Inhibition of carnitine palmitoyl-transferase 1A in hepatic stellate cells protects against fibrosis

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Data Availability Statement

The data associated with this paper are available upon request to the corresponding author.

Conflict of interest

The authors declare that they have no conflicts of interest related to the study.

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Authors' contributions

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Abstract

Background & Aims: The pathogenesis of liver fibrosis requires activation of hepatic stellate cells (HSCs); once activated, HSCs lose intracellular fatty acids but the role of fatty acid oxidation and carnitine palmitoyltransferase 1A (CPT1A) in this process remains largely unexplored.

Methods: CPT1A was found in HSCs of patients with fibrosis. Pharmacological and genetic manipulation of CPT1A were done in HSCs human cell lines and primary HCSs. Finally, we induced fibrosis in mice lacking CPT1A specifically in HSCs.

Results: Here we show that CPT1A expression is elevated in HSCs of patients with NASH, showing a positive correlation with the fibrosis score. This was corroborated in rodents with fibrosis, as well as in primary human HSCs and LX-2 cells activated by transforming growth factor β1 (TGFβ1) and fetal bovine serum (FBS). Furthermore, both pharmacological and genetic silencing of CPT1A prevent TGFβ1- and FBS-induced HSC activation by reducing mitochondrial activity. The overexpression of CPT1A, which can be induced by saturated fatty acids and reactive oxygen species, triggers mitochondrial function and the expression of fibrogenic markers. Finally, mice lacking CPT1A specifically in HSCs are protected against fibrosis, induced by a choline-deficient high fat diet, a methionine- and choline-deficient diet, or treatment with carbon tetrachloride.

Conclusions: These results indicate that CPT1A plays a critical role in activation of HSCs and is implicated in the development of liver fibrosis, making it a potentially actionable target for fibrosis treatment.

Keywords: CPT1A, NASH, fibrosis, metabolism, fatty acids, beta oxidation

Lay summary

We show that CPT1A located in HSCs is elevated in patients and mice models with fibrosis, and that CPT1A induces the activation of these cells. Inhibition of CPT1A ameliorates fibrosis by preventing the activation of HSCs.

Highlights

- CPT1A is overexpressed in HSCs cells from patients with fibrosis and positively correlates with fibrosis and NAS score.
- Fatty acid oxidation is increased in activated HSCs and the inhibition of CPT1A blunts the activation of HSCs by reducing mitochondrial activity.
- The specific deletion of CPT1A in HSCs of mice protects against methionine-and-choline-deficient diet (MCDD)-, carbon tetrachloride- and choline deficient + high fat diet (CDHFD)-induced fibrosis.

Introduction

One of the most common types of fibrotic liver is non-alcoholic fatty liver disease (NAFLD), which may develop into non-alcoholic steatohepatitis (NASH). Hepatic stellate cells (HSCs) are the primary fibrogenic cell type activated following liver injury, moving from a quiescent phenotype rich in vitamin A into activated myofibroblast-like cells with proliferative and migratory properties [1-3].

In NAFLD, hepatocytes ectopically store fatty acids as a consequence of excessive free fatty acids when the capacity to store lipids safely is exceeded. In contrast, activation of hepatic stellate cells (HSCs) leads to a loss of fatty acid content [4, 5]. This event has been mainly explained by the reduction in the expression of adipogenic transcription factors, which are abundant in quiescent HSCs, during the transdifferentiation of these cells [6, 7]. Accordingly, activation of HSCs is inhibited by peroxisome proliferator-activated receptor gamma (PPARγ) signaling, in part due to reduced WNT activity [8].

Proliferation and migration of HSCs require considerable energy requirements, triggering a cellular metabolic rewiring [9]. For instance, HSC activation relies on the stimulation of glycolysis [10], glutaminolysis [11], and *de novo* lipogenesis [12]. However, the potential role of fatty acid oxidation (FAO) in the decline of lipid droplets in activated HSCs remains largely unknown. Carnitine palmitoyltransferase 1A (CPT1A) is a mitochondrial enzyme that is the rate-limiting step in the beta oxidation of medium- and long-chain fatty acids to obtain energy in the form of ATP. The vast majority of research on CPT1A has used mouse models that are deficient for this enzyme in all liver cell types or only in hepatocytes. Notably, these results show that increasing the activity of CPT1A reduces diet-induced hepatic triglyceride levels [13-15]. Indeed, lipid accumulation in hepatocytes induces fibrogenic activation of HSCs. Despite the loss of fatty acids upon

HSC activation, however, the potential role of CPT1A in these specific cells has not been studied.

In the present study, we have investigated the function of CPT1A located within HSCs in liver fibrosis. Our findings show that CPT1A is highly expressed in HSCs from patients and rodents with fibrosis, as well as in human primary HSCs and LX-2 cells activated by transforming growth factor β1 (TGFβ1) and fetal bovine serum (FBS). Both pharmacological and genetic inhibition of CPT1A blunt TGFβ1- and FBS-induced HSC activation by reducing mitochondrial activity. Finally, the specific deletion of CPT1A in HSCs of mice protects against methionine- and choline-deficient diet (MCDD) as well as against carbon tetrachloride–induced fibrosis. Thus, CPT1A plays a critical role in the activation of HSCs, with implications in liver fibrosis development.

