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Inhibition of the activation of hepatic stellate cells by arundic acid via the induction of cytoglobin

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ABSTRACT

Background: The activation of hepatic stellate cells plays a central role in the development of liver fibrosis during chronic liver trauma. The aim of the present study was to identify a compound that inhibits the activation process of stellate cells.
Methods: Rat primary cultured stellate cells and a human stellate cell line (LX-2) were used. The effects of arundic acid on the expression of α-smooth muscle actin, collagen 1α1, and cytoglobin were evaluated.
Results: Arundic acid (300 μM) inhibited the activation of primary rat stellate cells, as determined by morphological transformation and α-smooth muscle actin expression, after both prophylactic and therapeutic treatment. The level of α-smooth muscle actin mRNA showed a dose-dependent decrease in response to arundic acid, and 50 μM arundic acid exhibited the maximum inhibition of collagen 1α1 mRNA expression. In contrast, arundic acid triggered an unexpected increase in mRNA and protein levels of cytoglobin, the fourth globin in mammals expressed exclusively in hepatic stellate cells. The effect of arundic acid on the level of α-smooth muscle actin mRNA was abrogated in HSCs treated with cytoglobin siRNA. Arundic acid decreased the expression of collagen 1α1 mRNA in LX-2 cells.
Conclusion: Arundic acid affects the activation process of hepatic stellate cells via the unexpected induction of cytoglobin.

1. Introduction

Liver fibrosis is characterized by an accumulation of extracellular matrix materials in response to chronic trauma induced by alcohol abuse, hepatitis viral infection, and fatty overload. Hepatic stellate cells (HSCs), which consist of hepatic sinusoids and are a liver-specific pericyte under physiological conditions, play a central role in hepatic inflammatory and fibrotic reactions. Liver injury initiates the activation of HSCs, which is characterized by the phenotypic transformation from a vitamin A-rich quiescent phenotype into type I collagen-producing and α-smooth muscle actin (α-SMA)-expressing myofibroblast-like cells. Activated HSCs secrete various cytokines, such as transforming growth factor β (TGF-β), insulin-like growth factor, and connective tissue growth factor, resulting in the augmentation of local fibrotic reactions around sinusoids [1,2]. Based on this knowledge, anti-fibrotic therapies for the liver could be achieved by the inhibition or reversion of HSC activation.

Arundic acid, also known as (R)-(−)-2-propyloctanoic acid or ONO-2506, was originally shown to possess an inhibitory effect on astrocyte activation. It was reported to suppress the synthesis of S100β in astrocytes and had protective effects in several neurological diseases, including ischemic stroke [3] and Alzheimer's disease [4]. Because astrocytes and HSCs share a number of similarities and contribute to scar formation and tissue repair in the brain and liver, respectively [5,6], a question arises of whether arundic acid could affect the activation process of HSCs.

Cytoglobin, which was originally described as stellate cell activation-associated protein, is a newly identified member of the globin family that buffers intracellular oxygen storage and transfer and scavenges nitric oxide and reactive oxygen species [7,8]. Previously, we reported that cytoglobin deficiency accelerated diethylnitrosamine-induced tumor formation in the liver [9]. Recently, the anti-fibrotic effect of cytoglobin was reported in vitro and in vivo; treatment with human recombinant cytoglobin attenuated thioacetamide-induced liver fibrosis in a rat model

Abbreviations: α-SMA, α-smooth muscle actin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HSC, hepatic stellate cell; PDGFR, platelet-derived growth factor receptor; TGF, transforming growth factor.

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and induced the apoptosis of HSC-T6 cells [10]. However, the role of cytoglobin in the activation of HSCs and its function in liver fibrosis remain largely unknown.

In this study, we show that arundic acid inhibits the activation of HSCs and collagen synthesis in rat primary cultured HSCs. The unexpected induction of cytoglobin may play a role in the pharmacological effect of arundic acid.

