Cyclic phosphatidic acid and lysophosphatidic acid induce hyaluronic acid synthesis via CREB transcription factor regulation in human skin fibroblasts

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ABSTRACT

Cyclic phosphatidic acid (cPA) is a naturally occurring phospholipid mediator and an analog of the growth factor-like phospholipid lysophosphatidic acid (LPA). cPA has a unique cyclic phosphate ring at the sn-2 and sn-3 positions of its glycerol backbone. We showed before that a metabolically stabilized cPA derivative, 2-carba-cPA, relieved osteoarthritis pathogenesis in vivo and induced hyaluronic acid synthesis in human osteoarthritis synoviocytes in vitro. This study focused on hyaluronic acid synthesis in human fibroblasts, which retain moisture and maintain health in the dermis. We investigated the effects of cPA and LPA on hyaluronic acid synthesis in human fibroblasts (NB1RGB cells). Using particle exclusion and enzyme-linked immunosorbent assays, we found that both cPA and LPA dose-dependently induced hyaluronic acid synthesis. We revealed that the expression of hyaluronan synthase 2 messenger RNA and protein is up-regulated by cPA and LPA treatment time dependently. We then characterized the signaling pathways up-regulating hyaluronic acid synthesis mediated by cPA and LPA in NB1RGB cells. Pharmacological inhibition and reporter gene assays revealed that the activation of the LPA receptor $LPAR_1$, $G_{i/o}$ protein, phosphatidylinositol-3 kinase (PI3K), extracellular-signal-regulated kinase (ERK), and cyclic adenosine monophosphate response element-binding protein (CREB) but not nuclear factor κB induced hyaluronic acid synthesis by the treatment with cPA and LPA in NB1RGB cells. These results demonstrate for the first time that cPA and LPA induce hyaluronic acid synthesis in human skin fibroblasts mainly through the activation of $LPAR_1$-$G_{i/o}$ followed by the PI3K, ERK, and CREB signaling pathway.

1. Introduction

In 1992, cyclic phosphatidic acid (cPA) was originally isolated from the myxoamoebae of a true slime mold, *Physarum polycephalum* [1]. Later, the presence of cPA was also found in mammalian tissues [2,3].

Although the chemical formula of cPA is similar to that of lysophosphatidic acid (LPA), cPA has a quite unique structure with a cyclic phosphate ring at the sn-2 and sn-3 positions of its glycerol backbone. These features provide cPA with biological functions that are distinct from or opposite to those of LPA [4–10]. To date, six LPA receptors, $LPAR_{1-6}$, have been reported, and cPA stimulates $LPAR_{1-5}$. To explain the distinct biological functions of cPA and LPA despite their activation of the same receptors, we speculated the following possibilities: (1) cPA also activates cellular targets distinct from those of LPA receptors, and (2) differences in the affinity and efficacy of cPA and LPA to bind and activate LPA receptors and the combination and expression level of LPA receptors in individual cells affect the final physiological output [11].

To date, we have found that cPA has distinct biological activities such as inhibiting autotaxin [6], suppressing the invasion and metastasis of cancer cells [5–7], blocking nociceptive responses by primary afferent C-fibers [8], and attenuating ischemia-induced delayed neuronal cell death in rat hippocampal CA1 regions [12]. Therefore, cPA is considered

to be a promising therapeutic agent for diseases such as cancer and neurodegeneration [8–12]. To develop this potential, we synthesized several metabolically stabilized derivatives of cPA. 2-Carba-cPA (2ccPA) is one of the compounds in which phosphate oxygen is replaced with a methylene group at the *sn*-2 position [6,10], and we showed that 2ccPA efficiently maintains numerous biological functions of cPA.

Recently, we found that 2ccPA induces hyaluronic acid synthesis in human osteoarthritis synoviocytes [13]. Hyaluronic acid is a predominant component of the extracellular matrix and is involved in cellular functions such as skin hydration, joint lubrication, and stimulation of the migratory response of keratinocytes in wound healing [14,15]. Hyaluronic acid is synthesized by specific enzymes, hyaluronan synthases 1, 2, and 3 (HAS1–3), and in skin dermis, HAS2 is shown to be the predominant isoform [14]. The *HAS2* promoter contains functional response elements for various transcription factors, including nuclear factor κB (NFκB), cyclic adenosine monophosphate response element-binding protein 1 (CREB1), specificity protein 1 (SP1), and signal transducer and activator of transcription 3 (STAT3) [16,17]. Some of these factors are known effector molecules downstream of LPA stimulation [18–20]. Upstream of these transcription factors, phosphatidylinositol-3 kinase (PI3K) [16,21], extracellular-signal-regulated kinase (ERK) [16,22], and Rho/Rho-associated kinase (ROCK) [22] are involved in the signaling pathway of HAS2 protein expression. Furthermore, cPA and LPA stimulate PI3K and ERK signaling [23–25], and LPA stimulates $G_{i/o}$/PI3K–Akt/NFκB signaling [18].

This study focused on hyaluronic acid synthesis in human skin fibroblasts. We showed that cPA and LPA increased HAS2 protein expression and thereby induced hyaluronic acid synthesis in the cells. Moreover, we showed that activation of LPAR₁, $G_{i/o}$ protein, PI3K, and ERK followed by HAS2 transcriptional activation through CREB activation was important for the HAS2-up-regulating signaling pathway mediated by cPA and LPA.

2. Materials and methods

2.1. Pharmacologic agents

cPA 18:1 was chemically synthesized as previously described [26]. Bovine serum albumin (BSA; fraction V, fatty acid-free) and 1-oleoyl-*sn*-lysophosphatidic acid (LPA 18:1) were purchased from Sigma-Aldrich (St. Louis, MO). LPA and cPA were dissolved in phosphate-buffered saline (PBS) containing 0.1% (w/v) BSA. The inhibitors Ki16425, U-73122 (Cayman Chemicals, MI), AG 490, CREB binding protein CBP–CREB interaction inhibitor (Calbiochem, CA), U0126 (LC Laboratories, MA), Y-27632 (Sigma-Aldrich), LY294002 (Cell Signaling Technology, Inc., MA), and BAY 11-7082 (Focus Biomolecules, PA) were dissolved in dimethyl sulfoxide and added to cells at a working concentration of 1 μM (AG 490) or 10 μM 30 min before the addition of cPA or LPA. Pertussis toxin (PTX; List Biological laboratories, Inc., CA) was dissolved in PBS and added to cells at a working concentration of 100 ng/ml 30 min before the addition of cPA or LPA.