Material and Methods

Human samples

The study population included 21 biopsy-proven NASH patients with different stages of fibrosis progression, who underwent a liver biopsy during bariatric surgery or with a diagnostic purpose at the Marqués de Valdecilla University Hospital (Santander, Spain) (Table S1). Liver samples with histologically normal liver obtained from 6 subjects during programmed cholecystectomy without steatosis were selected as healthy controls. Patients consumed <20 g alcohol/day, did not take potentially hepatotoxic drugs, had no analytical evidence of iron overload, and were seronegative for autoantibodies and for hepatitis B/C viruses and human immunodeficiency virus. Hepatic histopathological analysis was performed according to the scoring system of Kleiner et al. [16] . Minimal criteria for NASH included the combined presence of grade 1 steatosis, hepatocellular

ballooning, and lobular inflammation with or without fibrosis. This study was performed in agreement with the Declaration of Helsinki and with local and national laws. The Human Ethics Committee of the hospital approved the study procedures, and all participants voluntarily signed an informed written consent before inclusion in the study. Correlation analysis between amount of CPT1A in αSMA-positive cells and different variables of these patients are included in Table S2.

Animals and diets

8 weeks old male C57 BL/6J mice were kept under 12 hours light/dark cycle and had *ad libitum* access to standard diet, methionine-choline-deficient diet (MCDD) (A02082002BR, Research Diets), high fat diet (HFD) (D12492 60% fat, Research Diets), or choline deficient and high fat diet (CDHFD) (D05010402; 45% fat, Research Diets) for the specified times. Food intake and body weight were monitored weekly during experimental phase in all the experiments. Animal protocols were approved by the Committee at the University of Santiago de Compostela.

Results

CPT1A is upregulated in activated HSCs from patients and rodents with fibrosis

Immunofluorescence labeling in liver sections of healthy persons or patients with fibrotic NASH showed that CPT1A colocalized with α smooth muscle actin (αSMA) in fibrotic liver but not in healthy liver (Fig. 1A and Fig. S1). Furthermore, the area of colocalization positively correlated with the fibrosis score and the NAS score (Fig. 1A). Correlation of CPT1A- αSMA area with other variables is available in Table S2. To investigate the expression of CPT1A independent of their activation status, we also did correlations with

glial fibrillary acidic protein (GFAP) a marker of quiescent HSCs [2, 17, 18]. The colocalization of CPT1A with GFAP was very low (Fig. S2). Moreover, the merged CPT1A/GFAP staining was not correlated with fibrosis score (Fig. S2B-C). These findings indicate that the majority of CPT1A is expressed in activated HSC and proportionally to fibrosis score, while its levels in quiescent HSCs are comparatively very low.

We next investigated the immunofluorescence of CPT1A in livers from mouse models of diet-induced fibrosis. Mice were fed a high-fat diet (HFD) with 45% kcal fat content or a choline-deficient HFD (CDHFD) for 52 weeks. In these mouse models of fibrosis, we detected a clear colocalization of CPT1A and αSMA, which was significantly higher than in control mice fed a standard diet (SD) (Fig. 1B, Fig. S3A). We also used the mouse model of chemically-induced fibrosis using carbon tetrachloride; these results also showed an increased colocalization staining in carbon tetrachloride–treated mice as compared to non-treated control mice (Fig. S3B).

In corroboration with the histological data, we found that the levels of CPT1A (both at the protein and the mRNA levels), as well as of collagen alpha 1 (COL1α1) and collagen alpha 2 (COL1α2), were augmented in whole liver and in isolated hepatocytes (Fig. S3C) and isolated HSCs (Fig. 2A) from mice fed a CDHFD for 52 weeks. Similar results were observed for mice treated with carbon tetrachloride or fed a methionine- and choline-deficient diet (MCDD): these displayed augmented levels of CPT1A in liver as well as in isolated HSCs (Fig. 2B-C, Fig. S3D-E). While CPT1A expression was higher in isolated hepatocytes from mice fed a MCDD, it was decreased in mice treated with carbon tetrachloride (Fig. S3D-E). We next evaluated the potential alteration of CPT1A expression during the activation of primary human HSCs (phHSCs). Notably, treating with TGFβ1 (a potent fibrogenic inducer) increased the expression of CPT1A as well as

of the pro-fibrotic markers αSMA, COL1α1, and COL1α2 (Fig. 2D-E). Overall, these results indicated that CPT1A expression is consistently elevated in activated HSCs in both humans and rodents.