2. Methods

2.1. Cell culture

Primary HSCs were isolated from 12-week-old male Wistar rats as previously described in detail [11]. The isolated HSCs were cultured on uncoated plastic plates (Falcon, Lincoln Park, NJ, USA) or chamber slides (Lab-Tek, Naperville, IL, USA) in Dulbecco's modified Eagle's medium (DMEM) (Sigma, Missouri, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, CA, USA) in 5% CO_2/95% air at 37 °C at a density at 1×10^5 cells/cm^2. The cultured HSCs were treated with arundic acid (Ono Pharmaceutical Co., Ltd., Osaka, Japan; 50, 100, or 300 μM dissolved in DMSO) as indicated in the figure legends. LX-2 cells, a gift from Dr. Scott Friedman [12], were maintained in uncoated plastic flasks in DMEM supplemented with 10% FBS. After re-plating 1×10^5 cells/cm^2 in a 12-well plate, the cells were treated with arundic acid, which was reported to suppress the synthesis of S100β in astrocytes at the concentration range of 30–300 μM [13]. A clinical trial of arundic acid in acute ischemic stroke for 1 h a day at the dose of 2–12 mg/kg/h indicated that the range of maximum plasma concentration (Cmax) of arundic acid (MW 186.3) was 17.8–70.0 μg/mL (100–380 μM) [14]. No dose-related pattern of serious adverse events was reported to observe within the enrolled subjects [15]. From these previous reports, the concentrations of arundic acid used in the present study were determined to be 50–300 μM.

2.2. Real-time PCR

Total RNA was isolated from HSCs by the acid guanidinium thiocyanate/phenol/chloroform method. cDNA was prepared using 1 μg of total RNA, as described previously [16]. real-time PCR was performed using an ABI Prism 7500 real-time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR Premix Ex Taq (Takara Bio Inc., Shiga, Japan). The primers used for real-time PCR are shown in Table 1.

2.3. Immunoblot analysis

Proteins (10 μg) prepared from primary HSCs were separated by SDS–PAGE. Immunoblotting was performed as described previously [16]. Protein expression was quantified using the Fujifilm Image Reader LAS-3000 (Fuji Medical Systems, Stamford, CT, USA) and NIH ImageJ software (http://rsbweb.nih.gov/ij/). Monoclonal anti-α-SMA and anti-β-actin antibodies were purchased from Sigma, and the monoclonal anti-S100A4 antibody was purchased from Abcam plc (Cambridge, MA, USA). Polyclonal antibodies against rat cytoglobin were generated in our laboratory [7].

2.4. Immunocytochemistry

After washing with PBS, primary cultured HSCs on chamber slides were fixed with 4% paraformaldehyde (PFA) for 30 min at room temperature. Then, the slides were incubated with 0.5% Triton-X 100 in PBS for 15 min for cell permeabilization. After blocking, the culture was incubated with a monoclonal antibody against α-SMA (1:200, Sigma, St. Louis, MO, USA), followed by a secondary antibody (1:200, Invitrogen, Carlsbad, CA, USA). Signals were detected using Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) with fluorescence microscopy.

2.5. Transient transfection with siRNA

Transfection with siRNA was performed using Lipofectamine-RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Three days after isolation and culture, primary HSCs were transfected with siRNA (20 pmol/35-mm dish) targeted against cytoglobin (s139241, Ambion, Austin, TX, USA) or with non-silencing control siRNA (Invitrogen, Carlsbad, CA, USA). Cells were harvested after transfection for the determination of mRNA and protein expression.

2.6. Statistics

The data are expressed as the means ± SEM. The statistical analyses were performed with a one-way ANOVA. For multiple treatment groups, a post hoc multiple comparison (Dunnett's test) was used.

3. Results

3.1. Inhibition of the activation of primary rat HSCs by arundic acid

The effect of arundic acid on the culture-initiated activation of primary rat HSCs was tested. First, we performed our experiments with a prophylactic treatment schedule (Fig. 1A). The effect of arundic acid on morphological and cytological transition during HSC activation was evaluated. In the control group, HSCs underwent the well-known activation process, which consists of the loss of lipid droplets containing vitamin A and enlarged cytoplasmic processes at days 4 and 6. In contrast, HSCs maintained their cytoplasmic lipid droplets and dendritic and slender processes in response to treatment with 300 μM arundic acid (Fig. 1B). Immunocytochemistry revealed the expression of α-SMA, which is a representative marker of activated HSCs and forms stress fibers, in untreated HSCs at days 4 and 6, but its expression was markedly reduced in HSCs treated with 300 μM arundic acid (Fig. 1C). Additionally, the mRNA and protein levels of α-SMA were significantly suppressed by 300 μM arundic acid at 1, 4 and 6 days after beginning culture (Fig. 1D). Arundic acid exhibited a dose-dependent

Table 1
List of primers used in the present study.

