Collagen release by human hepatic stellate cells requires vitamin C and is efficiently blocked by hydroxylase inhibition

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Abstract
Liver fibrosis is characterized by the accumulation of extracellular matrix proteins, mainly composed of collagen. Hepatic stellate cells (HSCs) mediate liver fibrosis by secreting collagen. Vitamin C (ascorbic acid) is a cofactor of prolyl-hydroxylases that modify newly synthesized collagen on the route for secretion. Unlike most animals, humans cannot synthesize ascorbic acid and its role in liver fibrosis remains unclear. Here, we determined the effect of ascorbic acid and prolyl-hydroxylase inhibition on collagen production and secretion by human HSCs. Primary human HSCs (p-hHSCs) and the human HSC cell line LX-2 were treated with ascorbic acid, transforming growth factor-beta (TGFβ) and/or the pan-hydroxylase inhibitor dimethyloxalylglycine (DMOG). Expression of collagen-I was analyzed by RT-qPCR (COL1A1), Western blotting, and immunofluorescence microscopy. Collagen secretion was determined in the medium by Western blotting for collagen-I and by HPLC for hydroxyproline concentrations. Expression of solute carrier family 23 members 1 and 2 (SLC23A1/SLC23A2), encoding sodium-dependent vitamin C transporters 1 and 2 (SVCT1/SVCT2) was quantified in healthy and cirrhotic human tissue. In the absence of ascorbic acid, collagen-I accumulated intracellularly in p-hHSCs and LX-2 cells, which was potentiated by TGFβ. Ascorbic acid co-treatment strongly promoted collagen-I excretion and enhanced extracellular hydroxyproline concentrations, without affecting collagen-I (COL1A1) mRNA levels. DMOG inhibited collagen-I release even in the presence of ascorbic acid and suppressed COL1A1 and alpha-smooth muscle actin (αSMA/ACTA2) mRNA levels, also under hypoxic conditions. Hepatocytes express both ascorbic acid transporters, while p-hHSCs and LX-2 express the only SVCT2, which is selectively enhanced in cirrhotic livers. Human
1 | INTRODUCTION

Liver fibrosis is the common pathology associated with chronic liver diseases, which is characterized by excessive hepatic accumulation of extracellular matrix (ECM) proteins, including collagen and fibronectins. In a primary response to repair the injured liver tissue, hepatic stellate cells (HSCs) activate, migrate to the site of damage, and start a wound healing response by producing ECM proteins. If the source of damage is not resolved, HSCs-mediated deposition of ECM progresses causing liver fibrosis, which disrupts the normal liver architecture and impairs liver function. Liver fibrosis may further progress to irreversible cirrhosis. End-stage cirrhosis patients develop life-threatening complications, such as portal hypertension, variceal bleeding, ascites, liver failure, and are at increased risk to develop hepatocellular carcinoma. There is no cure for liver fibrosis and liver transplantation is the only treatment available for end-stage cirrhosis. Over 60% of all liver transplantsations are performed because of cirrhotic liver disease. In the healthy liver, HSCs are considered quiescent and are characterized by large intracellular lipid droplets. Upon activation, HSCs transdifferentiate into myofibroblasts (i.e., activated HSCs, aHSCs), lose their lipid content and become migratory, proliferative, express alpha-smooth muscle actin (αSMA) and overproduce extracellular matrix proteins.