Inhibition of CPT1A blocks *in vitro* HSC activation

Because CPT1A is upregulated upon HSC activation, we hypothesized that inhibiting CPT1A could reduce HSC activation by decreasing mitochondrial activity and FAO. As expected, a 24-h TGFβ1 treatment in phHSCs increased the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) indicative of the glycolytic flux (Fig. 3A). Strikingly, treating with etomoxir, a small molecule irreversible inhibitor of CPT1A, completely blocked TGFβ1-induced OCR without affecting ECAR (Fig. 3A). Quantification of the different parameters of mitochondrial function revealed that etomoxir blunted TGFβ1-induced basal respiration, ATP-linked respiration, and maximal respiration (Fig. 3B). Furthermore, TGFβ1-induced expression of fibrotic markers was abolished by etomoxir (Fig. 3C). Similar to pharmacological inhibition, genetic silencing of CPT1A in phHSCs also blocked the 24-h TGFβ1-induced OCR and decreased the expression of fibrotic markers (Figures 3D-G).

We next tested our conditions using LX-2 cells, a human HSC cell line that has been extensively characterized and retain key features of hepatic stellate signaling [19]. In an initial time-response experiment with TGFβ1, we found that as soon as 1 h after initiation of TGFβ1, mRNA expression of CPT1A, but not of CPT2, was significantly increased and remained at high levels at the 6-h and 12-h timepoints of treatment (Fig. S4A). This upregulation occurred before any detectable upregulation of COL1α1 and COL1α2 (Fig. S4B). Moreover, treatment with PDGF, another potent activator of HSCs, effectively increased the expression of proliferation markers, without altering CPT1A expression

levels (Fig. S4C). LX-2 cells treated with TGFβ1 showed a greater FAO rate than untreated ones (Fig. S5A). In agreement, 12-h TGFβ1 treatment increased the OCR and the expression of fibrotic markers, and these effects were blocked by etomoxir (Fig. S5B-C). Consistent with the activation of LX-2 cells, TGFβ1 also reduced the intracellular lipid content, while increasing the levels of ATP and of mitochondrial reactive oxygen species (ROS), as measured by MitoSOX; treatment with etomoxir also blocked these TGFβ1-induced actions (Fig. S5D-F). In the conditions studied, no differences were detected between LX-2 cells treated with TGFβ1 and those treated with TGFβ1 and etomoxir with respect in protein levels of cleaved caspase 3 or -7, beta-galactosidase staining or mRNA expression levels of P16 (Fig. S5G-H). We therefore concluded that the inhibitory actions of etomoxir on HSC activation occurred independently of apoptosis and senescence.

Similar to the pharmacological inhibition of CPT1 for 12 hours, the genetic repression of CPT1A in LX-2 cells (Fig. S6A) blocked the increased OCR, reduced lipid content, and increased levels of ATP and ROS provoked in the wild-type cells by 12-h TGFβ1 treatment (Fig. S6B-F), and these features were not related to senescence (Fig. S6G). The results obtained after the activation of LX-2 cells by 12-h TGFβ1, and the blockade of those effects after the genetic or pharmacological inhibition of CPT1A, were consistently maintained at longer periods of time. Specifically, after 48 hours, etomoxir was still able to partially block the higher OCR, lower lipid content, and increased levels of ATP and ROS induced by TGFβ1 treatment (Fig. S7).

To find out whether CPT1A could affect proliferation at long periods of time, we performed EdU assays in LX-2 cells manipulating CPT1A or administering FBS for 72 hours. The overexpression of CPT1A significantly increased the proportion of EdU stained cells (Fig. S8A). In addition, incubation of LX-2 cells with 10% FBS, which

promotes CPT1A expression (Fig. 4A), increased the proliferation of LX-2 cells (Fig. S8B). Contrarily, the administration of 10% lipid-depleted FBS, a condition that does not increase CPT1A expression (Fig. 4A), was unable to induce cell proliferation (Fig. S6B). Finally, we performed an EdU assay in LX-2 cells downregulating CPT1A with siRNAs. After 72 hours, we found a slight but non-significant reduction (p=0.07) in the proliferation of LX-2 cells (Fig. S8C). Thus, there is a long-term increase in proliferation of HSCs following the activation of CPT1A, and a tendency to decrease cell proliferation after downregulating CPT1A.

When isolated HSCs are grown in culture with DMEM supplemented with 10% fetal bovine serum (FBS), they spontaneously acquire an active pro-fibrogenic phenotype [20-23]. Using this experimental paradigm to measured FAO and CPT1A expression, we found that both aspects increased in LX-2 cells incubated with 10% FBS as compared to cells incubated in a control medium with 2% FBS (Fig. 4A). Further, LX-2 cells incubated with 10% FBS displayed a time-dependent increase in the expression of the pro-fibrogenic markers αSMA, COL1α1, and COL1α2 (Fig. 4B). However, if lipids were removed (using a 10% lipid-depleted FBS), the fibrogenic response to the 10% FBS was almost completely blunted (Fig. 4B). Treating the LX-2 cells with etomoxir gave a similar attenuated fibrogenic effect when was added to 10% FBS-treated cells (Fig. 4B). Consistent with these results, the expression levels of short-chain acyl-CoA dehydrogenase (SCAD), medium-chain acyl-CoA dehydrogenase (MCAD), or long-chain acyl-CoA dehydrogenase (LCAD) increased in cells incubated with 10% FBS and decreased in 10% lipid-depleted FBS (Fig. 4C). Also, the reduced lipid content and higher OCR associated with 10% FBS–induced activation were blocked after removing lipids from the medium or after etomoxir treatment (Fig. 4D-E). Altogether, these data indicated that HSC cells require lipid availability and a functional FAO pathway to be activated.