	Sense	Antisense
Human TGF-β1	AGCGACTCGCCAGAGTGGTTA	GCAGTGTGTTATCCCTGCTGTCA
Human PDGFR	CCCTTATCATCCTCATCATGC	CCTTCCATCGGATCTGGTAA
Human collagen1α1	CCCGGGTTTCAGAGACAACTTC	TCCACATGCTTTATTCCAGCAATC
Rat collagen1α1	AATGGTGAGACGTGGAAACCTG	GAGACCACGAGGACCAGAAG
Rat α-SMA	TCGCCCTGGATTTTGAGAATGA	GTGCCTCCAGACAGGACATT
Rat S100A4	CAACGAGGGTGACAAGTTCAAG	TGCAGGACAGGAAGACACAG

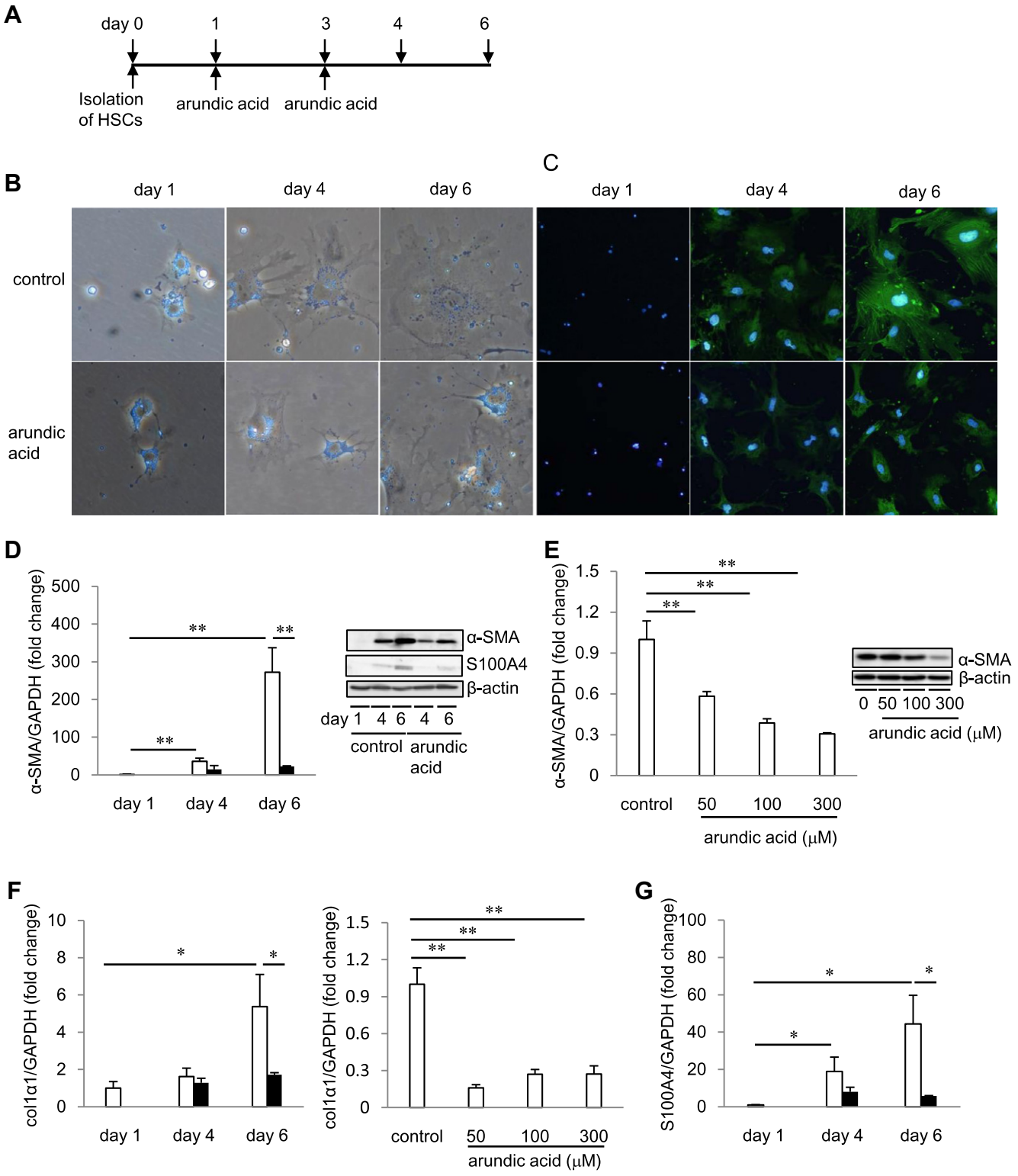

Fig. 1. Inhibition of the activation of primary rat HSCs by arundic acid. (A) Primary rat HSCs were treated with arundic acid on days 1 and 3 and observed until day 6. (B) After HSCs were treated with 300 μM arundic acid for 5 days, morphological changes were observed under phase-contrast microscopy. Magnification, 200×. (C) Primary HSCs were treated as in (B), and α-SMA immunocytochemistry was performed. Magnification, 200×. Note that arundic acid markedly suppressed α-SMA expression in HSCs. (D) After HSCs were treated with 300 μM arundic acid, total RNA was isolated on days 1, 4 and 6. The expression of α-SMA mRNA was measured with real-time PCR (left). Representative immunoblots of α-SMA, S100A4, or β-actin (right). AA, arundic acid. $^{**}p<0.01$. (E) After HSCs were treated with arundic acid at 50, 100, or 300 μM for 5 days, total RNA was isolated on day 6. The expression of α-SMA mRNA was measured with real-time PCR (left). Representative immunoblot of α-SMA (right). $^{**}p < 0.01$. (F) HSCs were treated as in D and E, total RNA was isolated and collagen 1α1 mRNA was measured by real-time PCR. col1α1, collagen 1α1. $^{*}p < 0.05$. $^{**}p < 0.01$. (G) After HSCs were treated with arundic acid (300 μM), total RNA was isolated on days 1, 4 and 6. The expression of S100A4 mRNA was measured by real-time PCR. $n = 3–4$. $^{*}p < 0.05$.

(50, 100, and 300 μM) decrease in the mRNA and protein levels of α-SMA (Fig. 1E). In addition, the compound significantly reduced the expression of collagen 1α1 mRNA 6 days after starting culture, with maximum inhibition at 50 μM (Fig. 1F). These findings

