3 phosphorylation was similar to those observed for ERK and PI3K/Akt signaling pathway activation. STAT3 phosphorylation in response to L-AHG was significantly increased from 0.25 to 1 h (Fig. 2e) and induced in a dose-dependent manner (Fig. 2f).

To investigate whether the pathways were involved in HAS2 expression by L-AHG, we pretreated PD98059 and LY294002, inhibitors of MEK1/2 and PI3K, respectively, 1 h before L-AHG treatment. HAS2 expression induced by L-AHG was abolished by inhibition of MEK1/2 (Fig. 3a) and PI3K (Fig. 3b).

The preceding results show that HAS2 upregulation in HaCaT cells by L-AHG could be mediated by the ERK, PI3K/Akt, and STAT3 signaling pathways.

3.3. AMPKα signaling pathway participates in L-AHG-mediated regulation of HAS2 expression in HaCaT cells

A recent study reported that AMPKα phosphorylation activates PPARα, which in turn significantly influences adiponectin-mediated

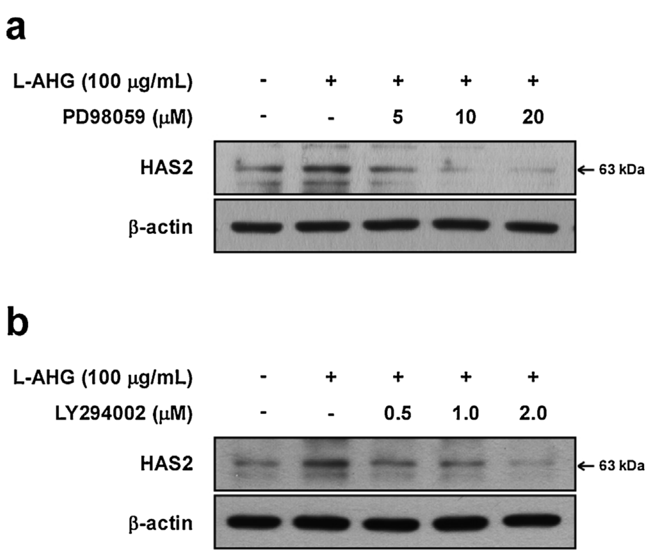

Fig. 3. Effects of ERK or PI3K/Akt signaling pathway inhibition on L-AHG-induced HAS2 expression in HaCaT cells. (a) Serum-starved cells were untreated or treated with inhibitor of MEK1/2 (PD98059) for 1 h, and incubated with L-AHG for 6 h. (b) Serum-starved cells were untreated or treated with inhibitor of PI3K (LY294002) for 1 h, and incubated with L-AHG for 6 h. Protein levels were determined by western blot. The data are representative of more than two experiments with similar results.

HAS2 upregulation in human dermal fibroblasts [23]. First, we investigated whether L-AHG increases AMPKα phosphorylation. It was noted here that L-AHG induced AMPKα phosphorylation in HaCaT cells. Maximum effect was observed after 0.25 h but decreased to the basal level after 3 h (Fig. 4a). We also examined whether L-AHG mediated PPARα activation and affects HAS2 expression. As shown in Fig. 4a, L-AHG activated PPARα in HaCaT cells after 0.5–2 h. L-AHG activated AMPKα/PPARα in a dose-dependent manner (Fig. 4b). To determine whether L-AHG-induced AMPKα/PPARα activation was associated with L-AHG-induced HAS2 upregulation, media were pretreated with Compound C or GW6471 to inhibit AMPKα or PPARα, respectively. L-AHG-induced HAS2 expression was abolished by Compound C or GW6471 in a dose-dependent manner (Fig. 4c and d). Furthermore, we inhibited AMPKα activation by pretreatment with Compound C for 1 h then applied L-AHG to confirm whether L-AHG-mediated PPARα activation is the result of L-AHG-induced AMPKα phosphorylation. L-AHG-mediated PPARα activation was hampered by AMPKα inhibition in a manner dependent on the dose of Compound C (Fig. 4e). Therefore, the results suggest that L-AHG-mediated HAS2 upregulation could be also influenced by the AMPKα/PPARα signaling pathway.