2.2. Cell culture and treatment

Normal human skin fibroblasts, RIKEN original (NB1RGB) were provided by RIKEN BRC through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The cells were cultured in minimum essential media-alpha containing 5.6 mM D-glucose (#11900-024, Life Technologies Corp., CA) supplemented with 10% fetal bovine serum (Biowest, FL), 0.2% NaHCO₃, and 0.06% glutamine. Cells were grown at 37 °C in a humidified incubator containing 5% CO₂. For all the experiments, human fibroblasts were seeded into a 6-well plate (5×10^3 cells/well) and incubated for 8 h with culture media containing 10% fetal bovine serum. As it has been reported that hyaluronic acid production was generally higher in subconfluent compared with confluent cultures [27], we seeded cells in very low density to detect clearly the effects of cPA and LPA on

hyaluronic acid synthesis. The cells were then subjected to serum starvation for 16 h with serum-free minimum essential media-alpha. All subsequent incubations were performed under serum-starved conditions.

2.3. Particle exclusion assay

Particle exclusion assay was carried out according to a protocol described previously [28]. Briefly, sheep erythrocytes (Nippon Biotest Laboratories Inc., Tokyo, Japan) were fixed in 1.5% formaldehyde in calcium- and magnesium-free PBS (CMF-PBS) overnight at 37 °C, washed with CMF-PBS 4 times, reconstituted in CMF-PBS (5×10^8 cells/ml) and used for the particle exclusion assay. For the assay, serum-starved fibroblasts (3×10^4 cells/6 cm-dish) were incubated with 3 μM cPA or LPA in serum-free culture media for 24 h. And cells were treated for 30 min at 37 °C in the absence or presence of 16 units/ml of hyaluronidase "Amano" from *Streptomyces hyalurolyticus* (Amano enzyme Inc., Tokyo, Japan) in serum-free media as described previously [29]. Then, fixed sheep red blood cells (1×10^7 cells) in 3 ml PBS containing 0.1% BSA were added. The red blood cells were allowed to settle for approximately 10 min. The fibroblasts were then observed and photographed with a Nikon ECLIPSE TS 100 phase-contrast microscope (NIKON Corp., Tokyo, Japan). The hyaluronic acid pericellular coat to cell area ratios were measured using ImageJ software [30].

2.4. Determination of hyaluronic acid concentration in culture supernatants of fibroblasts

To study the effects of cPA or LPA on human fibroblast hyaluronic acid synthesis, we removed the serum-free media, then we preincubated serum-starved cells for 30 min with or without inhibitors for signal transduction pathways or transcription factors, and then vehicle or 3 μM cPA or LPA was added. Thereafter, the cells were cultured for 24 to 72 h. The cells were counted and media were collected to measure hyaluronic acid using an enzyme-linked immunosorbent assay kit (R&D Systems, Inc., MN) according to the manufacturer's instructions. The obtained data were analyzed and converted to concentration values (ng/1×10^3 cells) or (ng/ml) using the analysis software GraphPad Prism (GraphPad Software, San Diego, CA).

2.5. Agarose-gel electrophoresis of hyaluronic acid

Serum-starved cells were incubated with vehicle, 3 μM cPA or LPA for 72 h, then 20 ml media were collected and concentrated to 30 μl using centrifugal filters (Amicon Ultra-30 K membrane, Millipore, MA). Agarose-gel electrophoresis of concentrated media from vehicle, 3 μM cPA or LPA treated cells was performed as described by Mary K. Cowman et al. [31,32]. Concentrated media (30 μl/lane) and 15 μl/lane of DNA marker (#Marker 6, Nippon gene Co., Ltd., Tokyo, Japan) were loaded on the gel. And electrophoresis was carried out at a constant voltage of 20 V for 30 min and then 50 V for 1 h. Then the gel was stained with 0.005% Stains-All (Sigma-Aldrich) in 50% ethanol/water for 16 h. Destaining was done with 10% ethanol/water. Then, a strong blue band was detected as hyaluronic acid band [33]. The gel image was obtained using a GE Healthcare ImageQuant LAS 4000.

2.6. Western blot analysis

Cells were collected and subjected to western blot analysis to detect phospho-ERK (pERK), ERK, HAS2, and β-actin expressions. Proteins were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to an Immobilon-P Transfer Membrane (Millipore). Using anti-HAS2 (1:500 dilution, #HAS2 (H-60): sc-66916, Santa Cruz Biotechnology, Inc., CA), anti-β-actin (1:1000 dilution, Medical & Biological Laboratories Co., Ltd., Aichi, Japan), anti-pERK or anti-ERK (1:1000 dilution, Cell Signaling Technology, Inc., MA) antibodies and horseradish peroxidase-conjugated anti-rabbit immunoglobulin G

(1:10,000 dilution; Kirkegaard & Perry Laboratories Inc., MD), we performed immunodetection using "Can Get Signal" immunoreaction enhancer solutions (TOYOBO Co., LTD., Osaka, Japan) and an enhanced chemiluminescence system (Bio-Rad Laboratories, Inc., CA).

2.7. RNA isolation and real-time polymerase chain reaction (PCR)

Total RNA was extracted from human fibroblasts using Isogen reagent (Nippon gene) as a template for subsequent complementary DNA synthesis with oligo dT primers using an Omniscript RT Kit (Qiagen, CA). Messenger RNA (mRNA) levels were quantified using an ABI 7300 real-time PCR machine (Foster City, CA) and SYBR *Premix Ex Taq* II (Takara Bio Inc., Shiga, Japan). Gene-specific primer sets for HAS1 were used as previously reported [34], as were primers for β-actin, $LPAR_1$, $LPAR_4$, $LPAR_5$, $LPAR_6$, p2y5, and p2y10 [12].

The following primer sets were used: HAS2, 5′-GCCTCATCTGTGGAGA TGGT-3′ (F) and 5′-ATGCACTGAACACACCCAAA-3′ (R); $LPAR_2$, 5′-GAGG CCAACTCACTGGTCA-3′ (F) and 5′-GGCGCATCTCAGCATCTC-3′ (R); $LPAR_3$, 5′-GAAGCTAATGAAGACGGTGATGA-3′ (F) and 5′-AGCAGGAACC ACCTTTTCAC-3′ (R); and GPR87, 5′-AAATCCAGCAGGCAATTCAT-3′ (F) and 5′-CCCTGATGCTCTGGTTATGTT-3′ (R).