Overexpression of CPT1A induces *in vitro* HSC activation in an ATP-dependent manner

After demonstrating that inhibition of CPT1A was sufficient to prevent HSC activation, we next performed a gain-of-function experiment by overexpressing CPT1A in LX-2 cells with different doses of plasmid CPT1A (1 and 10 ng) (Fig. 5A) to investigate whether this gene could activate HSCs. Our results showed that the ectopic plasmid expression of CPT1A at both doses stimulated OCR and the expression of fibrotic markers (Fig. 5B–D). Thus, abnormally high levels of CPT1A are enough to activate HSCs. Moreover, overexpressing CPT1A in LX-2 cells previously treated with TGFβ1 led to an enhanced increase of OCR and expression of fibrotic markers (Fig. 5E–G), indicating that CPT1A overexpression may have a summatory effect to TGFβ1 in the activation of HSCs.

To determine whether ATP availability plays a direct role mediating the effects of FAO activation in fibrogenic activity, we first performed a dose-response with the ATP synthase/Complex-V inhibitor named oligomycin in LX-2 cells overexpressing CPT1A. Oligomycin inhibited CPT1A-induced OCR in a dose dependent manner, with a complete suppression of OCR at concentrations above 2000 nM (Fig. S9A). The dose of oligomycin which reduced the CPT1A-induced OCR to similar levels to the control baseline was 330 nM. This means that with this dose of oligomycin, the overexpression of CPT1A could not stimulate OCR but just maintain mitochondrial respiration at the same extent as quiescent HSCs. Next, we measured intracellular ATP levels in LX-2 cells under three different treatments: controls, overexpression of CPT1A and CPT1A + oligomycin (330 nM). Our results indicated that the overexpression of CPT1A was indeed increasing intracellular ATP levels, while the cotreatment with oligomycin was able to blunt

CPT1A-induced ATP and maintain it at baseline levels (Fig. S9B). Finally, we directly measured the mRNA levels of different fibrogenic markers. Whereas expression of αSMA, COL1α1 and COL1α2 was upregulated following the overexpression of CPT1A, this increase was almost completely suppressed in LX-2 overexpressing CPT1A and treated with oligomycin (Fig. S9C). Thus, our data point to mitochondrial ATP production as a major mediator of the effects of CPT1A overexpression on HSC activation.

Production of ROS by hepatocytes activates HSC

We next aimed to study if ROS, generated by an exacerbated FAO of hepatocytes in the fibrotic liver, could affect HSC activation. To induce ROS, we forced FAO in human HepG2 hepatocytes by: i) overexpressing CPT1A; ii) administering an overload of oleic acid; and iii) both overexpressing CPT1A and treating hepatocytes with oleic acid. Indeed, the three groups showed an elevated expression of CPT1A, being the combination of oleic acid and overexpression of CPT1A the most effective (Fig. S10A). After 12 hours, culture media was replaced by fresh growth media to avoid a potential direct effect of oleic acid in stellate cells. 12 hours later, culture media were collected, and ROS levels were determined. As expected, ROS levels were increased in culture media of FAO-induced hepatocytes cells in the three scenarios (Fig. S10B). Then, we used this conditioned media from hepatocytes to treat human HSCs LX-2 cells and, after 30 hours, we analyzed the activation status. The expression of fibrogenic markers αSMA, COL1α1 and COL1α2 was induced in LX-2 cells receiving the culture medium from hepatocytes overexpressing CPT1A, oleic acid, and the combination of overexpression of CPT1A plus oleic acid (Fig. S10C). Therefore, our results indicate that the induction of FAO in

hepatocytes stimulates the generation of ROS, which subsequently induce the activation of HSCs.

To assess the causal role of hepatocyte-generated ROS in the activation of HSCs, we prevented ROS production with N-acetyl-cysteine (NAC), a well-known antioxidant and precursor of glutathione [24], in FAO-stimulated hepatocytes. As expected, we found that NAC strongly inhibited the ROS production in culture media (Fig. S10B). However, the culture media of NAC-treated cells was partially able to blunt the activation of HSCs (Fig. S10C). This indicates that the profibrogenic effect of FAO-induced hepatocytes over the HSCs can be partially mediated by ROS production.