3.4. L-AHG upregulates HAS2 expression via EGFR activation in HaCaT cells

We also attempted to identify the upstream signal triggered by L-AHG in HAS2 upregulation. Here, we demonstrated that L-AHG upregulate HAS2 expression via the ERK, PI3K/Akt, STAT3, and AMPKα signaling pathways. Previous studies reported that the ERK and Akt signaling pathways are activated by EGFR induction in HaCaT cells [16,17]. It was also reported that EGFR activates STATs and AMPKα [18,31]. Previous studies showed that EGF, the ligand of EGFR, significantly enhanced HA production by upregulating HAS2 expression in keratinocytes [14,15]. Therefore, we investigated the role of EGFR in L-AHG-induced HAS2 expression and related signaling pathways. First, we determined whether L-AHG activates EGFR in HaCaT cells. Our results showed that EGFR was most highly activated after 0.25 h at 100 μg/mL L-AHG treatment and that the upregulation decreased with time (Fig. 5a). In HaCaT cells, L-AHG increased EGFR activation in a dose-dependent manner (Fig. 5b). Our results are consistent with previous studies which indicated that the signal termination of EGFR activation is driven by a series of steps including dephosphorylation, endocytosis, and degradation [32]. Our findings indicate that L-AHG activates EGFR as an early response.