The data were analyzed using the delta cycle threshold method. The expression level of each LPA receptor was normalized to β-actin expression as previously described [12,35]. The expression levels were normalized to those of $LPAR_1$ and expressed in terms of mean ± SE values.

2.8. Reporter gene assay

Reporter gene assay was performed as described [36]. Briefly, cells were transfected with phospho-cyclic adenosine monophosphate response element (pCRE)-secreted alkaline phosphatase (SEAP) vector (Mercury™ pathway profiling system, Clontech Laboratories Inc., CA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Then, after 24 h of incubation, cells were seeded into 6-well plates and incubated for 8 h followed by 16 h of serum starvation. Vehicle or 3 μM cPA or LPA was added, and cell culture media were collected after 4, 8, 12, and 24 h of incubation. SEAP activities in culture media were measured using a Great EscAPe SEAP chemiluminescence kit (Clontech Laboratories Inc.). The SEAP activities were calculated as the ratio of SEAP activity of ligand-stimulated cells to that of vehicle controls.

2.9. Statistical analysis

All values are reported as means ± standard error. The data were analyzed using the Student's *t*-test. A *P* value less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. cPA and LPA induce hyaluronic acid synthesis

Hyaluronic acid synthesis is important to the health and function of joints, eyes, and skin. However, hyaluronic acid synthesis declines with age and maintenance and regulation of the function of these organs are gradually lost. We have revealed that a metabolically stabilized cPA derivative, 2ccPA, remarkably induces hyaluronic acid synthesis in human osteoarthritis synoviocytes. This compound also relieves osteoarthritis pathogenesis in vivo [13]. Because skin aging is mainly triggered by loss of hyaluronic acid in the dermis, we focused on hyaluronic acid synthesis in human skin fibroblasts.

We initially studied the effects of cPA and LPA on hyaluronic acid synthesis in human fibroblasts, NB1RGB cells. Particle exclusion assay was used to detect pericellular hyaluronic acid coats and we observed an obvious increase in pericellular hyaluronic acid coat formation after cPA and LPA stimulation (Fig. 1). And these coats were removed after hyaluronidase treatment, suggesting that the coats surrounding the cells were composed by hyaluronic acid but not by other extracellular matrix components. The pericellular coat/cell area of cPA- or LPA-treated cells was approximately 3-fold higher than that of vehicle-treated cells (Table 1).

To measure hyaluronic acid concentration in media, we performed enzyme-linked immunosorbent assay. cPA treatment increased the amount of hyaluronic acid with incubation time (Fig. 2A). Before addition of cPA and LPA, we measured hyaluronic acid concentration in media (i.e. 0 time), but we could not detect any hyaluronic acid in culture media. After 72 h, 3 μM cPA and LPA was the most effective concentration among various doses (0.1–3 μM). To assess the dose dependency of cPA and LPA, we treated NB1RGB cells with various concentrations of cPA or LPA. As shown in Fig. 2B, the treatment with cPA or LPA for 72 h stimulated hyaluronic acid synthesis in a bell-shaped, dose-dependent manner. At the most effective dose of 3 μM of cPA or LPA, each concentration of hyaluronic acid was approximately 2.7 or 4.7 times higher
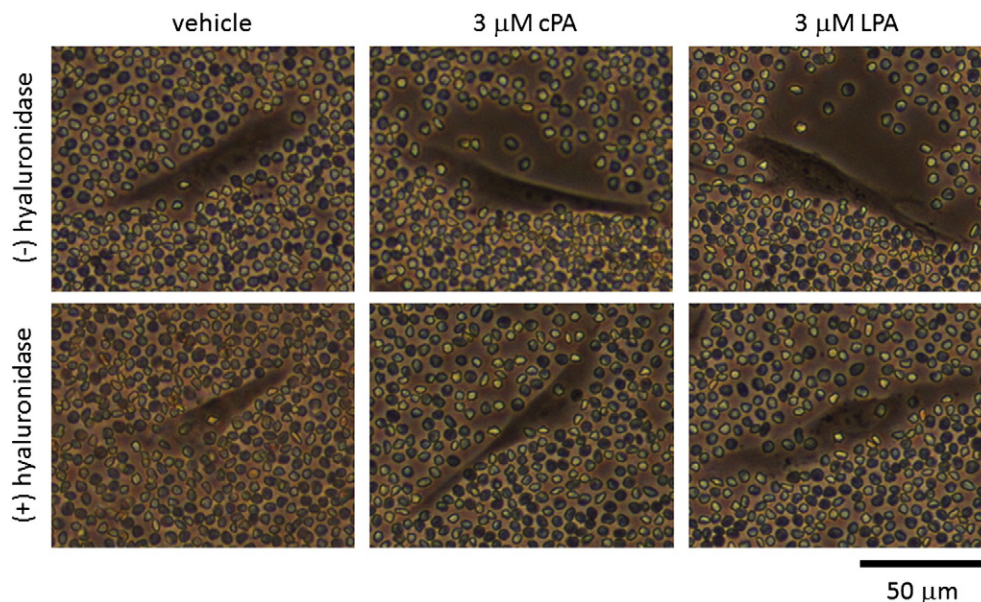

Fig. 1. Particle exclusion assay. Particle exclusion assay was performed to visualize hyaluronic acid coats surrounding cells. NB1RGB cells were treated with vehicle or 3 μM cyclic phosphatidic acid (cPA) or lysophosphatidic acid (LPA) for 24 h. Then cells were incubated with or without hyaluronidase for 30 min.

Table 1
Hyaluronic acid pericellular coat/cell area.

Treatment	Area ratio[a]
Vehicle	1.2 ± 0.1
cPA	3.3 ± 0.2
LPA	3.5 ± 0.2

[a] Values are means $\pm$ standard error (SE) ($n = 20$).

than that in the vehicle-treated group, respectively. Hyaluronic acid is produced at the plasma membrane, however other glycosaminoglycans are synthesized in the Golgi apparatus [37]. To verify whether the effects of cPA and LPA on hyaluronic acid synthesis are specific or not, we examined the effect of cPA and LPA on the synthesis of glycosaminoglycan. Serum-starved cells were incubated for 72 h with vehicle, 3 μM of cPA or LPA and glycosaminoglycan were measured with Blyscan Sulfated Glycosaminoglycan Assay kit (Biocolor Ltd., Northern Ireland, U.K.). We found that cPA and LPA did not increase the synthesis of other glycosaminoglycan under the experimental conditions (data not shown). So, results suggested that the effects of cPA and LPA were specific to hyaluronic acid synthesis.