We also assessed the role of ROS within HSCs. For this, we ectopically expressed CPT1A in LX-2 cells and then treated them with either vehicle or NAC[24]. As expected, the overexpression of CPT1A increased the levels of ROS, measured as MitoSOX staining area, whereas the administration of NAC in these cells abolished CPT1A-induced ROS production after 24 hours (Fig. S11A). However, the suppression of ROS in LX-2 cells had a minor impact in pCPT1A-induced fibrogenesis, with a (non-significant) reduction of approximately 20% in the expression of the fibrogenic markers (Fig. S11B). Overall, these results indicate that, *in vitro*, FAO-derived ROS within HSCs plays a quantitatively lesser relevant role than exogenous (from hepatocytes) ROS.

Saturated fatty acids are induced by TGFβ1 and induce HSC activation via CPT1A

We next investigated the signal pathway linking TGFβ1 with CPT1A gene regulation. It has been reported that peroxisome proliferator activated receptor α (PPARα) and PPAR gamma coactivator (PGC-1α) are able to induce CPT1A in certain contexts [25, 26]. Therefore, we measured the expression of both genes in LX-2 cells treated with TGFβ1

but failed to detect significant changes (Fig. S12A). In addition, it has been also shown that long-chain fatty acids (LCFs) regulate liver CPT1 expression through a PPARα-independent pathway [27]. Thus, we investigated the potential role of different fatty acids. First, we performed lipidomics analysis in LX-2 cells treated with TGFβ1 and found an increase in total fatty acids following the administration of TGFβ1 (Fig. 6A). As depicted in the first heatmap, saturated fatty acids, and to a much lesser extent mono- and poly-unsaturated fatty acids, contributed to the observed increase. Next, we measured the levels of specific species of saturated fatty acids, and found that 14:0, 15:0, 16:0 and 18:0 fatty acids were increased in LX-2 treated with TGFβ1, as represented in the second heatmap (Fig. 6B). The quantification of these lipid species in the TGFβ1+etomoxir group allowed us to detect a further accumulation of species 14:0, 15:0, 16:0 and 17:0, but not 18:0, compared to the cells treated with TGFβ1 alone (Fig. 6B). This agrees with the hypothesis that species 14:0, 15:0, 16:0 and 17:0 are being used to fuel beta oxidation in response to TGFβ1 administration.

Finally, to evaluate if these fatty acids could be able to trigger the activation of HSCs, we performed a functional experiment administering palmitic acid (16:0) at different doses to LX-2 cells. Palmitic acid significantly upregulated CPT1A expression as well as the levels of fibrogenic markers (Fig. 6C). Next, we treated LX-2 cell with palmitic and also silenced CPT1A. While this fatty acid increased the expression of fibrogenic markers, this effect was blocked when CPT1A was inhibited (Fig. 6D). Contrary, the administration of unsaturated fatty acids such as oleic and linoleic acid did not alter CPT1A expression, nor fibrogenic markers (Fig. S12B). These results indicate that 1) TGFβ1 increases the levels of total fatty acids and specially saturated species, which are fueling beta oxidation of activated HSC, and 2) palmitic acid, but not unsaturated fatty acids as oleic or linoleic acids, is able to induce a fibrogenic response via CPT1A.

Inhibition of CPT1A in HSCs protects against fibrosis

After showing that CPT1A is increased in patients and animal models of NASH with fibrosis, and that the manipulation of CPT1A plays a critical role in HSC activation in vitro, we next undertook the in vivo functional relevance of our findings. For this, we crossed CPT1A-floxed mice with bacterial artificial chromosome (BAC) transgenic mice in which Cre expression is driven by lecithin-retinol acyltransferase (Lrat) [28], to generate mice with a CPT1A knockout specifically in HSCs (HSC-CPT1A-KO) (Fig. 7A). After feeding mice a MCDD for 6 weeks, CPT1A was clearly colocalized with αSMA in WT mice but not in HSC-CPT1A-KO mice, confirming the efficiency of the recombination (Fig. 7A, Fig. S13). As expected, MCDD caused a significant induction in circulating levels of markers of liver damage AST and ALT, increased collagen deposition and hydroxyproline levels in the liver, and increased expression of fibrotic markers (Fig. 7B-E). Importantly, all these MCDD-induced effects were ameliorated in HSC-CPT1A-KO mice (Fig. 7B-E). In addition, the expression levels of inflammatory genes, including F4/80, TNFα, and IL1β, were upregulated by MCDD in WT mice but not significantly or to a lower degree in HSC-CPT1A-KO mice (Fig. 7E); this agrees with the decreased amount of inflammatory infiltrates measured by F4/80 staining (Fig. 7F).