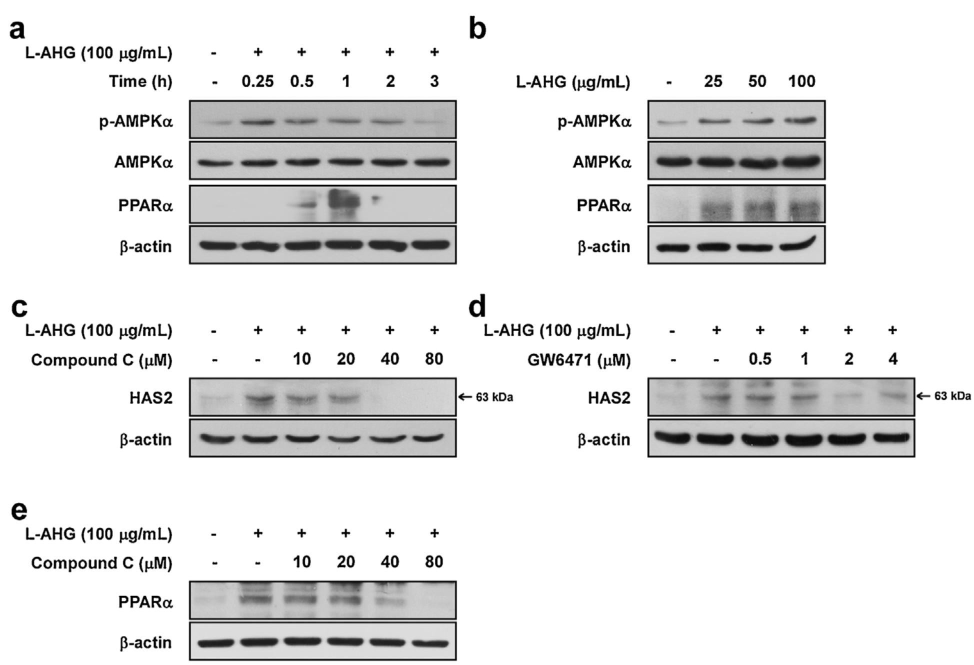

Fig. 4. Effects of AMPKα signaling pathway on L-AHG-induced HAS2 expression in HaCaT cells. (a) Cells were starved in serum-free DMEM for 24 h then subjected to 100 μg/mL L-AHG for 0.25, 0.5, 1, 2, or 3 h. (b) Cells were starved in serum-free DMEM then subjected to 25, 50, or 100 μg/mL L-AHG for 1 h. (c, d) Cells were starved with serum-free DMEM, treated with the indicated inhibitor concentrations for 1 h, and incubated with 100 μg/mL L-AHG for 6 h. (e) Cells were starved with serum-free DMEM, treated with the indicated inhibitor concentrations for 1 h, and incubated with 100 μg/mL L-AHG for 1 h. Protein levels were determined by western blot. The data are representative of more than two experiments with similar results.

We pretreated the media with the EGFR inhibitor gefitinib before applying L-AHG to the HaCaT cells to establish the role of EGFR in L-AHG-induced HAS2 expression. HAS2 upregulation by L-AHG was suppressed by gefitinib-induced EGFR inhibition in a dose-dependent manner (Fig. 5c). We explored whether EGFR activation affects L-AHG-induced signaling pathways in HaCaT cells. In HaCaT cells, EGFR inhibition abolished L-AHG-activated ERK and Akt but only weakly inhibited STAT3 activation (Fig. 5d). However, EGFR inhibitor did not suppress L-AHG-mediated AMPKα phosphorylation. Overall, these results suggest that EGFR plays a significant role in L-AHG-induced HAS2 upregulation. Our findings indicate that L-AHG-induced activation of the ERK/Akt, STAT3, and AMPKα signaling pathways are fully, partially, and not dependent on EGFR, respectively. Moreover, we have confirmed that the effect of L-AHG on HAS2 mRNA level is significantly eliminated by inhibition of ERK, PI3K/Akt, AMPKα and EGFR (Fig. 5e).

3.5. L-AHG enhances the content of HA in 3D reconstructed human skin

We demonstrated the mechanisms of L-AHG-mediated HA upregulation in HaCaT cells. However, the commonly used monolayer cell culture model does not physiologically approximate the human body. Therefore, we validated the effects of L-AHG on HA production using 3D reconstructed human skin with properties similar to those of living human skin. The model consisted of a lower dermis with normal human dermal fibroblasts and an upper epidermis with normal human epidermal keratinocytes. The epidermis was composed of basal, spinous, granular, and cornified layers simulating the multilayered and differentiated tissues characteristic of normal human epidermis [33]. The 3D reconstructed human skin was exposed to L-AHG for 7 days. Relatively low L-AHG concentrations were used for long-term treatments. The cell/tissue layers were observed by H&E staining and HA staining results by bHABP revealed that L-AHG enhanced HA production in the 3D reconstructed human skin model (Fig. 5f).