Moreover, we measured hyaluronic acid mass synthesized after cPA or LPA stimulation. Staining with Stains-All [31–33], a strong blue band of hyaluronic acid was detected and the hyaluronic acid mass was measured as $>2 \times 10^6$ Da (Fig. 2C). These results indicate that cPA and LPA similarly stimulate high molecular weight hyaluronic acid synthesis in human fibroblasts. Hyaluronic acid has several functions depending on its molecular weight. The low molecular weight hyaluronic acid ($<0.5 \times 10^6$ Da) was reported to induce inflammatory responses in inflammatory cells [38]. Here, we show that cPA and LPA stimulate NB1RGB to induce high molecular weight hyaluronic acid synthesis.

To investigate whether cPA and LPA affect HAS1–3 expression, we first examined HAS1–3 mRNA expression levels for 72 h by using quantitative real-time PCR. As shown in Fig. 3A, treatment with 3 μM of cPA and LPA increased mRNA expression of HAS2, a predominant isoform in skin dermis, with a maximum increase between 4 and 8 h. Conversely, HAS1 mRNA expression did not change in the presence of cPA and LPA (Fig. 3A). We could not detect HAS3 mRNA expression in NB1RGB cells (data not shown). It has been reported that HAS2 gene and its natural antisense RNA HAS2-AS1 exhibited coordinated expression and HAS2-AS1 stabilizes or augments HAS2 mRNA expression via RNA/mRNA heteroduplex formation [39]. Coordinated temporal profiles of transcriptional induction of HAS2-AS1 and HAS2 were observed in biphasic response to stimulation with interleukin-1β. On the other hand, transforming growth factor-β1 up-regulated both HAS2-AS1 and HAS2 transcripts for 72 h. In our study, we did not analyze HAS2-AS1 expression and the contribution of HAS2-AS1 expression for HAS2 mRNA expression stimulated by cPA- and LPA-treatment remains unclear, but we observed that HAS2 mRNA expression stimulated by cPA and LPA increased between 4 and 8 h and then decreased gradually afterwards. These results indicate that cPA and LPA treatment increases HAS2 mRNA expression and, in turn, hyaluronic acid synthesis and secretion into culture media.

We examined the protein expression of HAS2 with western blot analysis (see Fig. 3B). As shown in Fig. 3C, HAS2 protein expression was time-dependently increased for 72 h with 3 μM cPA and LPA treatment. These data show that the significant increase of hyaluronic acid in culture media mediated by cPA and LPA is due to up-regulation of HAS2 mRNA and protein expression. Although LPA stimulated HA synthesis much higher than cPA did (Fig. 2B), LPA up-regulated HAS2 mRNA and protein expression at the same level with cPA. So far, 4 kinds of covalent modification of HAS2 protein have been reported [37,40–42]. 1) Phosphorylation of HAS2 at threonine 110 by adenosine monophosphate activated protein kinase (AMPK) decreases its activity [43], 2) O-GlcNAcylation at serine 221 of HAS2 is involved in its stabilization [44], 3) Mono-ubiquitination at lysine 190 of HAS2 is required for dimer formation and its activity [45], and 4)
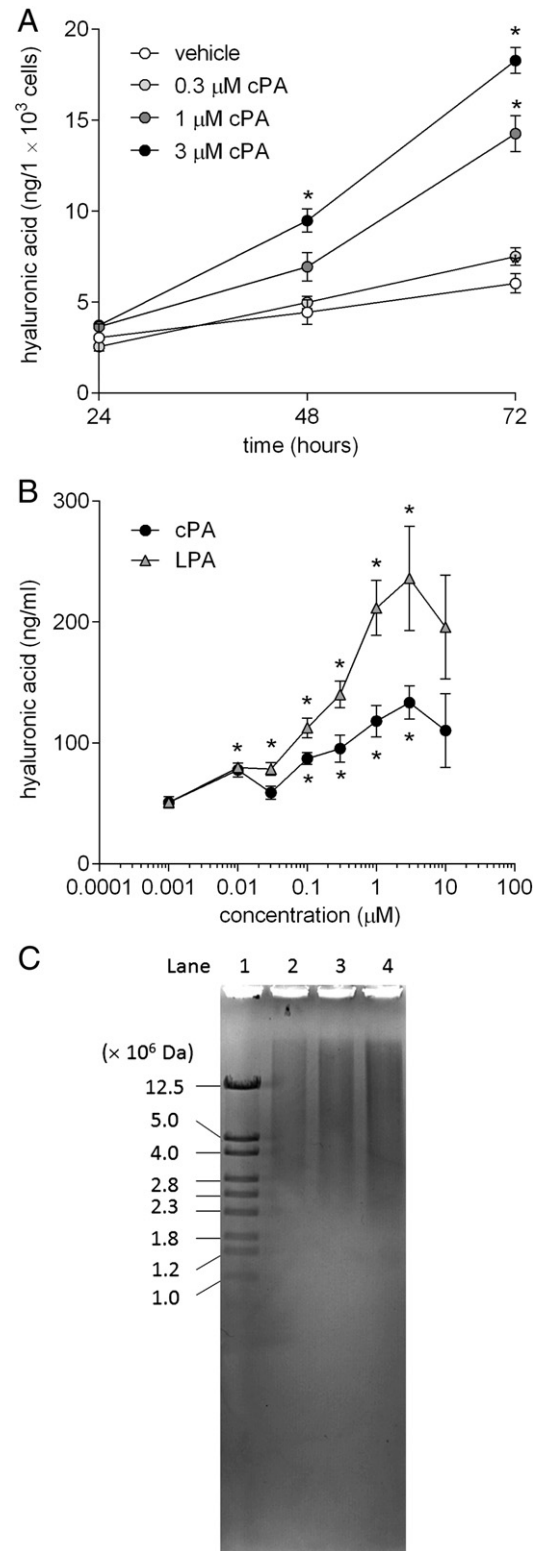