Collagen-I and III are the most abundant ECM components in the fibrotic liver and their production is strongly induced in HSCs by TGFβ. Collagens are synthesized as procollagen precursor molecules, which are subjected to multiple post-translational modifications while being synthesized, including hydroxylation of L-proline and L-lysine residues that stabilize collagen triple helices. Procollagen accumulates in the endoplasmic reticulum when hydroxylation is impaired. Following hydroxylation and secretion, the propeptide is endoplasmically reticulum when hydroxylation is impaired. Following hydroxylation and secretion, the propeptide is cleaved and collagen molecules assemble into collagen fibers. Collagen procollagen hydroxylases and l-lysyl hydroxylases stoichiometrically decarboxylate 2-oxoglutarate during hydroxylation and need ascorbic acid as cofactor. The main biological function of ascorbic acid in the collagen hydroxylase reactions is to serve as an oxygen acceptor in order to (re)activate the mentioned enzymes. Hydroxyproline has an essential role in providing the collagen triple helices with thermal stability. Without hydroxyprolines, the triple helix denatures at body temperature. Hydroxylysine is involved in the formation of collagen cross-links required to stabilize collagen fibrils, and additionally functions as an attachment site for carbohydrate units. Ascorbic acid deficiency disturbs proper collagen stabilization and secretion, as described in detail for dermal fibroblasts and evidently observed in scurvy. Advanced liver fibrosis impairs blood flow (portal hypertension) and oxygen supply to the liver. This inhibits prolyl-hydroxylases thereby stabilizing hypoxia-inducible factors-α 1 and 2 (HIF-1α and 2α), transcription factors that are known to activate aHSCs.

In addition to controlling the post-translational processing of collagen, ascorbic acid affects collagen gene transcription in various cell types, including COL1A1 in skin fibroblasts, tendon cells, and adipocytes. However, its role in liver fibrosis is controversial, as ascorbic acid is also a potent antioxidant to which anti-fibrotic properties have been assigned. Conversely, also pro-fibrotic effects of ascorbic acid have been reported in animal models. Ascorbic acid is an essential nutrient for humans, as we cannot synthesize it ourselves. In contrast, most animals, including laboratory rodents, can endogenously synthesize ascorbic acid through the action of gulonolactone oxidase. The corresponding GULO gene is non-functional in humans and guinea pigs. As a consequence, typical laboratory animals and animal-derived cell lines are unsuited to determine the relevance of ascorbic acid for liver fibrosis in humans.

Here, we investigated the effect of ascorbic acid on procollagen hydroxylation and collagen secretion by LX-2 cells and primary human hepatic stellate cells, as well as the expression of the two dedicated ascorbic acid transporters SVCT 1 and 2, in purified human liver cell fractions and in healthy and cirrhotic human liver tissue. Moreover, we studied the effect of inhibiting hydroxylases with dimethyloxalyl glycine (DMOG) on collagen secretion and HSCs activation to determine its potential as a new target to treat liver fibrosis.

2 | MATERIALS AND METHODS

2.1 | Ethical statement

The liver specimens for analysis were obtained according to Dutch legislation and the Code of Conduct for responsibly
dealing with human-derived material in the context of health research, the use of human material was approved by the Medical Ethical Committee of the University Medical Center Groningen (UMCG). The use of coded-anonymous human tissue enabled to refrain the need for written consent for “further use” human material.

The liver specimens for cell isolation were obtained from fresh tumor resections. All patients provided written documentation of informed consent. The Ethics Committee (Institutional Review Board) of the Otto-von-Guericke-University approved the study (reference number: 208/17). The study protocol conformed to the ethical guidelines of the declaration of Helsinki.

2.2 Reagents

Ascorbic acid (L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate, A8960, Sigma Aldrich, St. Louis, Missouri, United States). TGFβ1 (recombinant human transforming growth factor-beta 1, 240-B, R&D Systems, Minneapolis, Minnesota, United States). DMOG (Dimethyloxalylglycine, D3695, Sigma Aldrich), Bovine serum albumin (BSA, Merck Millipore, Darmstadt, Germany). Triton-X-100 (Merck).

2.3 Human liver material

Clinically healthy tissue was obtained from donors undergoing organ donation. Clinically cirrhotic tissue was obtained from explant livers scheduled for liver transplantation (Table S1). Liver tissue was stored in an ice-cold University of Wisconsin (UW) tissue preservation solution until further use.

2.4 Cell culture

LX-2 cells (SCC064, Merck) described by Xu et al.23 were used in passages 22-30. DMEM high glucose (Sigma Aldrich) supplemented with 10% fetal calf serum (Invitrogen, Breda, The Netherlands) and 1% antibiotics (Penicillin/Streptavidin/ Fungizone (p/s/f) Lonza, Basel Switzerland) was used for culturing them. Cells were passed by trypsinization and medium was refreshed every 3 days or when necessary. Cells were grown in 5% CO₂ and 37°C in ambient air (21% O₂) or in hypoxia (1% O₂).