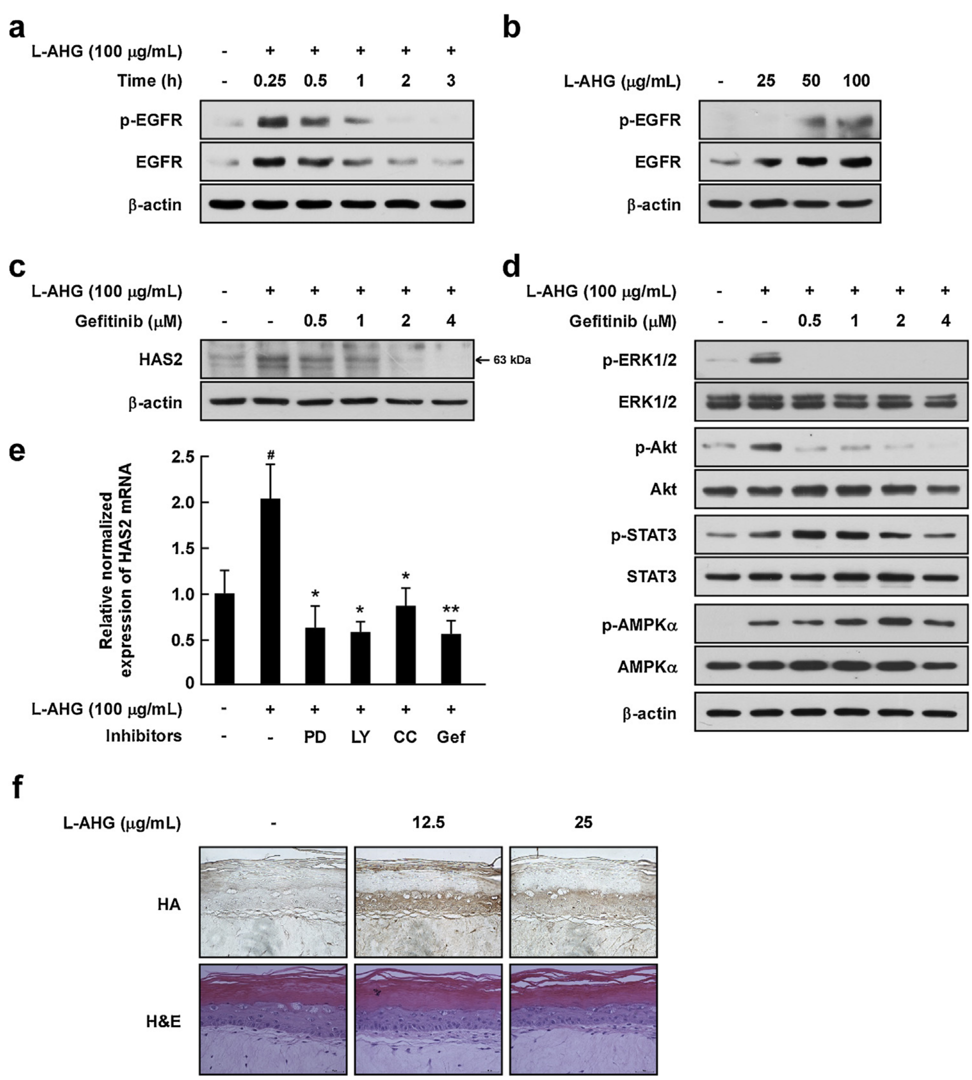

Fig. 5. Effects of EGFR activation on L-AHG-induced HAS2 expression in HaCaT cells. (a) Cells were starved in serum-free DMEM for 24 h then subjected to 100 μg/mL L-AHG for 0.25, 0.5, 1, 2, or 3 h. (b) Cells were starved in serum-free DMEM and subjected to 25, 50, or 100 μg/mL L-AHG for 0.25 h. (c) Cells were subjected to indicated gefitinib concentrations for 1 h then 100 μg/mL L-AHG for 6 h. (d) Cells were subjected to indicated gefitinib concentrations for 1 h then 100 μg/mL of L-AHG for 1 h. Phosphokinase, total kinase, HAS2, and β-actin expression were determined by western blot and the data are representative of more than two experiments with similar results. (e) Serum-starved HaCaT cells untreated or treated with PD98059 (20 μM; PD), LY294002 (2 μM; LY), Compound C (80 μM; CC) or gefitinib (4 μM; Gef) for 1 h then 100 μg/mL of L-AHG for 6 h. Data represents means ± SED of relative expression of HAS2 mRNA normalized to GAPDH (n = 3). #, $P < 0.05$ vs. control; *, $P < 0.05$ and **, $P < 0.01$ vs L-AHG. (f) Effects of L-AHG on HA production in 3D reconstructed human skin model. The 3D reconstructed human skin models were treated with L-AGH for 7 days. HA was stained brown as described in the Materials and Methods. Bar = 50 μm.