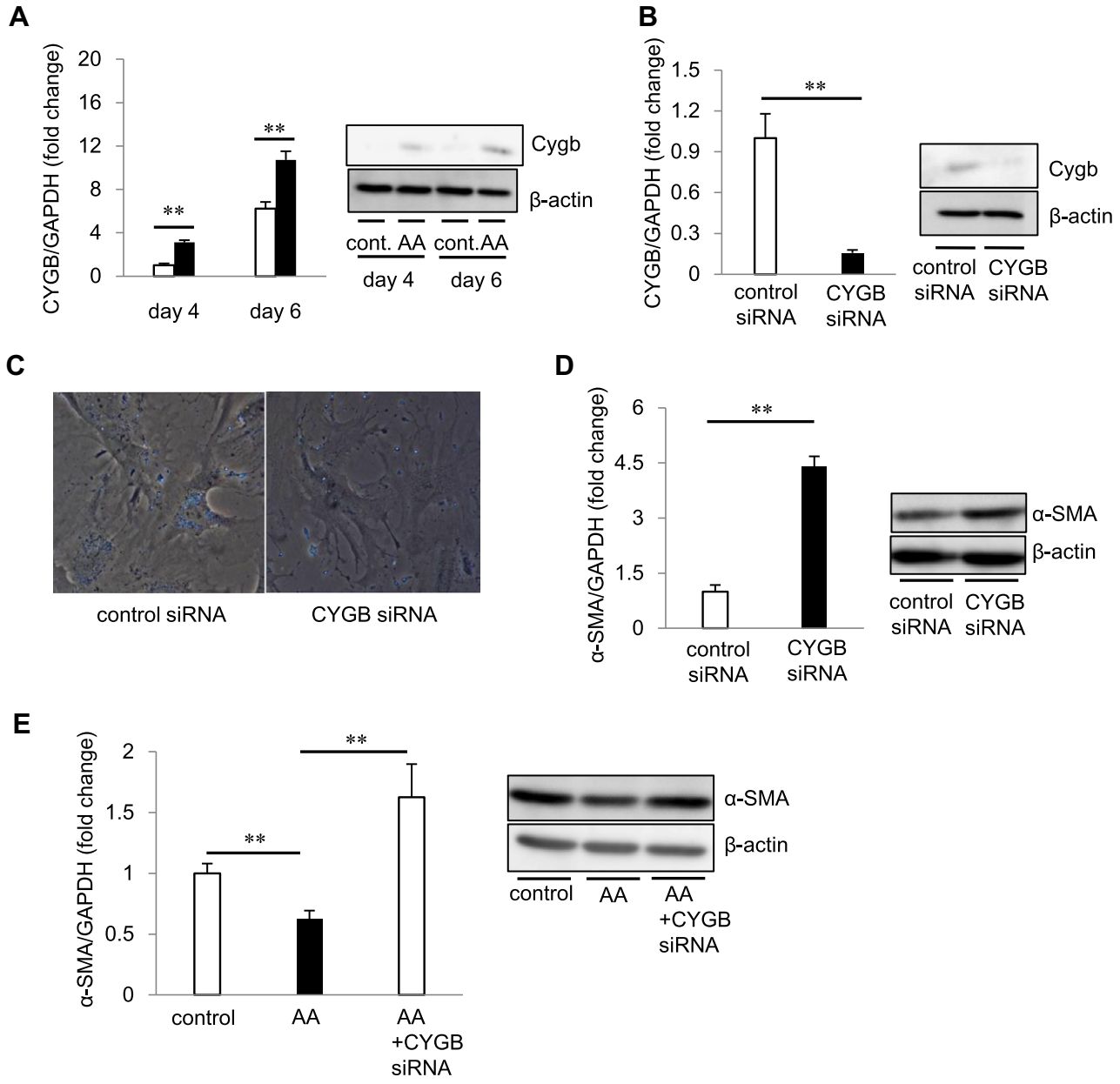

Fig. 2. Involvement of cytoglobin in the effect of arundic acid on the activation of primary HSCs. (A) After HSCs were treated with arundic acid (300 μM), total RNA was isolated on days 4 and 6. Cytoglobin mRNA was measured by real-time PCR (left). A representative immunoblot of cytoglobin (right). Note that arundic acid increased the expression of cytoglobin mRNA and protein in primary HSCs. cont., control. AA, arundic acid. $^{**}p < 0.01$. (B) HSCs were transfected with control siRNA or cytoglobin siRNA (20 pmol/per 35-mm plate) 3 days after starting primary culture. At 24 h after transfection, cells were washed to remove transfection reagent. Then, total RNA was isolated, and cytoglobin mRNA levels were determined (left). After culture for an additional 24 h, cells were harvested, and immunoblotting of cytoglobin was performed (right). Cygb, cytoglobin. $^{**}p < 0.01$. (C) Morphological changes were observed by phase-contrast microscopy 48 h after transfection. Magnification, x 200. CYGB, cytoglobin. (D) After HSCs were transfected with cytoglobin siRNA (20 pmol/per 35-mm plate), the cells were harvested, and the expression of α-SMA mRNA (left) and protein (right) was evaluated. CYGB, cytoglobin. $^{**}p < 0.01$. (E) After HSCs were transfected with cytoglobin siRNA (20 pmol/per 35-mm plate) for 24 h, the cells were treated with vehicle, arundic acid (300 μM), or arundic acid (300 μM) + cytoglobin siRNA for 48 h. The cells were harvested for real-time PCR or immunoblotting. $N = 3–4$. CYGB, cytoglobin. AA, arundic acid. $^{**}p < 0.01$.

indicate that arundic acid is potent in inhibiting the activation of primary rat HSCs in culture. Toxic effect of arundic acid (50–300 μM) to primary rat HSCs was negligible as judged from the no detachment of treated HSCs from the culture plates and from the no difference in the MTT assay between treated and non-treated HSCs (data not shown).