In a second model of fibrosis, we injected carbon tetrachloride for 6 weeks into both WT and HSC-CPT1A-KO mice. In WT mice, and as expected, carbon tetrachloride increased the serum levels of AST and ALT, hepatic collagen deposition, hydroxyproline levels, expression of fibrotic and inflammatory markers, and inflammatory infiltrates (Fig. S14A-F). However, all carbon tetrachloride–induced effects were clearly attenuated in HSC-CPT1A-KO mice (Fig. S14A-E). Likewise, expression analysis in isolated HSCs

from HSC-CPT1A-KO mice revealed that fibrogenic activation following carbon tetrachloride treatment was also clearly attenuated (Fig. S14F).

We next used a third model of fibrosis that resembles the characteristic whole-body metabolic deregulation of NASH using a feeding regime of CDHFD versus SD for HSC-CPT1A-KO mice. At 10 weeks, both HSC-CPT1A-KO and WT mice fed a CDHFD equally increased their body weight as compared to those fed a SD, without changes in food intake (Fig. 8A). Notably, however, the expected increases in serum levels of AST and ALT, hepatic collagen deposition, and hydroxyproline levels, and upregulation of fibrotic and inflammatory markers that were induced by CDHFD in WT mice were significantly attenuated in HSC-CPT1A-KO mice (Fig. 8B-D). Overall, these results indicate that the lack of CPT1A in HSCs protects against fibrosis development.

Discussion

We show here that CPT1A is upregulated in HSCs of patients with fibrosis and fibrosis animal models, and that CPT1A induces the activation of these cells, thereby contributing to the progression of liver fibrosis. Further, pharmacological and genetic inhibition of CPT1A ameliorates fibrosis by preventing the activation of HSCs. Notably, mice genetically lacking CPT1A in HSCs are protected against fibrosis.

HSC activation depends on the metabolic activation of glycolysis [10], glutaminolysis [11], and *de novo* lipogenesis [12]. An important number of the current drugs to treat NASH aim to inhibit *de novo* lipogenesis. For instance, inhibition of acetyl CoA carboxylase (ACC), the enzyme that catalyzes the rate-limiting step of *de novo* lipogenesis by converting acetyl co-enzyme A to malonyl-CoA, suppresses the activation of HSCs [12]. Clinical trials have shown that inhibitors of ACC [29, 30] or fatty acid synthase [31] are efficient in patients with NASH for reducing hepatic fat content, fibrosis markers, and liver stiffness. However, the potential role of FAO in HSCs has remained largely unexplored. This is of importance, given the coordinate regulation of fatty acid metabolism with malonyl-CoA, a main intermediate of fatty acid synthesis, which is the main allosteric inhibitor of CPT1A and therefore FAO [32]. In hepatocytes, increased glucose metabolism raises levels of malonyl-CoA, which is a potent allosteric inhibitor of CPT1 and blocks FAO, thereby favoring lipid storage. For this reason, some preclinical studies have aimed to promote FAO to reduce NAFLD [14, 15, 33]. However, in response to hepatic injury, HSCs are completely different to hepatocytes. When they are activated, HSCs convert from inactive, vitamin A–rich cells into myofibroblast-like cells with proliferative, migratory, and fibrogenic properties [1, 34]. These features are somewhat similar to the ones displayed by cancer cells, for which it is well established that fatty acids sustain their rapid proliferative rate and provide an essential energy source [35, 36].

Our results showing that CPT1A is overexpressed in HSCs cell in patients with fibrosis and fibrosis animal models, and that FAO is increased in activated HSCs, are in agreement with the hypothesis that activated HSCs lose their intracellular lipid droplets to provide fatty acids for activation and transdifferentiation. These results are functionally corroborated by genetic overexpression of CPT1A, which is not only sufficient to activate HSCs *per se* but also enhances TGFβ-induced fibrosis. In line with the cell-specific effects that CPT1 may play, intrahepatic CD4+ T cells or mice exposed to linoleic acid show elevated levels of CPT, while the pharmacological blockade of CPT decreases apoptosis of intrahepatic CD4+ T cells and inhibits HCC tumor formation [37]. These results highlight that each cell type has a specific metabolic rewiring in response to injury, and that activating CPT1A does not always exert a beneficial action in the liver.

A descriptive study has previously shown that the expression of *PPARβ* is increased in activated primary HSCs [6]. Activation of *PPARβ* was initially related to its adipogenic role, but this gene is also known to induce FAO [38], even though its capacity to regulate HSC activation via FAO has not been tested. To our knowledge, our study is the first to demonstrate that CPT1A is consistently elevated in both LX-2 cells and phHSCs activated by different stimuli, as well as in animal models and patients with NASH. Moreover, we also show that CPT1A is functionally relevant, as both genetic and pharmacological inhibition of CPT1A ameliorated fibrosis *in vitro* and *in vivo*. In line with this, one report indicated that the mouse stellate JS1 cell line treated with etomoxir induces lipid droplet accumulation in these cells and reduces the expression of fibrotic markers [23]. However, this result was obtained by using etomoxir at a relatively high dose (20 μM; note that we used 4 μM for our experiments). This is a relevant fact, as high doses of etomoxir are known to promote FAO independently of CPT1A and to exert off-target effects [39, 40], so it is difficult to draw a solid conclusion concerning the role of CPT1A from a single