Primary human HSCs (p-hHSCs) and hepatocytes were isolated from macroscopically normal liver specimens obtained from fresh tumor resections by an “all-in-one” liver cell purification procedure, as described previously.23 Freshly isolated quiescent human HSCs were culture-activated to activated HSCs for up to 2 weeks by allowing these cells to attach and proliferate. Briefly, p-hHSCs were seeded in cell culture plates, medium was refreshed 4 hours after isolation and every 3 days for up to 2 weeks until plates were confluent and HSCs are fully activated (aHSCs). Activated HSCs were passaged by trypsinization and passages 2-5 were used for experiments (from three independent isolations). Freshly isolated primary human hepatocytes were seeded and after 4 hours harvested.

Primary rat HSCs were isolated by 2-step perfusion of the liver with pronase (Merck Millipore) and collagenase-P (Roche, Almere, the Netherlands). Afterward, cells were separated by Nycodenz (Axis-ShieldPOC, Oslo, Norway) gradient centrifugation, as described before.26 Freshly isolated HSCs were culture-activated to activated HSCs for 7 days. Cells were harvested 4 hours, 1-, 3-, and 7-days after isolation to observe the time- and culture-dependent activation of HSCs in vitro.

2.5 Treatments

Cells were seeded to 80% confluence in 6- and 12-well plates (Gibco) (250,000 cells/well and 100,000 cells/well, respectively) and allowed to attach for 24 hours in complete medium. After attachment, cells were starved with 0.5% serum-containing medium for 18 hours, then cells were treated with vehicle (PBS), 10 ng/mL of TGFβ, 0.17 mmol/L of L-ascorbic acid or TGFβ plus ascorbic acid (10 ng/mL, 0.17 mmol/L, respectively) for 2 or 6 days (to allow collagen-I release and accumulation into the medium) for further analysis in serum-free medium. DMOG (1 mmol/L) treatment was performed 24 hours after attachment in serum-free medium for 48 hours in combination with TGFβ plus ascorbic acid (10 ng/mL, 0.17 mmol/L, respectively) under normal (21% O₂) or hypoxic (1% O₂) conditions.

2.6 RNA isolation, cDNA synthesis, and real-time quantitative PCR

Two-step reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed as previously described.25 Briefly, RNA was isolated from cell cultures using TRIzol reagent according to the supplier’s instruction (Thermo Fisher Scientific, Amsterdam, The Netherlands). RNA quality and quantity were determined using a NanoDrop 2000c UV-vis spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized from 2.5 μg RNA using random nonamers and M-MLV reverse transcriptase (Invitrogen). TaqMan primers and probes were designed using Primer Express 3.0.1 and are shown in Table S2. All target genes were amplified using the
Q-PCR core kit master mix (Eurogentec, The Netherlands) on a 7900HT Fast Real-Time PCR system (Applied Biosystems Europe, The Netherlands). SDSV2.4.1 (Applied Biosystems, Europe) software was used to analyze the data. Expression of genes is presented in 2-delta CT or fold induction and normalized to 18S.
2.7 Protein isolation, protein quantification, and western blot analysis

Protein samples were prepared for Western blot analysis as described previously. Protein concentrations were quantified using a protein assay (Bio-Rad, Hercules, CA, USA) with BSA as a standard. Thirty micrograms of protein were separated on Mini-PROTEAN TGX precast 4%-15% gradient gels (Bio-Rad) and transferred to nitrocellulose membranes using the Trans-Blot Turbo transfer system (Bio-Rad). Primary antibodies details and dilutions are listed in Table S3 and appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000; DAKO, Agilent, Santa Clara, CA, United States) were used for detection. Proteins were detected using the Pierce ECL Western blotting kit (Thermo Fisher Scientific). Images were captured using the ChemiDoc XRS system and Image Lab version 3.0 (Bio-Rad). Bands were quantified using the densitometry method with ImageJ software (public domain, developed at the National Institutes of Health).