Fig. 2. Effects of cPA and LPA on the synthesis of hyaluronic acid. (A) NB1RGB cells were incubated with 0.3, 1, or 3 μM cPA for the time indicated. The concentrations of hyaluronic acid in culture media were determined with enzyme-linked immunosorbent assay (ELISA). The data represent mean $\pm$ SE values from triplicate independent experiments (*$P < 0.001$ vs. the vehicle-treated group). (B) NB1RGB cells were incubated with vehicle or various concentrations of cPA or LPA for 72 h. The concentrations of hyaluronic acid in culture media were determined with ELISA. The data represent mean $\pm$ SE values from quadruplicate independent experiments (*$P < 0.001$ vs. the vehicle-treated group). (C) Electrophoresis of hyaluronic acid on 0.5% agarose-gel. Lane 1: DNA marker; lane 2: concentrated medium from vehicle treated cells, lane 3: concentrated medium from 3 μM cPA treated cells, and lane 4: concentrated medium from 3 μM LPA treated cells.
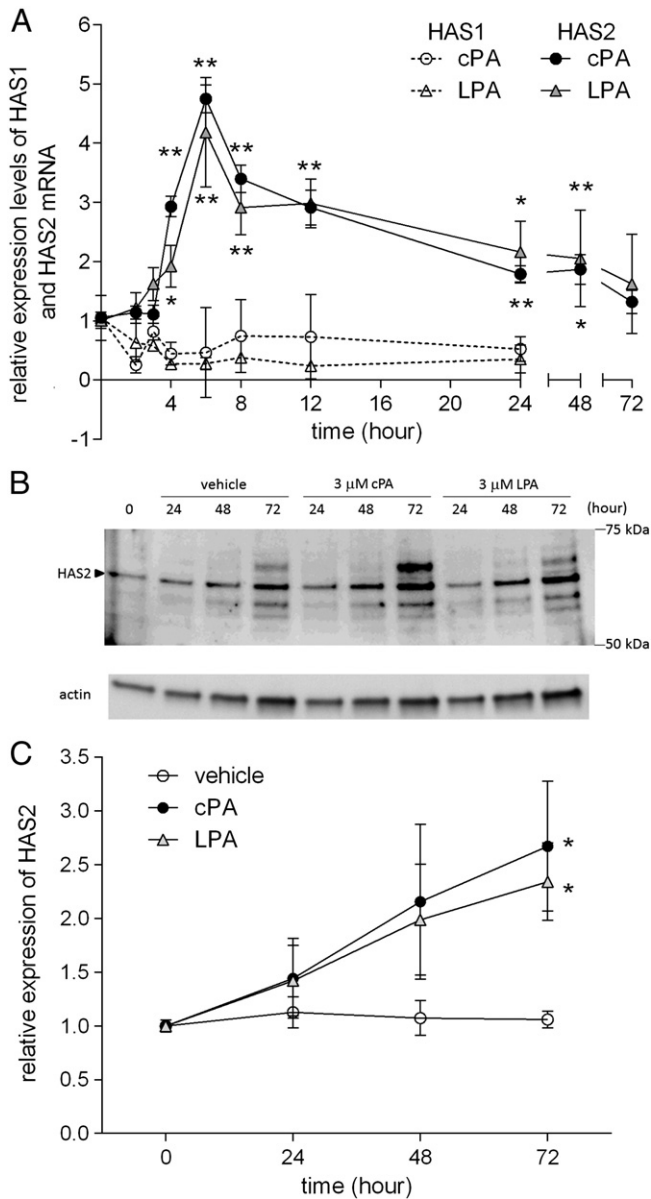
Fig. 3. Effects of cPA and LPA on the expression of hyaluronan synthase (HAS) 1 and 2 messenger RNA (mRNA) and HAS2 protein. (A) NB1RGB cells were incubated with 3 μM cPA (open or closed circles) or LPA (open or closed triangles) for the time indicated. mRNA levels of HAS1 (dashed line) and HAS2 (solid line) were determined with quantitative real-time polymerase chain reaction (PCR). The data represent the mean ± SE values from triplicate independent experiments (*$P < 0.005$ and **$P < 0.001$ vs. time 0). (B) NB1RGB cells were incubated with vehicle or 3 μM cPA or LPA for the time indicated. Protein levels of HAS2 and β-actin were determined with western blot analysis. (C) Expression levels of HAS2 and β-actin were determined using a densitometer, and the HAS2/β-actin ratio was calculated and normalized to cells without any treatment at time 0. The data represent mean ± SE values from triplicate independent experiments (*$P < 0.05$ vs. the vehicle-treated group).

Phosphorylation of HAS2 by ERK at the amino acid residues, which is different from that modified by AMPK, increases its activity [46]. Taking those results into account, cPA and LPA might have different post-translational regulation of HAS2 activity, and as a result the cPA and LPA showed different levels of hyaluronic acid synthesis.

3.2. cPA and LPA increase HAS2 protein expression by activating CREB transcription factor

To explore which signaling pathways stimulate hyaluronic acid synthesis in response to cPA and LPA, we first investigated the expression of
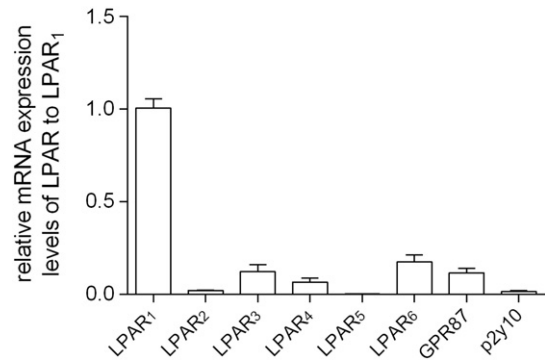

Fig. 4. Expression of LPA receptors (LPARs) in human fibroblasts. Total RNA was extracted from NB1RGB cells, and the expression level of each LPAR was determined using quantitative real-time PCR. The expression levels were normalized to those of LPAR$_1$ and expressed in terms of mean ± SE values ($n = 3$).

LPA G protein-coupled receptors in NB1RGB cells. As shown in Fig. 4, quantitative real-time PCR analysis showed that NB1RGB cells strongly expressed LPAR$_1$, which induces cellular signaling through three G proteins, G$_{i/o}$, G$_q$, and G$_{12/13}$ [47].