experiment using this inhibitor. It is also necessary to take into consideration that, in some cell types, oxidative metabolism induced by etomoxir at concentrations exceeding 5 μM is independent of its effects on FAO, and that concentrations induce acute production of ROS with associated evidence of severe oxidative stress [41]. Our experiments with etomoxir at a dose of 4 μM showed a clear amelioration of HSC activation by different stimuli. Importantly, this beneficial action was not associated with changes in ROS levels. We also failed to detect any alteration in proton leaks in our OCR analysis, indicating that etomoxir did not damage the mitochondrial membrane. Critically, all of our findings obtained from the pharmacological inhibition of CPT1A with etomoxir were reproduced by genetic silencing.

The mechanism activating CPT1A involved saturated long-chain fatty acids as palmitic acid, which induces HSC activation in a CPT1A-dependent manner, while unsaturated fatty acids failed to exert such effects. This is in agreement with a previous study showing that long-chain fatty acids can directly regulate liver CPT1 expression [27]. CPT1A-induced fibrogenesis is highly dependent of ATP availability since the inhibition of ATP synthase blunted the upregulation of fibrogenic markers upon the ectopic expression of CPT1A in HSCs. However, the role of ROS generated within HSC seems to be less relevant for the actions of CPT1A.

The causal effects of *in vitro* CPT1A inhibition were corroborated in mice specifically lacking CPT1A in HSCs. These mice were subjected to three different fibrosis-induced conditions: MCDD, carbon tetrachloride, and CDHFD. In all cases, CPT1A deficiency in HSCs protected the animals against fibrosis and inflammation. Importantly, our results indicate that CPT1A-induced effects were not due to effects in cell death or senescence.

These results support the hypothesis that activated HSCs display an enhanced metabolism that accommodates the accelerated biological functions, and thereby increased energy

requirements, of activated HSCs. FAO and mitochondrial activity seem to play a key role in generating an energy source for activation and proliferation of HSCs; in turn, when CPT1A is inhibited, HSCs exhibit a more inactive phenotype with suppressed metabolism. Altogether, these findings indicate that compounds specifically targeting CPT1A in HSCs may open a new avenue to treat liver fibrosis.

Figure legends

Fig. 1. CPT1A is upregulated in activated hepatic stellate cells (HSCs) of fibrotic livers. (A) Representative colocalized immunofluorescence for αSMA (red), expressed in the cytoplasm of activated HSCs, with CPT1A (green) in healthy (n = 6) and NASH patients with fibrosis (n = 21). Nuclei were stained with DAPI (blue). Correlation of αSMA-CPT1A merged area with the fibrosis score and NAS score is shown. (B) Representative colocalized immunofluorescence for αSMA with CPT1A in liver sections from mice fed a standard diet (SD), a high-fat diet (HFD) or a choline deficient, high-fat diet (CDHFD) for 52 weeks (n = 4).

Fig. 2. CPT1A is upregulated in activated primary cultures of HSCs from mice or human patients. (A–C) Mice were *i*) fed a choline deficient high-fat diet (CDHFD) or (as a control) a standard diet (SD) for 52 weeks (A), *ii*) fed a SD diet and treated with carbon tetrachloride (0.6 ml/kg i.p.) or (as a control) with vehicle once a week for 6 weeks (B), or *iii*) fed a methionine- and choline-deficient diet (MCDD) or a SD for 4 weeks (C). Representative microphotographs are shown of haematoxylin and eosin (H&E; upper panel) and Sirius red staining (lower panel) of liver sections. Expression of COL1α1, COL1α2, and CPT1A expression in isolated HSCs is shown (n = 3 or 4). (D) Primary human HSCs (phHSCs) from a donor were treated with 8 ng/ml TGFβ1 for 24 h (n=8). Expression of fibrotic markers and CPT1A is shown. (E) Correlation between CPT1A expression and αSMA, COL1α1, and COL1α2. HPRT was used to normalize mRNA levels. Data are mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001

Fig. 3. Either pharmacological or genetic inhibition of CPT1A attenuates the metabolic and fibrogenic activation induced by TGFβ1 in primary human HSCs (phHSCs). (A) Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in phHSCs treated with TGFβ1 and 4 μM etomoxir for 24 h (n = 4). Arrows indicate the timepoint at which mitochondrial respiration modulators (oligomycin [Oligo], phenylhydrazone [FCCP], or rotenone/antimycin A

[Rot/AA]) were added to the assay. Right, graph depicting the effect of TGFβ1 or etomoxir treatments on quiescent or energetic metabolic states, based on quantification of glycolysis and oxygen consumption rate during basal metabolism. (B) Parameters of mitochondrial function (n = 4). (C) Expression of fibrotic markers (n = 4). (D) Expression of CPT1A in phHSCs with CPT1A silencing and 24-h TGFβ1 treatment (n = 4). (E) OCR, ECAR, and basal metabolism in phHSCs with CPT1A silencing and 24-h TGFβ1 treatment (n = 4). (F) Parameters of mitochondrial function (n = 4). (G) Expression of fibrotic markers (n = 4). mRNA levels were normalized to the housekeeping gene HPRT. Data are mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001