Because arundic acid was reported to suppress the activation of astrocytes by inhibiting the expression of S100β in a rat stroke model, we investigated whether arundic acid could affect the level of S100A4, which is a member of the S100 protein family expressed in HSCs, also known as fibroblast-specific protein 1 (FSP1). We found that S100A4 was decreased at both the mRNA and protein levels in response to 300 μM arundic acid (Fig. 1D and G).

3.2. Involvement of cytoglobin in the anti-activation effect of arundic acid on HSCs

Cytoglobin is a cytoplasmic globin that is specifically expressed in HSCs in the liver, and its function is largely unknown. We recently reported that cytoglobin deficiency promotes tumorigenesis of the liver accompanied by augmented fibrosis in a diethylnitrosamine-induced mouse liver cancer model (Thuy le et al. [9]),

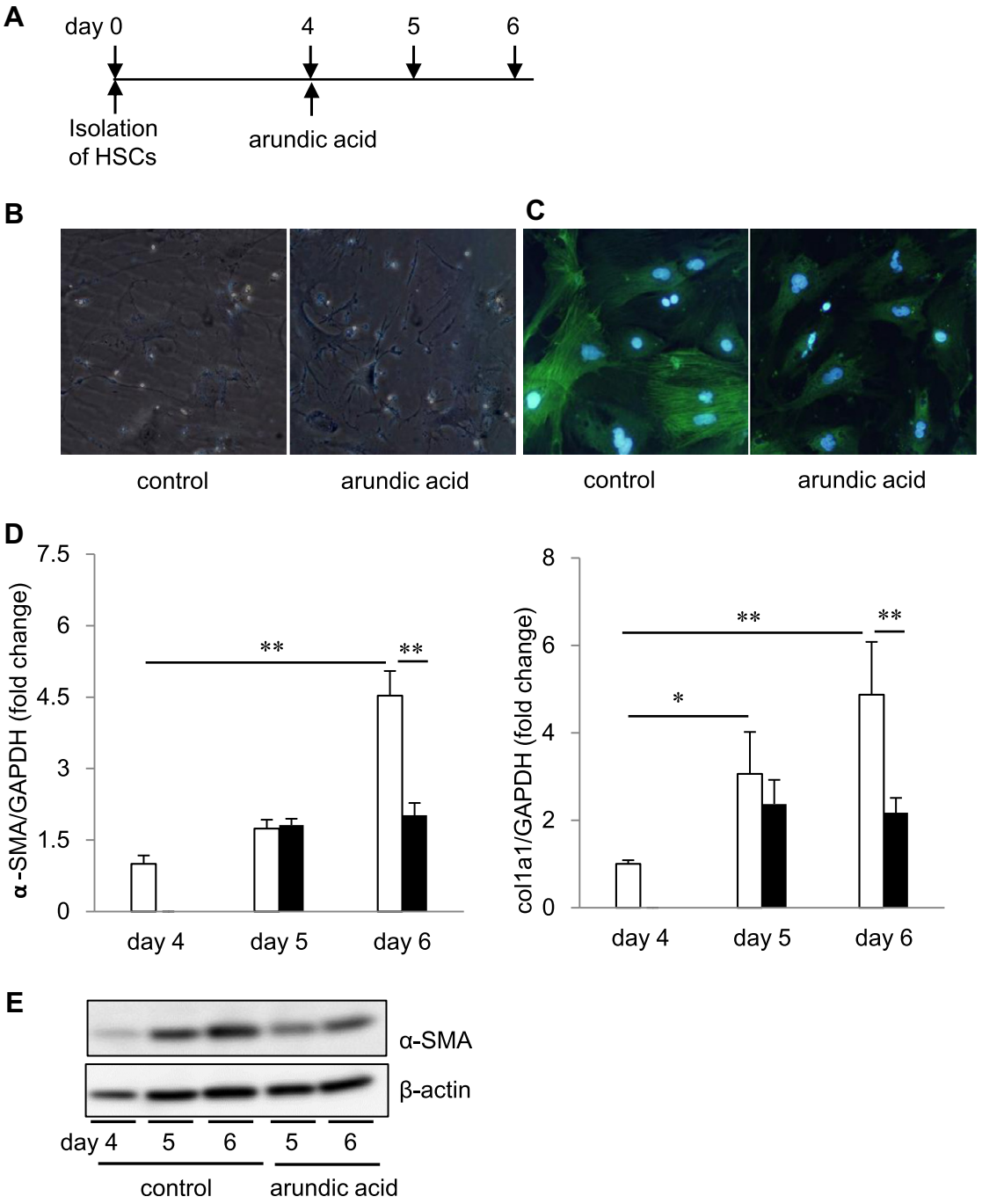

Fig. 3. Therapeutic effect of arundic acid on culture-activated HSCs (A) Isolated HSCs were kept untreated for 4 days to induce spontaneous activation. Then, they were treated with arundic acid (300 μM) for the following 2 days. The evaluation of morphological changes (B) and α-SMA immunostaining (C) were performed at day 6. Magnification, 200×. (D) After HSCs were treated with arundic acid (300 μM) for 48 h, they were harvested, and the level of α-SMA and collagen 1α1 mRNA was determined by real-time PCR on days 4, 5 and 6. $^{**}p < 0.01$. (E) Representative immunoblot of α-SMA. col1α1, collagen 1α1. $n = 4$. $^{*}p < 0.05$. $^{**}p < 0.01$.