2.8 Immunofluorescence microscopy

Cells were seeded in 12-well plates containing glass coverslips. After specified treatments (see results section), cells-containing coverslips were washed, fixed (4% PFA, 10 min), and permeabilized (0.1% Triton X-100, 10 min, to analyze intracellular collagen accumulation) or not (to analyze collagen deposition outside of cells) prior to non-specific blocking (2% BSA, 30 min). Coverslips were then incubated with primary antibodies (1 h, at RT) (Table S3), washed three times and incubated with rabbit anti-goat Alexa Fluor 488 (Thermo Fisher Scientific) (30 min, at RT) and covered from light. Coverslips were then washed three times and mounted with Vectashield antifade mounting medium with DAPI (Vector Laboratories, Gdynia, Poland). Coverslips were air-dried, sealed, stored at 4°C, and covered from light until further use. Images were obtained in a Zeiss 410 inverted laser scan microscope (Leica Microsystems, Wetzlar, Germany) with 16X or 40X magnification objectives using immersion oil and processed using ImageJ software (public domain, developed at the National Institutes of Health).

2.9 Hydroxyproline quantification

Hydroxyproline concentrations were quantified in collected the medium from 2- or 6-day treated cells according to Palmerini et al. Briefly, culture media (of 2- or 6-day-treated cells) were centrifuged (400 g for 10 min at 4°C) and protein was precipitated with trichloroacetic acid (Merck) (10% final concentration) for 20 min on ice. Protein pellets were hydrolyzed in 6 N HCl (Merck) (overlaid with mineral oil to prevent evaporation) for at least 20 hours at 85°C. Subsequently, the hydrolyzed protein was derivatized upon incubation in 0.1 M boric acid (Merck) (pH to 9.5) supplemented with O-phthalaldehyde (Thermo Fisher Scientific) and conjugated by incubating 3 min with 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (Sigma Aldrich). Samples included 2-10 µM internal standard (3,4-dehydroproline (Sigma Aldrich) and were measured by high-performance liquid chromatography (HPLC, Thermo Fisher Scientific) using a fluorescence detector (excitation 470 nm/emission 530 nm). Hydroxyproline (Sigma Aldrich) concentrations, relative to the internal standard, were determined from a 4-hydroxyproline (0-16 µM) standard curve.

2.10 Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). The significance of differences between groups was tested by one-way ANOVA or unpaired t test. Calculations were made using the software of GraphPad Prism 5. Results were considered statistically different when the P value < .05.

3 RESULTS

3.1 Ascorbic acid is required for effective collagen release by human HSCs

LX-2 cells (Figure 1) and primary human HSCs (p-hHSCs) (Figure 2) were treated with TGFβ (10 ng/mL) with and without ascorbic acid (AA, 0.17 mmol/L) to determine whether ascorbic acid affects collagen release in human HSCs. Activation of LX-2 by TGFβ was confirmed by increased...
αSMA protein and mRNA (ACTA2) expression (Figure S1A,B). As expected, collagen-I mRNA and cellular protein levels were significantly enhanced in TGFβ-exposed LX-2 cells after 2 (Figure 1A,B) and 6 days (Figure 1C,D). Two- and 6-day exposure to ascorbic acid did not significantly change basal and TGFβ-induced COL1A1 mRNA levels (Figure 1A,D). Interestingly, and despite the enhanced COL1A1 mRNA expression after TGFβ and AA co-treatment, cellular collagen-I protein levels were significantly decreased at 2- and 6-day TGFβ-cotreated conditions (Figure 1B,D). Immunofluorescence microscopy on Triton-X100-permeabilized (top panels, Figure 1E,F) and non-permeabilized (bottom panels, Figure 1E,F) LX-2 cells revealed that collagen-I accumulated mostly intracellularly under control conditions, which was strongly enhanced by TGFβ, both after 2-days (Figure 1E) and 6-days (Figure 1F) exposure. Only minor (extracellular) collagen-I staining was observed in non-permeabilized TGFβ-treated LX-2 cells after 2- and 6- days (bottom panels, Figure 1E,F, respectively). AA-treated LX-2 cells showed low intracellular collagen-I and a slight increase in extracellular collagen-I staining after 6 days (Figure 1F). Cotreatment with AA strongly increases