4. Discussion

Marine macroalgae (seaweeds) are abundant, rapidly growing photosynthetic organisms rich in carbohydrates and producing multiple annual harvests [34]. Industries using marine macroalgae are expected to generate high-value products from them. The beneficial effects of various carbohydrates derived from marine macroalgae on skin health have been extensively studied [35]. However, only the skin-whitening efficacy of L-AHG has been explored in depth [36]. Adequate moisture is essential for normal skin function and is a crucial factor in maintaining skin health [12]. HA is a major skin moisture factor and has been used in many cosmetic skin hydration formulations because of its remarkable ability to retain large amounts of water [4]. However, since HA is a large, bulky polymer, it is poorly absorbed by the deeper skin layers and is active only on the skin surface. Therefore, we sought a compound to increase HA production in the skin cells themselves. Our results showed that L-AHG significantly upregulates HA production and HAS2 expression in cultured HaCaT cells. Previous studies showed that HAS2 upregulation increases HA production in skin cells. In HaCaT cells, glucosamine stimulated HA synthesis over 24 h and increased HAS2 mRNA levels after 2 h [37]. UDP-glucose elevated HAS2 mRNA via STAT3 Tyr705 phosphorylation and HA synthesis in HaCaT cells [11]. In *fro/fro* mouse fibroblasts, Akt signal pathway activation enhanced HAS2 mRNA which was associated with increased HA production [38]. In HaCaT cells, Compound K activated the ERK and Akt pathways which in turn upregulated *Has2* and stimulated HA synthesis [19,20]. Therefore, the present study proposes that L-AHG may enhances maintenance of water in skin by augmenting HA production in skin cells.

As shown in Fig. 1d, HAS2 upregulation by L-AHG occurred within a short induction time. L-AHG-induced HAS2 expression increased for up to 12 h but was attenuated after 24 h. This result corroborates a previous study which reported that HASs have short half-lives [39]. An earlier study indicated that the Akt, ERK, and STATs signal pathways mediate HAS upregulation [13]. We demonstrated that L-AHG also activated these signal pathways involving HAS2 expression in HaCaT cells. The induction times of these signal pathways (0.25–2 h) were shorter than that for HAS2. We established that L-AHG participates in the ERK, Akt, and STAT3 signal pathways. Furthermore, we anticipated that L-AHG may mediate HAS2 expression by other pathways in certain cases [21–23], despite the well-known mechanism of HAS2 expression in skin cells. A recent study reported that adiponectin promotes HAS2 expression by activating PPARα via AMPKα in human dermal fibroblasts [23]. However, no such role was indicated for the AMPKα/PPARα signaling pathway in HAS2 expression in human keratinocytes. The present study suggests the role of the AMPKα/PPARα signaling pathway in L-AHG-mediated HAS2 upregulation. To the best of our knowledge, this study is the first to report the role of the AMPKα signaling pathway in the regulation of HAS2 expression in human keratinocytes. Although Compound C is the most commercially available selective inhibitor of AMPKα, it may also affect the activation of several other kinases in keratinocytes. Therefore, the effect of other pathways on HAS2 expression regulated by L-AHG can also be considered. Another previous study has demonstrated a conflicting role of AMPKα in HA production [40]. According to this study, activated AMPKα increases phosphorylation of HAS2 at Thr-110 residue, which inhibits the enzymatic activity, consequently leading to the reduction of HA in human aortic smooth muscle cells. Our results showed the increase of HA production following HAS2 mRNA and protein levels, but the effect of this signaling pathway mediated by L-AHG in terms of HAS2 enzymatic activity needs to be confirmed.

Certain studies suggested that the human *Has2* promoter region has binding sites for STAT3, CREB, and NF-κB [15,41]. Therefore, activation of the STAT3, ERK, and PI3K/Akt signaling pathways by L-AHG promoted the STAT3, CREB, and NF-κB transcription factors, which in turn induced *Has2* transcription. In contrast, it is as yet unknown whether PPARα is a transcriptional factor for the *Has2* promoter. For this reason, further research is needed to determine whether AMPKα-mediated PPARα activation directly induces HAS2 mRNA transcription or activates another transcription factor.