indicating the anti-fibrotic and tumor suppressive function of cytoglobin. To investigate the involvement of cytoglobin in the observed anti-activation effect of arundic acid on primary HSCs, we treated HSCs with arundic acid and determined the expression level of cytoglobin. As shown in Fig. 2A, surprisingly, 300 μM arundic acid markedly increased cytoglobin expression at both the mRNA and protein levels. Next, we introduced siRNA targeting cytoglobin in HSCs. siRNA targeting cytoglobin markedly reduced the levels of cytoglobin mRNA and protein (Fig. 2B). Morphologically, HSCs treated with cytoglobin siRNA showed a prominently activated phenotype of HSCs, with a marked loss of lipid droplets (Fig. 2C). The knockdown of cytoglobin by siRNA significantly increased the mRNA and protein levels of α-SMA (Fig. 2D). The inhibitory effect of arundic acid on α-SMA expression in HSCs was abrogated by siRNA targeting cytoglobin (Fig. 2E). These results demonstrated that arundic acid could exert its pharmacological action on the activation of HSCs via the up-regulation of cytoglobin.

3.3. Therapeutic effect of arundic acid on activated HSCs

Primary cultured HSCs undergo spontaneous activation after 4 days of culture. To investigate whether arundic acid could

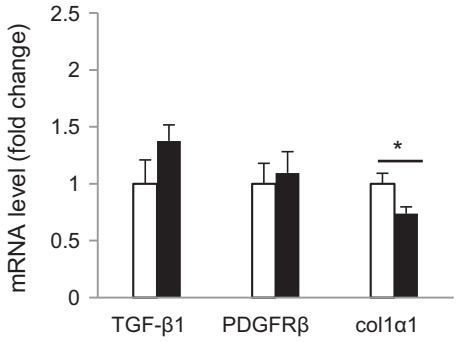

Fig. 4. Suppression of the expression of the collagen 1α1 gene in LX-2 cells by arundic acid. LX-2 cells were incubated with arundic acid (300 μM) for 48 h. The expression of TGF-β1, PDGFRβ and collagen 1α1 mRNA was measured by real-time PCR. □, vehicle control (DMSO). ■, test compound. col1α1, collagen 1α1. $N = 4–8$ per group. $^{*}p < 0.05$.