![Image](image_url)

**Figure 2** Ascorbic acid is necessary for collagen release in primary human hepatic stellate cells. Primary human activated hepatic stellate cells (p-HSCs) were treated with vehicle, TGFβ, and/or L-ascorbic acid for 48h. A, mRNA expression of COL1A1 after 48h treatments. B, Left: Representative Western blots of collagen-I and GAPDH (loading control); Right: Quantification of three independent experiments. C, Representative images of collagen-I (green) and DAPI (blue) staining of permeabilized (top panels) and non-permeabilized (bottom panels) p-HSCs. Scale bar, 50 µm top panels and 100 µm bottom panels. D, Quantification of hydroxyproline levels normalized per µg protein (µmol/L/µg) in spent medium of 2-day treated cells. n = 3 mean ± SEM *P < .05, **P < .001, ***P < .0001 (One-way ANOVA)
the amount of extracellular collagen-I in TGFβ stimulated LX-2 cells, both a 2-day (Figure 1E) and 6 days (Figure 1F) of treatment. Hydroxyproline concentrations (Figure 1G) and collagen-I protein levels (Figure 1H) in the medium were significantly enhanced when LX-2 cells were cotreated with TGFβ and AA for 6 days when compared to the single treatments and control cells. These data suggest that AA promotes the secretion of collagen-I in LX-2 cells, in particular under TGFβ-stimulated conditions.

As expected, similar findings were obtained with p-hHSCs and were already evident after 2 days of AA co-treatment (Figure 2). TGFβ enhanced collagen-I mRNA and cellular collagen-I protein levels (Figure 2A,B). Ascorbic acid did not affect basal and TGFβ-induced COL1A1 mRNA or protein levels (Figure 2A,B). In line, ascorbic acid reduced the intracellular staining of collagen-I (top panels, Figure 2C) and promoted the extracellular staining of collagen-I, as evident by immunofluorescence microscopy with non-permeabilized liver tissues.

**FIGURE 3** Ascorbic acid transporters expression in human liver cells and healthy and cirrhotic human livers. A, Liver tissue, purified human hepatocytes, purified human HSCs, and LX-2 cells were analyzed to assess the expression of SLC23A1 and SLC23A2 by QPCR. B, Western blot analysis to assess the expression of SVCT2, collagen-I, αSMA, and GAPDH in p-hHSCs and LX-2 cells. C, Healthy and cirrhotic human liver tissues were analyzed to assess the mRNA expression of SLC23A1, SLC23A2, ACTA2, and COL1A1. D, Representative image of Sirius Red staining on healthy and cirrhotic liver tissue, with quantification of five healthy and four cirrhotic tissues. The right panel shows the correlation analysis between the Sirius red staining and SLC23A2 expression. E, Representative Western blots of SVCT2, αSMA, and PEX14p (used as loading control) in primary rat HSCs after 4 h (D0), 1- (D1), 3- (D3), and 7- (D7) days culture-activation. n = 3-6, mean ± SEM *P < .05, **P < .001, ***P < .0001 (t test)
3.2 Human HSCs express one dedicated ascorbic acid transporter, SLC23A2, which is enhanced in liver cirrhosis