Fig. 4. Beta oxidation mediates the activation of HSC LX-2 cells induced by FBS. (A) Fatty acid oxidation rate in LX-2 cells treated with FBS 10% (n = 6), and expression of CPT1A in LX-2 cells treated with 10% FBS or 10% lipid-depleted FBS for 1 h (n = 5 or 6). (B) Expression of αSMA, COL1α1, and COL1α2 in LX-2 cells treated for 1, 2 and 12h with 10% FBS or 10% lipid-depleted FBS in presence of etomoxir or vehicle (n = 3–6). (C) Expression of beta oxidation markers in LX-2 cells (n = 5–6). (D) Representative microphotographs of oil red O (ORO) staining in LX-2 cells. Lipids (red area) were quantified and normalized to the total number of nuclei per field (n = 5–6). (E) Oxygen consumption rate (OCR) in LX-2 cells treated with 10 % FBS; 10% FBS and etomoxir; or 10% lipid-depleted FBS (n = 3). mRNA levels were normalized to the housekeeping gene HPRT. Data are mean ± standard error mean (SEM). *P < 0.05, **P < 0.01, ***P < 0.001

Fig. 5. The genetic overexpression of CPT1A activates human HSC LX-2 cells and increases the metabolic activation induced by TGFβ1. (A) Expression of CPT1A in LX-2 cells transfected with either 1ng or 10ng of plasmid encoding CPT1A (n = 4). (B) Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). Arrows indicate the timepoint at which

mitochondrial respiration modulators, of oligomycin (Oligo), phenylhydrazone [FCCP], or rotenone/antimycin A (Rot/AA), were added to the assay. Right, graph depicting the effect of the upregulation of CPT1A on quiescent or energetic metabolic states based on quantification of glycolysis and OCR during basal metabolism. (C) Parameters of mitochondrial function (n = 5-8). (D) Expression of fibrotic markers (n = 4). (E) OCR, ECAR, and basal metabolism in LX-2 cells overexpressing CPT1A following the administration of TGFβ1 for 12 h (n = 6). (F) Parameters of mitochondrial function (n=3). (G) Expression of fibrotic markers (n = 5). mRNA levels were normalized to the housekeeping gene HPRT. Data are mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001

Fig. 6. Increased levels of palmitic acid induce CPT1A and the fibrogenic response in LX-2 cells. (A) Lipidomics analysis of different classes of fatty acids in human LX-2 HSCs treated with TGFβ1 or vehicle for 12 hours (n=5). (B) Levels of specific species of saturated fatty acids in LX-2 activated by TGFβ1 and following the administration of etomoxir (n=5). (C) Expression of CPT1A and fibrogenic (αSMA, COL1α1, COL1α2) markers in LX-2 incubated with palmitic acid at doses of 1μM and 10μM for 4 hours (n=4). (D) Expression of CPT1A and fibrogenic markers in LX-2 silencing CPT1A and incubated with 10μM palmitic acid for 4 hours (n=4). mRNA levels were normalized to the housekeeping gene HPRT. Data are mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001

Fig. 7. CPT1A deficiency in HSCs protects against liver fibrosis in mice fed a methionine- and choline-deficient diet (MCDD). HSC-CPT1A knock-out (KO) or wild-type (WT) mice were fed a standard diet (SD) or a MCDD diet for 6 weeks (n = 4–7). (A) Representative colocalized immunofluorescence for αSMA (red) with CPT1A (green). Nuclei were stained with DAPI (blue). (B) Serum levels of AST and ALT. (C) Liver sections stained with H&E (top), ORO (middle), or Sirius red (bottom); stained areas were quantified. (D) Hepatic hydroxyproline levels.

(E) Expression of fibrotic (αSMA, COL1α1, COL1α2, TGFβ) and inflammatory (F4/80, TNFα, and IL1β) markers in liver. (F) F4/80 immunohistochemistry staining. Stained areas were quantified. mRNA levels were normalized to the housekeeping gene HPRT. Data are mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001

Fig. 8. CPT1A deficiency in HSCs protects against fibrosis induced by choline-deficient, high-fat diet (CDHFD) in mice. HSC-CPT1A-KO or WT mice were fed a CDHFD or a standard diet (SD) for 10 weeks (n = 6 or 7). (A) Cumulative body weight gain and food intake. (B) Serum levels of AST and ALT. (C) Liver sections stained with H&E (top), ORO (middle), or Sirius red (bottom). Staining areas were quantified. (D) Hepatic hydroxyproline levels. (E) Expression of fibrotic and inflammatory markers in the liver. mRNA levels were normalized to the housekeeping gene HPRT. Data are mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001

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