decrease or reverse HSC activation, we used a therapeutic treatment schedule of arundic acid by adding it 4 days after beginning culture (Fig. 3A). We found that activated HSCs reversed their phenotype from a flattened cell shape to a shape with dendritic processes in response to treatment with 300 μM arundic acid for 48 h (Fig. 3B). This change was accompanied by a decrease in α-SMA expression, as determined by immunocytochemistry (Fig. 3C). The protein and mRNA levels of α-SMA and the mRNA level of collagen 1α1 remained low after treatment with arundic acid from day 4 to day 6 (Fig. 3D and E). These data indicated that arundic acid decreased the further activation of HSCs in primary culture.

3.4. Suppression of collagen 1α1 gene expression in LX-2 cells by arundic acid

To determine whether the inhibitory effect of arundic acid on the function of rat stellate cells was reproduced in human stellate cells, we investigated the effect of arundic acid on collagen gene expression in LX-2 cells. The levels of collagen 1α1 but not TGF-β1 or PDGF receptor β (PDGFRβ) mRNA in LX-2 cells were significantly decreased in response to treatment with 300 μM arundic acid (Fig. 4).

4. Discussion

In this study, we demonstrated that arundic acid attenuates the activation of HSCs. The principal findings obtained in this study include the following points: (1) the inhibition of HSC activation in primary culture by arundic acid; (2) possible involvement of the up-regulation of cytoglobin in the suppressive effect of arundic acid in HSC activation; (3) a decrease in the further activation of rat HSCs by arundic acid; and (4) the suppression of collagen synthesis in LX-2 cells by arundic acid. These data indicated that arundic acid could be a potent therapeutic and prophylactic compound against liver fibrosis that targets the activation process of HSCs.

We demonstrated that cytoglobin is up-regulated by arundic acid in primary cultured rat HSCs when they were treated from either day 1 or day 4. Previously, we reported that the expression of cytoglobin is induced in primary cultured HSCs in a time-dependent fashion [7]. Although cytoglobin is a hemo-protein and has hydrogen peroxidase activity and lipid peroxidase activity at the purified recombinant protein level, it was reported to exhibit anti-oxidative activity in several cell types [17,18], and oxidative stress contributes to the activation of HSCs [19,20]. When HSCs were treated with cytoglobin siRNA, we found an increase in morphological transition and a marked increase in α-SMA expression. Additionally, the reduced α-SMA expression level induced by arundic acid was significantly up-regulated in HSCs treated with cytoglobin siRNA. These results clearly indicate for the first time that cytoglobin may be induced in HSCs to decrease HSC activation, as represented by the induction of α-SMA expression after cytoglobin reduction by a specific siRNA. The anti-fibrotic role of arundic acid on HSCs is caused at least in part by increasing the cytoglobin level in the cells. To the best of our knowledge, arundic acid is the first compound demonstrated to trigger an increase in the cellular cytoglobin level.

S100A4, a member of the calcium-binding protein S100 family, is used as a marker of fibroblasts. Several studies have demonstrated the involvement of S100A4 in the promotion of cell motility and invasion in cancer, and thus, this molecule is also called metastatin-1 [21–23]. The metastatic activity of S100A4 is regulated through interaction with Smad3, an essential mediator of TGF-β signaling [24,25]. In the present study, the induction of S100A4 was observed in culture-activated HSCs, and this induction was down-regulated by treatment with arundic acid (Fig. 1D and G). However, no difference in the phosphorylation of Smad3 between control and arundic acid-treated groups was observed (data not shown). Additionally, S100A4-positive cells in the liver were reported to be macrophages, not HSCs or fibroblasts [26]. Thus, further study is necessary to clarify the role of S100A4 in the process of liver fibrosis.

In conclusion, arundic acid suppressed the activation of HSCs and collagen synthesis in primary rat HSCs. The effect of arundic acid on HSCs is possibly mediated by the induction of cytoglobin. Thus, arundic acid may become a novel therapeutic drug for the treatment of liver fibrosis.

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