Human cells are able to take up ascorbic acid either via active (SVCT1 and SVCT2, encoded by SLC23A1 and SLC23A2, respectively) and passive (GLUT1, 3, and 4) transport. hHSCs and LX-2 cells did not express SLC23A1 (SVCT1), while SLC23A2 mRNA and the corresponding protein SVCT2 are readily detectable (Figure 3A,B). Interestingly, SLC23A2 mRNA levels were slightly downregulated by TGFβ treatment in LX-2 cells; however, protein levels were not affected (Figure S1A,C). In primary HSCs, the SVCT2 protein and SLC23A2 mRNA levels were significantly downregulated with TGFβ and ascorbic acid (~60% reduction in combined TGFβ + AA treatment; Figure S1D,F). Human hepatocytes expressed both dedicated AA transporters (Figure 3A). In addition, hHSCs and LX-2 cells expressed GLUT1 and GLUT3, both of which seem to be enriched in hHSCs when compared to total liver and human hepatocytes (Figure S2A). GLUT4 gene expression was not detectable in human liver extracts or purified human liver cells (data not shown). Interestingly, SLC23A2 mRNA levels were significantly elevated in human cirrhotic livers (characterized by enhanced levels of ACTA2 and COL1A1), when compared to healthy liver tissue (Figure 3C). Interestingly, the SLC23A2 mRNA levels in human liver samples correlated significantly with the level of fibrosis, as determined by the tissue area stained by Sirius red (Figure 3D). In contrast, SLC23A1 mRNA levels appeared reduced in cirrhotic livers; however, this was not significant (Figure 3C). The isolation and purification protocol for human HSCs includes a culture period that makes it difficult to compare SVCT2 expression in quiescent HSCs vs fully activated HSCs in vitro. In order to compare SVCT2 expression between quiescent and activated HSCs, we used rat HSCs freshly isolated from a healthy liver (Day 0 = D0) that was culture-activated for seven days (D7; Figure 3E). SVCT2 protein levels strongly increased during the 7 day culture period, similar to the HSCs activation marker αSMA (Figure 3E). Overall, these results suggest that transdifferentiating HSCs increase their capacity to take up ascorbic acid during liver fibrosis progression and thereby sustain collagen-I production.

3.3 Hydroxylase inhibition impairs collagen release and suppresses TGFβ-induced HSCs activation

Ascorbic acid is a cofactor for hydroxylases that act in several biological processes, including collagen production, maturation, and secretion. Therefore, we next analyzed the effect of the pan-hydroxylase inhibitor dimethylglyoxal glycine (DMOG) on collagen production and deposition by LX-2 cells. DMOG effectively blocked the ascorbic acid-induced collagen-I deposition by TGFβ-treated LX-2 cells (bottom panels, Figure 4A); however, this was not accompanied by an evident increase in intracellular collagen-I staining (top panels, Figure 4A). Notably, DMOG also reduced both basal and TGFβ-induced mRNA levels of ACTA2 and COL1A1 in the absence and presence of ascorbic acid (Figure 4B). These results suggest that hydroxylase inhibition by DMOG not only targets collagen release, but also suppresses other pathways involved in HSCs activation.

3.4 DMOG, unlike hypoxia, has antifibrotic effects on LX-2 cells and prevents collagen release under hypoxia

Advanced liver fibrosis impairs blood flow (portal hypertension) and oxygen supply to the liver. This inhibits prolyl hydroxylases thereby stabilizing hypoxia-inducible factors 1 and 2 (HIF-1 and 2), transcription factors that are known to activate ACTA2 and COL1A1, among others. DMOG also inhibits the prolyl hydroxylases that reduce the turnover of HIFs and it is, therefore, important to differentiate between hypoxia/HIF-dependent and DMOG-dependent effects on HSCs activation. Both DMOG and hypoxia (1% O₂) upregulated the expression of HIF-1α-target genes (PGK1 and EGLN3) in LX-2 cells, even though HIF1A mRNA levels were downregulated (Figure 5A), a compensatory mechanism also reported by others. As observed by others, hypoxia enhanced COL1A1 mRNA levels under TGFβ-treated conditions (both with and without ascorbic acid co-treatment; Figure 5B, left panel). In contrast, DMOG inhibited the TGFβ-induced COL1A1 expression, most effectively when co-treated with ascorbic acid (Figure 5B, left panel). Similarly, DMOG reduced TGFβ-induced ACTA2 levels, both in the absence and presence of ascorbic acid (Figure 5B, right panel). Comparable to normoxia (shown in Figure 1), ascorbic acid promoted extracellular collagen-I deposition by LX-2 cells grown under hypoxic conditions, which was strongly reduced by DMOG (Figure 5C, compare bottom panels TGFβ + AA vs DMOG + TGFβ+AA). This was accompanied by a clear accumulation of intracellular collagen-I in DMOG-treated LX-2 cells (Figure 5C, compare top panels TGFβ + AA vs DMOG + TGFβ+AA). Similar
to observed under normoxic conditions (Figure 4), DMOG suppressed basal and TGFβ-induced ACTA2 and COL1A1 mRNA levels in LX-2 cells grown in hypoxia (Figure 5D). Taken together, these data show that ascorbic acid is an essential cofactor for hydroxylases in human HSCs to promote collagen-I production and secretion at multiple levels, which can be blocked and modulated by prolyl-hydroxylase inhibition even under (pathophysiologic) hypoxia.