To investigate the role of LPAR$_1$ on the regulation of hyaluronic acid synthesis, we used Ki16425, an LPA receptor antagonist that specifically acts on LPAR$_1$ and LPAR$_3$. As shown in Fig. 5, the cPA- and LPA-induced increases in hyaluronic acid synthesis were partially suppressed by Ki16425 treatment, suggesting the involvement of LPAR$_1$ in their signaling pathways. We then investigated whether the PTX-sensitive G$_{i/o}$-dependent pathway was involved in cPA- and LPA-mediated hyaluronic acid synthesis. When cells were pretreated with 100 ng/ml PTX before cPA or LPA stimulation, cPA- and LPA-mediated hyaluronic acid synthesis was inhibited by PTX. This result suggests that cPA and LPA upregulate hyaluronic acid synthesis and HAS2 mRNA expression via the LPAR$_1$-mediated G$_{i/o}$ signaling pathway.

To gain more insight into the signaling pathways involved in the stimulation of hyaluronic acid synthesis in response to cPA and LPA via LPAR$_1$, we used several specific inhibitors of downstream signaling molecules at non-cytotoxic concentrations confirmed by counting the cell number after 72 h of incubation (data not shown). The phospholipase C-γ inhibitor U73122 did not inhibit the effects of cPA and LPA, suggesting that the downstream signaling pathway of LPAR$_1$ is not G$_q$/phospholipase C-γ dependent (Fig. 5). The hyaluronic acid synthesis stimulated by cPA and LPA was suppressed by PI3K inhibitor YL294002, ERK1/2 inhibitor U0126, and ROCK inhibitor Y27632, whereas the hyaluronic acid synthesis in vehicle-treated cells was also suppressed by YL294002, U0126, and Y27632. ERK and PI3K signaling pathways were reported to be necessary for the regulation of hyaluronic acid synthesis by platelet-derived growth factor-BB in human skin fibroblasts [16]. ROCK signaling and nuclear translocation of pERK have also been linked to the regulation of hyaluronic acid synthesis [22]. Therefore, the activity of these inhibitors on cPA- and LPA-stimulated hyaluronic acid synthesis might be due to the decline of potential hyaluronic acid synthesizing capability in NB1RGB cells. Although it is clear that cPA and LPA activate LPAR$_1$ and the G$_{i/o}$ signaling pathway, cPA and LPA might also stimulate hyaluronic acid synthesis via PI3K, ERK, and ROCK signaling pathways.

The binding sites for STAT3, NFκB, and CREB are located in the promoter region of HAS2 [16]. We investigated the effects of AG490 (STAT3 inhibitor), Bay11-7082 (NFκB inhibitor), and CBP–CREB interaction inhibitor on hyaluronic acid synthesis via HAS2 mRNA expression. As shown in Fig. 6, Bay11-7082, AG490, and CBP–CREB interaction inhibitor suppressed hyaluronic acid synthesis with vehicle treatment. However, the addition of Bay11-7082 and AG490 did not suppress cPA- and LPA-stimulated hyaluronic acid synthesis. From these results, we excluded the possibility of the involvement of NFκB and STAT3 in the cPA- and LPA-stimulated HAS2 mRNA expression signaling
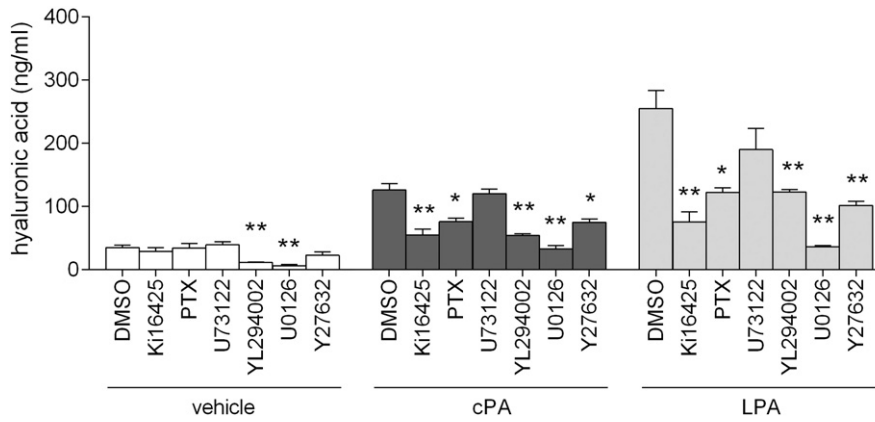
Fig. 5. Effects of signal transduction inhibitors on cPA- and LPA-induced hyaluronic acid synthesis. (A) NB1RGB cells were pretreated with or without 10 μM of Ki16425, U73122, YL294002, U0126, and Y27632 or 100 ng/ml of pertussis toxin for 30 min. Then, the cells were incubated with vehicle or 3 μM cPA or LPA for 72 h. The concentrations of hyaluronic acid in culture media were determined using ELISA. The data represent mean ± SE values from triplicate independent experiments (*$P < 0.05$ and **$P < 0.001$ vs. dimethyl sulfoxide [DMSO]).

pathway. Conversely, we found that the CBP–CREB interaction inhibitor significantly suppressed cPA- and LPA-stimulated hyaluronic acid synthesis. These results indicate that the CREB signaling pathway but not the STAT3 and NFκB signaling pathways has a major role in cPA- and LPA-induced hyaluronic acid synthesis. Since we found that the treatment with cPA and LPA increased HAS2 mRNA expression at 6 h (Fig. 3A), we investigated the effects of the CBP–CREB interaction inhibitor on cPA- and LPA-stimulated HAS2 mRNA expression at 6 h. Approximately 78% and 74% of cPA- and LPA-stimulated HAS2 mRNA expression were inhibited by CBP–CREB interaction inhibitor treatment (Fig. 7). These results were in good agreement with those of our pharmacological inhibition assay (Fig. 6).