We also sought the upstream signal involved in L-AHG-regulated HAS2 expression. It was found that EGFR is also activated by L-AHG in HaCaT cells and mediates the ERK, Akt, and STAT3 signaling pathways but not the AMPKα signaling pathway. EGFR is transactivated by G protein-coupled receptors (GPCRs) whose types and downstream responses are very diverse [17,42]. Ca^{2+} signaling downstream of GPCR activation may induce AMPKα and EGFR in mammalian cells [43,44]. The association between EGFR and Ca^{2+} signaling in human keratinocytes is unclear. However, Ca^{2+} signaling may mediate L-AHG-regulated HAS2 expression in HaCaT cells.

In summary, we identified influence of L-AHG on HA upregulation and its molecular mechanisms in HaCaT cells (Fig. 6). L-AHG upregulates HA synthesis and HAS2 expression by activating the ERK, PI3K/Akt, and STAT3 signaling pathways in HaCaT cells. L-AHG also induces the AMPKα/PPARα signaling pathway which participates in L-AHG-induced HAS2 expression in HaCaT cells. Our data suggest that in HaCaT cells, the L-AHG-mediated ERK, Akt, and STAT3 signaling pathways are EGFR-dependent, whereas the L-AHG-induced AMPKα signaling pathway is not.

In various cell types containing epidermal keratinocytes, HA regulates cell physiology with HA receptors such as CD44 and RHAMM. The stimulation by HA has been reported in the attachment, apoptosis, proliferation, differentiation, migration, tumorigenesis, inflammation and so on [45–47]. Interestingly, describing the effects of HA according to molecular size, small and middle molecular fragments of HA have pro-inflammatory effects, whereas high molecular HA have anti-inflammatory effects [48,49]. Therefore, we expect to improve skin moisturizing ability of L-AHG with the effect on increase of HA production, but we should not exclude the impact of the other side.

Nevertheless, we propose the clinical application of L-AHG for skin hydration effect because we confirmed that L-AHG upregulated HA production in a 3D reconstructed human skin model simulating the physiological characteristics of living skin tissue. To suggest the possibility of L-AHG as a cosmetic material, it is

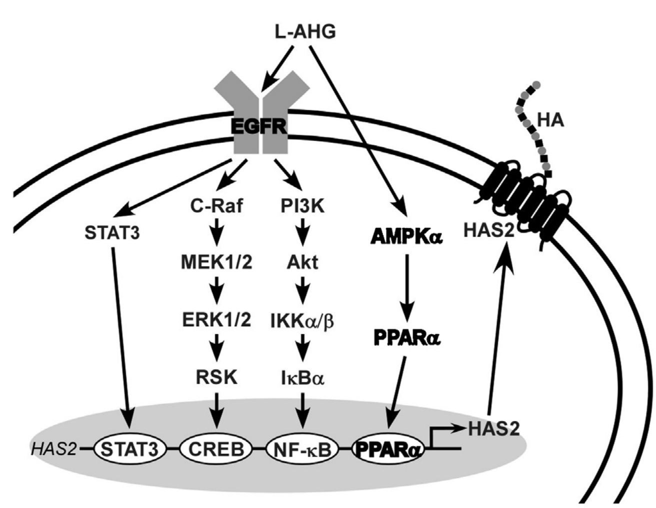

Fig. 6. Schematic diagram explaining skin moisturizing mechanisms of L-AHG. L-AHG enhances HA synthesis in HaCaT cells by inducing HAS2 expression, which in turn is upregulated by EGFR-mediated activation of the ERK, Akt, and STAT3 pathways and by EGFR-independent activation of the AMPKα signal pathway.

necessary to investigate whether L-AHG reaches the epidermis in human skin to upregulate HA production and, whether the regulation has a significant effect on skin hydration.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdermsci.2019.10.005.

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