4 | DISCUSSION

In this study, we show that human hepatic stellate cells critically depend on ascorbic acid to effectively hydroxylate and secrete collagen-I. Human HSCs only express one dedicated ascorbic acid transporter, SLC23A2/SVCT2, which is enhanced in cirrhotic livers, while human hepatocytes express both SLC23A1/SVCT1 and SLC23A2/SVCT2. Pharmacological inhibition of hydroxylase activity prevents collagen-I deposition, even under ascorbic acid-supplementation, and additionally suppresses COL1A1 and ACTA2 (encoding αSMA) mRNA levels. Thus, despite anti-oxidant actions, ascorbic acid supplementation may also promote liver fibrosis. Selective inhibition of ascorbic acid uptake by HSCs and/or the hydroxylase activity may hold promise for the treatment of liver fibrosis.

The process leading to fibrosis in different tissues and organs is relatively well-defined, including myofibroblast activation and extracellular matrix production. For most tissue-derived fibroblasts it has been shown that ascorbic acid plays a central role in promoting collagen release, acting as a co-factor for hydroxylases that post-translationally modify the collagen polypeptides. In addition, ascorbic acid may also stimulate myofibroblasts activation as shown for skin and cardiac fibroblasts. It is surprising, though, that the role of ascorbic acid in human HSCs activation and liver fibrosis has so far not been studied in detail, not in vitro nor in vivo.

Most data on HSCs activation and liver fibrosis are generated by in vitro and in vivo models using animal-derived...
Early studies showed that ascorbic acid promotes proliferation and collagen synthesis in primary rat HSCs cultures, while more recent studies reported the opposite effect in rat HSCs cell lines. The latter study focused on the antioxidant effects of ascorbic acid, without analyzing a possible effect on collagen production. Similarly, the presumed anti-oxidant properties of ascorbic acid, in combination with alpha-tocopherol, reduced ethanol-induced...
hepatic fibrosis in rats. Nevertheless, such results cannot be translated to human liver fibrosis, because rodents can endogenously produce ascorbic acid while humans cannot. The senescence marker protein-30 (SMP30)/gluconolactonase (GNL in mice/GULO in humans) knockout (KO) mice cannot endogenously produce ascorbic acid and show reduced CCl_{4}-induced liver fibrosis, while the supplementation of ascorbic acid aggravated liver fibrosis in these mice. As humans also lack a functional GULO gene, these data imply that ascorbic acid can potentially promote liver fibrosis in patients with liver disease. Culture media for human fibroblasts, also for HSCs, do not routinely include ascorbic acid (supplementation) and thus may limit the optimal secretion of collagen, leading to its intracellular accumulation in the endoplasmic reticulum. Ascorbic acid itself is rather labile in culture media at 37°C and is dissipated within 12-24 hours under these conditions. L-ascorbic-acid-phosphate is a stable form of ascorbic acid that is protected from oxidation (85% active ascorbic acid remains after 1 week) and was used in our study in accordance with recent studies analyzing skin fibroblasts. Similar to observed for skin fibroblasts, these culture conditions lead to efficient hydroxylation and secretion of collagen-I by human HSCs and LX-2 cells within 2 days in the presence of L-ascorbic-acid-phosphate.