Moreover, we examined whether ERK activation in skin fibroblasts is induced by cPA and LPA stimulation. After cPA and LPA stimulation, ERK activity (pERK/total ERK) was increased in a time-dependent manner, with a maximum between 5 and 30 min (Fig. 8). These results were consistent with those of previous studies in other cell lines [23,24].
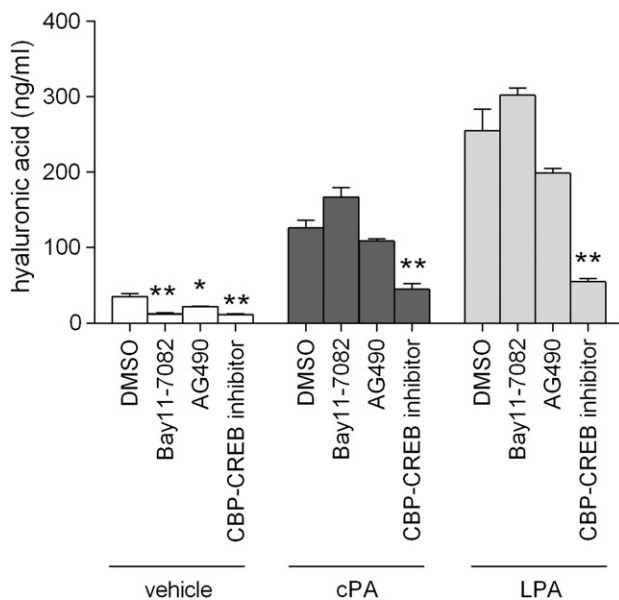
Previously, it has been reported that ErbB2–ERK signaling activated hyaluronic acid synthesis via phosphorylation of HAS2 and the stimulation decreased the molecular size of hyaluronic acid [46]. Although cPA and LPA increased the high molecular size of hyaluronic acid, cPA- and LPA-induced ERK phosphorylation might phosphorylate HAS2.

Finally, to determine the effect of cPA and LPA on CRE-dependent transcription, the activation of cyclic adenosine monophosphate-dependent gene transactivation in NB1RGB cells was examined using pCRE-SEAP, a reporter construct consisting of three copies of the CRE-binding sequence driving the expression of the SEAP gene. As shown in Fig. 9, cPA and LPA transactivated SEAP activity in a time-dependent manner. Taken together, these results suggest that cPA- and LPA-induced hyaluronic acid synthesis is a consequence of HAS2 mRNA upregulation mediated via LPAR$_1$/G$_{i/o}$ followed by the PI3K, ERK, ROCK, and CREB cascades in NB1RGB cells as shown in Scheme 1. cPA and LPA first stimulate the LPAR$_1$/G$_{i/o}$ pathway, and PI3K and ERK are subsequently activated. Although PI3K was reported to activate ERK signaling in MDA-MB-231 breast cancer cells [24], whether ERK activation in human skin fibroblasts is mediated directly or indirectly by PI3K remains unclear. Moreover, the ROCK family of Rho-associated protein kinases is reportedly essential for F-actin assembly, and phosphorylation of cofilin and actin assembly caused translocation of pERK in the nucleus [22]. LPAR$_1$
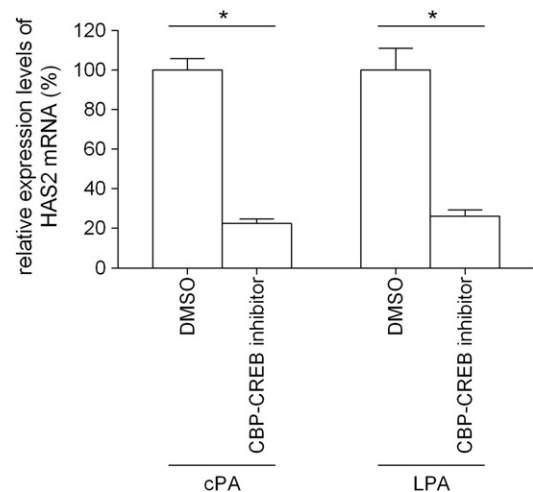

Fig. 6. Effects of signal transcription factor inhibitors on cPA- and LPA-induced hyaluronic acid synthesis. NB1RGB cells were pretreated with or without 10 μM Bay11-7082, 1 μM AG490, or 10 μM cyclic adenosine monophosphate response element binding protein (CREB)-binding protein (CBP)–CREB interaction inhibitor for 30 min. Subsequently, the cells were incubated with vehicle, 3 μM cPA or LPA for 72 h. The concentrations of hyaluronic acid in culture media were determined with ELISA. The data represent mean ± SE values from triplicate independent experiments. DMSO, dimethyl sulfoxide.

Fig. 7. Effects of CBP–CREB interaction inhibitor on cPA- and LPA-stimulated HAS2 mRNA expression. Serum-starved NB1RGB cells were preincubated with or without 10 μM CBP–CREB interaction inhibitor for 30 min. Then cells were incubated with vehicle, 3 μM cPA or LPA for 6 h, and mRNA levels of HAS2 were determined with qPCR. HAS2 mRNA levels of cPA- or LPA-treated cells at 6 h were expressed as 100%. The data represent the mean ± SE values from triplicate independent experiments (*$P < 0.001$ vs. DMSO).
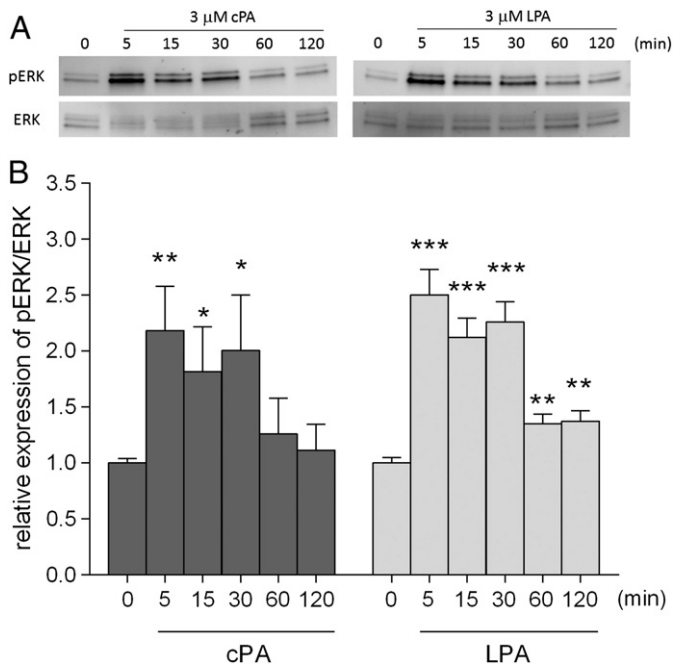
Fig. 8. Effects of cPA and LPA on extracellular-signal-regulated kinase (ERK) phosphorylation. (A) NB1RGB cells were incubated with 3 μM cPA or LPA for the time indicated. Protein levels of phospho-ERK (pERK) and total ERK were determined with western blot analysis. (B) The expression levels of pERK and total ERK were determined using a densitometer, and the pERK/ERK ratio was calculated and normalized to cells without any treatment at time 0. The data represent mean ± SE values from triplicate independent experiments (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs. time 0).