Interestingly, human HSCs express only one of the two dedicated ascorbic acid transporters, ie, SVCT2, while hepatocytes express both. SVCT2 is upregulated in cirrhosis, suggesting that the uptake of ascorbic acid, specifically by HSCs, is enhanced to maintain collagen-I production. These observations are in line with animal studies. Slc23a2 was selectively upregulated in liver fibrosis that developed after bile duct ligation (BDL) in mice/rats, while Slc23a1 levels were not affected or even reduced after 8 weeks. The latter study also analyzed mRNA abundance of ascorbic acid transporters in patients with liver disease, including hepatocellular cholestasis, primary biliary cirrhosis, hemochromatosis, and non-alcoholic steatohepatitis and found both SLC23A1 and SLC23A2 mRNA levels were enhanced compared to healthy livers. This partly aligns with our findings, since we observe a specific upregulation of SLC23A2, but a trend to SLC23A1 downregulation. We speculate that the differences found in our studies derive from the different etiologies of liver diseases, and/or stage of disease, and future work needs to clarify this. Still, the common ground of our—and these earlier—studies is that selective inhibitors for SVCT2 may hold therapeutic potential for the treatment of liver fibrosis, compounds that still need to be discovered.

As an alternative for blocking ascorbic acid uptake in HSCs, we analyzed the effect of inhibiting the hydroxylase activity using DMOG, which led to a decrease in collagen-I deposition even in the presence of ascorbic acid. In addition, DMOG treatment reduced mRNA levels of ACTA2 and COL1A1, indicating a strong in vitro anti-fibrotic effect on both collagen production and secretion by human HSCs. Indeed, also other pharmacological inhibitors of hydroxylase activity have promising effects on collagen deposition and fibrosis development in rodents providing further support for the therapeutic potential for treating liver fibrosis.

Liver fibrosis, and especially the progression to cirrhosis, leads to decreased oxygen supply to the parenchyma, activating the hypoxia-inducible factors HIF-1α and −2α. HIF activity is suppressed by prolyl-hydroxylases (PHDs) in the presence of oxygen, iron, and 2-oxoglutarate. DMOG, therefore, simultaneously suppresses collagen maturation and activates HIF signaling. Indeed, DMOG stimulated the HIF-1α pathway in human HSCs to comparable levels to HSCs grown under hypoxic conditions (1% O_{2}). However, hypoxia did not reduce collagen production and/or secretion by HSCs, when compared to normoxic conditions. In fact, hypoxia further enhanced TGFβ-induced ACTA2 and COL1A1 mRNA levels in human HSCs, compared to normoxia. Thus, the DMOG-induced suppression of ACTA2 and COL1A1 gene expression and intracellular collagen accumulation, even in hypoxia, suggests a HIF-1α-independent anti-fibrotic mechanism. This was also observed for intestinal fibrosis in mice, where DMOG showed an anti-fibrotic effect independent of HIF-1α activation. Instead, DMOG inhibited ERK-mediated TGFβ-signaling, thereby suppressing ACTA2 and COL1A1 transcription. We hypothesize that DMOG acts in a similar fashion in hHSCs, but this needs further confirmation.

In conclusion, we show for the first time that human hepatic stellate cells require ascorbic acid to efficiently secrete collagen. Human HSCs express SVCT2, which is upregulated during HSCs activation and liver cirrhosis. Both blocking SVCT2 activity or hydroxylase activity in HSCs hold promise for the treatment of liver fibrosis, which ideally could be combined with ascorbic acid supplementation to provide the other liver cells with sufficient amounts of this important antioxidant vitamin.
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CONFLICT OF INTEREST

The authors certify that they have NO affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials described in this manuscript.

AUTHOR CONTRIBUTIONS

N. Smith-Cortinez, R. Bank, C. Paulusma, K.N. Faber; performed the conceptualization and design of the study. N. Smith-Cortinez, R. R. Fagundes, V. Gomez, D. Kong, D. de Waart, S. Sydor, V. de Meijer, P. Olinga acquired the data. N. Smith-Cortinez, R. R. Fagundes, V. Gomez, C. Taylor, C. Paulusma, K.N. Faber analyzed the data. N. Smith-Cortinez, K.N. Faber drafted the manuscript. N. Smith-Cortinez performed the statistical analysis. N. Smith-Cortinez, K.N. Faber obtained funding. D. de Waart, J. Heegsma did technical assistance. C. Paulusma, K.N. Faber did the study supervision.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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