Scheme 1. Schematic model for hyaluronan synthase 2 (HAS2) gene expression via activation of cyclic adenosine monophosphate response element-binding protein (CREB). Cyclic phosphatidic acid (cPA) and lysophosphatidic acid (LPA) interact with LPAR1, which activates Gi/o then mediates the activation of phosphatidylinositol-3 kinase (PI3K) and extracellular-signal-regulated kinase (ERK). Activation of ROCK leads to ERK activation, too [22]. Activated ERK increases the activity of HAS2 via HAS2 protein phosphorylation [46]. Activated CREB stimulates the transcriptional activation of the HAS2 gene.

activates the $G_{12/13}$-Rho signaling pathway [47]. In addition, ERK activation was reported to be involved in LPA receptor/G_{12}-dependent activation of the CREB pathway in ovarian cancer cells [20]. Therefore, ERK may also be activated via LPAR1/$G_{12/13}$-ROCK signaling. Finally, activated ERK activates CREB, inducing HAS2 mRNA transcription.

Hyaluronic acid has a critical role in the physiology and pathology of several mammalian tissues. Increase of hyaluronic acid is involved in inflammation, cancer, fibrosis and diabetes, and decrease of hyaluronic acid is related to joint disorders. Previously, it has been reported that LPA level was increased in chronic hepatitis C patients, and was positively correlated with serum hyaluronic acid amount [48]. And LPAR1
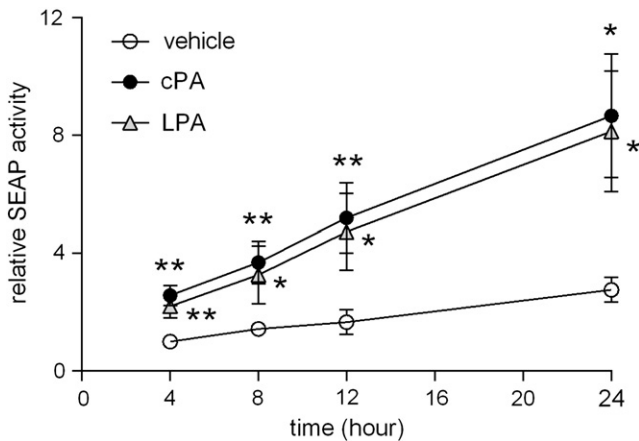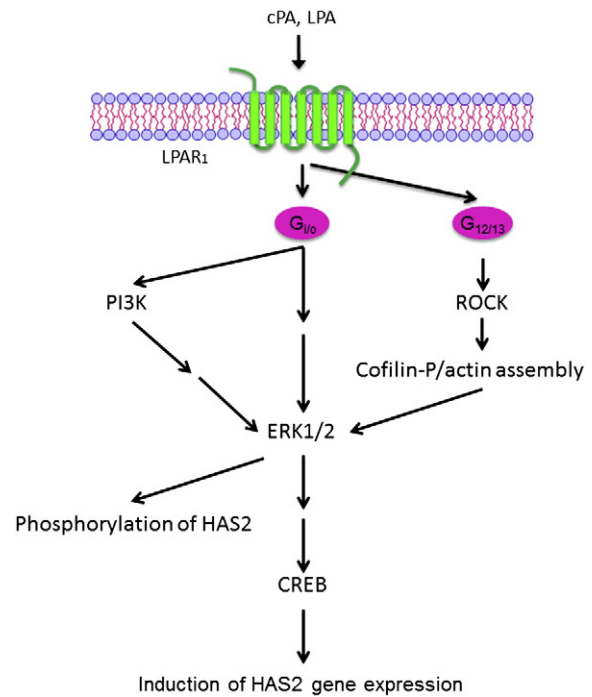
antagonist inhibited lung fibrosis in the mouse bleomycin model [49]. Here, we observed that LPA up-regulated hyaluronic acid via LPAR1 stimulation, suggesting that increased LPA might stimulate LPAR1 and caused hyaluronic acid accumulation. However, our data were obtained using skin fibroblasts, therefore, the relationship between increased LPA and hyaluronic acid accumulation in the diseases remains unresolved.

Our findings on the up-regulation of dermis hyaluronic acid synthesis by cPA and LPA offer useful information for maintaining the health of the skin. So far, the correlations between cPA and the diseases are unknown, thus, cPA would be available as a therapeutic agent for the control of aging of skin.

4. Conclusion

Hyaluronic acid synthesis is important for the maintenance of healthy joint, eye, and skin functioning. 2ccPA, a cPA derivative, increases hyaluronic acid synthesis in human osteoarthritis synoviocytes [13]. In this study, we focused on the maintenance of skin health and examined the effect of cPA and LPA on hyaluronic acid synthesis by using human skin fibroblasts. The study revealed the signaling pathways involved in the stimulation of hyaluronic acid synthesis in response to cPA and LPA. This is the first report that cPA and LPA induce hyaluronic acid synthesis as a consequence of HAS2 mRNA up-regulation in human skin fibroblasts mainly through activation of LPAR1/$G_{i/o}$ followed by PI3K, ERK, ROCK, and CREB signaling (see Scheme 1). This study suggests that cPA could suppress the age-related decline of hyaluronic acid synthesis in the skin.

Fig. 9. Effects of cPA and LPA on cyclic adenosine monophosphate-responsive gene activation via CREB. Cells transiently transfected with phospho-cyclic adenosine monophosphate response element-secreted alkaline phosphatase (pCRE-SEAP) expression vector were treated for 4, 8, 12, and 24 h with vehicle or 3 μM cPA or LPA. Relative SEAP activity is shown and fold inductions are calculated in reference to values in the vehicle-treated group. The data represent mean ± SE values from triplicate independent experiments (*$P < 0.05$ and **$P < 0.01$ vs. vehicle-treated group).

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